Fascinating discoveries have been made in the last 60 years in cancer research. Yet, these discoveries have not changed the main methods of standard cancer treatment. Surgery, radiotherapy and chemotherapy were developed in the first half of the 20th century. At this time nothing was known about the biology of cancer metastasis and about the role of the immune system in the fight against cancer. This book is a plea for an update of cancer treatment by including biology-based treatments, such as immunotherapy and oncolytic virotherapy. These treatments have a higher tumor selectivety and lower side effects than radio-or chemotherapy. In addition they can induce long-lasting effects which are based on cancer-specific immunological memory. We now know that epigenetic mechanisms allow tumors to develop therapy-resistant variants. Therefore, efficacy of future cancer therapy could be improved by targeting cancer-associated epigenetic mechanisms. The tumor microenvironment represents an important support system for cancer growth, invasion and metastasis. Future strategies targeting the tumor microenvironment are considered as promising. The book addresses all people concerned with cancer

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978-620-2-05545-1

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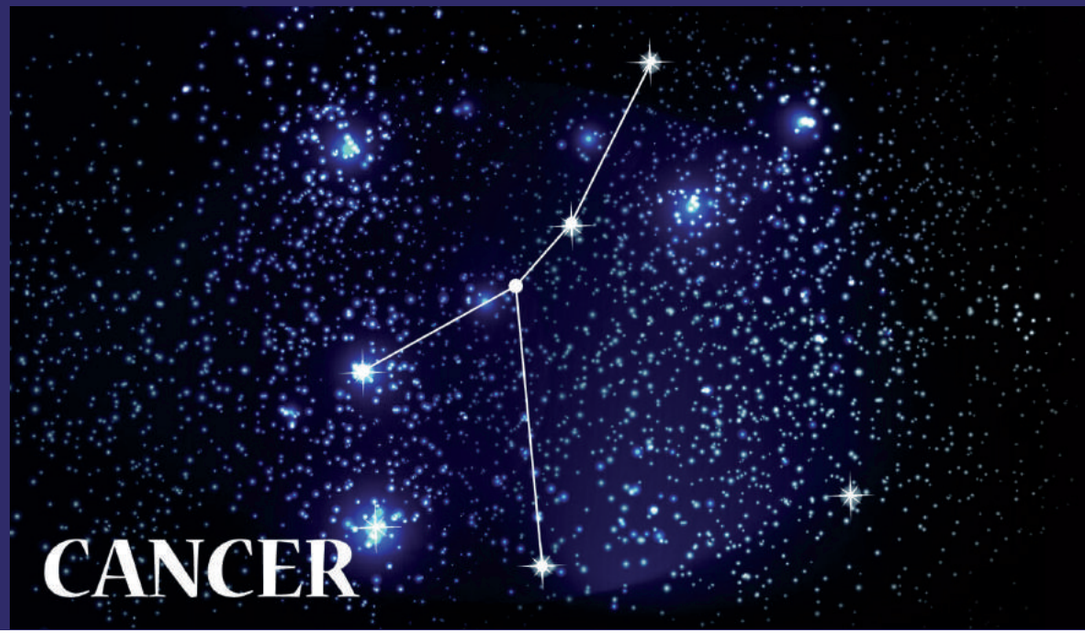
Schirrmacher



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Cover image: www.ingimage.com

Publisher:
LAP LAMBERT Academic Publishing
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International Book Market Service Ltd., member of OmniScriptum Publishing Group
17 Meldrum Street, Beau Bassin 71504, Mauritius

Printed at: see last page
ISBN: 978-620-2-05545-1

QUO VADIS CANCER THERAPY ?

Fascinating discoveries of the last 60 years

A plea for more immunotherapy, lower side effects and higher efficacy

Prof Volker Schirrmacher*

Keywords

Cancer research, immunotherapy, oncolytic viruses, monoclonal antibodies, small molecule inhibitors, memory T cells, anti-cancer vaccines, checkpoint inhibitors, side effects, efficacy

*** Immunological and Oncological Center Cologne, Germany**

This book is dedicated to my wife *Barbara*

to our daughters *Tanja* and *Elise*

to my sister *Antje*

and to our grandchildren:

Jonas, Julian, Luna

Helene, Franz, Gustav

QUO VADIS CANCER THERAPY?

FASCINATING DISCOVERIES OF THE LAST 60 YEARS:

A plea for more immunotherapy, lower side effects and higher long-lasting efficacy

QUO VADIS CANCER THERAPY ?

Fascinating discoveries of the last 60 years

A plea for more immunotherapy, lower side effects and higher efficacy

INTRODUCTION

This book is directed at all people concerned with cancer and interested in its pathogenesis, biology and treatment possibilities. Each mode of treatment has its history which is being reviewed.

The first Chapter deals with the development of conventional treatments such as surgery, radiotherapy, chemotherapy and hormone therapy. Each treatment category is explained and the effects and side effects summarized.

Targeted therapies represent new types of drugs based on molecular science and rational drug design. To understand their scientific basis, development and mode of function, Chapter II describes the many advances in molecular biology which formed a basic platform for targeted therapies.

Targeted therapies, described in Chapter III, are directed towards defined cancer-associated targets. They can be small molecules inhibiting intra-cellular enzymes of signal transduction. Or they can be larger molecules like monoclonal antibodies (mabs) targeting membrane expressed receptors.

Immunotherapy is described in Chapter IV. Progress in immunology and virology in the second half of the last century has led to new modes of treatment which are based on biology rather than physics (radiotherapy) or chemistry (chemotherapy). The first half is devoted to antibodies, which meanwhile have entered the clinical practice while the second half deals with T-cell mediated immunity and adoptive cellular immunotherapy.

Chapter V describes milestones of virological research with relevance to cancer. Its main focus is directed towards oncolytic viruses and virus-modified anti-cancer vaccines.

Chapter VI describes combinations of biological therapies and standard therapies. For a cancer patient it is important that the therapy has as low side effects as possible and should affect overall survival (OS). Biologic therapies (e.g. vaccination immunotherapy or oncolytic virus therapy) have a mode of action that is different from cytostatic drugs. Their effects are host mediated and usually not immediate but delayed. Their side effects are much less pronounced than those of chemotherapy. The combinations described point towards low dose applications with reduced side effects.

Physiological regulatory systems are explained in Chapter VII. Cancer can be characterized as a disease of dysregulation at many levels, within the cancer cell itself and outside in its tissue environment. A physiological process such as wound healing is complex but optimized by nature and goes without side effects. Graft rejection by the immune system is another example. We will provide an example that dysregulations associated with cancer, even in late-stage disease with cachexia, are principally reversible. In this example it is a specially potent immune response, which can be transferred from an immunoresistant mouse strain into the tumor-bearing strain. So it is worth studying further, how to reverse cancer-associated dysregulations.

Chapter VIII discusses a change of paradigm. Rather than focusing on the tumor cells, one could target therapies on the stromal support network and the host cells involved with it. One could target for instance host cells helping extravasation and metastatic niche formation. Or one could target the invasion front with its invadopodia, invasion enzymes and ECM. Also the communication between "seed" (disseminated tumor cells) and "soil" (target organ of metastasis) could be worth targeting.

The final Chapter IX reflects the topic of this book: Quo vadis cancer therapy ? It will compare immunotherapy with other therapies and then discuss in general how to reduce side effects and how to improve efficacy.

What this book is not aiming at is to cover all aspects of cancer. The focus is more on the concepts behind the different modes of treatment and the search for possible synergistic effects for future improvements. Another aspect concerns the enormous advancement of knowledge in the sciences of biology and medicine within the last century. The step-wise elucidation of the functioning of our body, as witnessed by Nobel Prizes in Physics, Chemistry

and Physiology or Medicine, is satisfying. So this book also aimes at transmitting the progress made in human sciences to the general public.

I witnessed and participated in cancer research for nearly 50 years. The most fascinating discoveries made in this time period will be explained. There are also auto-biographical aspects which are separated in boxes.

Each Chapter is rounded up by a list of key points and by a list of references.

CHAPTER I. STANDARD THERAPY

Cancer is a disease of cells in a multicellular organism and thus older than mankind. This knowledge goes back to Rudolf Virchow, who in the 19th century formulated the cellular theory of cancer. Leukemia was for the first time considered as a disease of white blood, thus the name. Hyperplasia was defined as a growth based on the increase of cell division while hypertrophia was defined as a growth due to the increase of cell volume.

In 1910, W B Coley, J Ewing and E Codman in New York treated sarcomas from bone by a mixture of bacterial toxins. The positive effects observed in some but not all patients were likely due to stimulation of the immune system. One of Coleys toxins became much later known as the Tumor Necrosis Factor alpha (TNF-α). But surgeons and oncologists at the time did not take much notice of those induced anti-cancer reactions.

A. SURGERY

Operations of cancerous growths were performed at the end of the 19th century even before it was known that cancer is a disease of abnormal cells and not of pus. With more experience, surgeons became aware that they were successful only when the disease was early discovered and localized. Often they were not successful. From leukemia it was known since about 1900 that there exist different varieties: Those that are chronic and indolent such as chronic myeloid leukemia (CML) and those that are acute and aggressive, such as acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML).

The following description of the development of surgery in the 20th century is based on two important books. One is written by S Mukherjee (1), published in 2010 and presents a very personal biography of cancer, entitled "The Emperor of All Maladies." The other is written by KH Bauer (2), the founder of the German Cancer Research Center in Heidelberg (Germany). In 1963, he wrote a comprehensive textbook about cancer and the state-of-the-art of treatment at the time.

i) THE DOGMA OF RADICAL, ULTRA-RADICAL AND SUPRA-RADICAL SURGERY

Decades of surgical cancer treatment in the first half of the 20th century passed without a major success. So surgeons became more and more frustrated. The fight against other diseases such as smallpox, typhus and tuberculosis had been far more successful. Thus, surgeons gradually increased the aggressiveness of cancer operations. Already in 1890, WS Halsted from the Johns-Hopkins University (USA) had introduced the concept of radical mastectomy to treat breast cancer (3). Without understanding cancer metastasis as the main reason for the failures of surgical treatment of cancer, Halsted continued with surgery and increased the size and extent of operations.

In 1930 radical surgery had become a dogma worldwide and Halsted had become the Hero of cancer surgery. He had formulated the "centrifugal theory" which later turned out to be wrong. Gradually "radical" mastectomy became "super-radical" and finally "ultra-radical".

ii) DISPROVAL OF THE DOGMA AFTER 90 YEARS

But there were others like G Keynes from London (UK). He doubted the concept of radical surgery and tested combinations of conventional surgery with radiotherapy. Also, in the USA, G Crile Jr in the 1950s decided to give up radical mastectomy. Based on 40 years of clinical experience , Keynes and Crile had apparently similar positive experiences without radical mastectomy.

It was still a long way to disprove a prevailing dogma. This required new statistical methods, the concept of statistical power and high numbers of cases. So, even in 1973 Crile had not been able to disprove Halsteds theory of radical mastectomy. Nevertheless, he sent an urgent appeal to breast cancer patients, namely to refuse radical mastectomy.

The surgeon B Fisher from Pennsylvania (USA) was eventually successful in disproving the prevailing dogma. It lasted 10 years until he could convince 1765 patients to participate in a clinical study performed at 34 clinical centers in the USA and Canada. It was a 3-armed study. Arm 1: Radical mastectomy, arm 2: Conventional mastectomy, arm 3: Lymphnodectomy plus radiation. The results were published in 1981. There were no differences in the frequency of recurrencies, metastases and mortality. Group 1 was not better than the other groups but had to pay with high morbidity. Systemic adjuvant therapy was proven as good as radical mastectomy (4).

The dogma of radical mastectomy, although wrong, had prevailed for as long as 90 years, from 1891 to 1981. It has been estimated that about 500 000 women worldwide underwent this procedure. Many of them became disabeled. Many had no idea that their treatment of cancer could have been done in a different, less aggressive, way. This incredible story was described in a book in 2001 (5) and a biography about WS Halsted appeared in 2010 (6).

When Fisher`s study results became published (4), radical surgery collapsed immediately. This was a collapse of a prevailing dogma. The dogma made people believe that cancer with metastases could be cured by surgery alone. The medical truth was that a cancer`s ability to invade healthy tissue, to disseminate via the blood and lymphatic vessels and to grow at distant sites into metastases destroyed all the efforts of the surgeons. There had been an ignorance about the basic biology and complexity of systemic metastatic disease. In addition, there was hardly any research in tumor immunology studying the complex interactions between a tumor and the host`s immune system.

In 2008 B Fisher formulated the following summary about cancer surgery:

" Surgery in the 20th century was dominated by the principles of William S. Halsted, who contended that the bloodstream was of little significance as a route of tumor cell dissemination; a tumor was autonomous of its host; and cancer was a local-regional disease that spread in an orderly fashion based on mechanical considerations. Halsted believed that both the extent and nuances of an operation influenced patient outcome and that inadequate surgical skill was responsible for the failure to cure" (7).

iii) A NEW ERA OF CANCER SURGERY

A new surgical era arose in 1957, when cancer surgery began to be influenced by laboratory scientific research and clinical studies. The results were in contrast to the Halsted principles. Clinical trials supported the thesis that operable cancer can already be in a state of systemic disease. In this situation, variations in local-regional therapy were unlikely to substantially affect survival. Complex tumor-host relationships were shown to affect every aspect of cancer and, contrary to Halsted`s thesis, the bloodstream was found to be of considerable importance for tumor cell dissemination. Clinical trials had shown that less radical surgery was justified.

In addition, studies had shown that improved survival can be achieved with systemic therapy after surgery. Such therapy could reduce both the incidence of distant disease and the tumor recurrence at the tumor site after minimal surgery. The use of systemic therapy in patients who had no identifiable metastatic disease was a drastic departure from previous strategies (7).

In 1962, the German surgeon KH Bauer published in german language a more than 1 000 page book about the state-of-art of cancer therapy, including the latest surgical techniques (2). He concluded among others:

a) Cancer therapy consists basically on three methods: operation, radiotherapy and chemotherapy.

b) Surgical treatment has the strongest impact. It should be performed, however, only when the disease is localized or maximally regionally disseminated.

c) Operations of cancer have to be carefully planned and carried out. Because of the possibility of regional cancer cell dissemination, cancer has to be operated within a rim of healthy surrounding tissue.

d) Advances in cancer surgery are made possible through advances in general surgery: Electrosurgery, modern procedures of anesthesia, chemoprophylaxis of wound infections, etc.

e) Operations of recurrencies are more difficult than those of primaries. Also the rates of cures are far lower.

f) Operations of metastases make sense only when these are solitary metastases or metastases restricted in space to lung- or liver-lobuli.

g) Operations in a palliative situation are performed only to prevent direct danger, such as ileus, suffocatio, blindness due to intracranial liquor pressure, etc. They may also serve to reduce cancer-associated pain.

h) A special situation exists with regard to operative endocrinotherapy. This relates to orchi-, ovary- or adrenalectomy in cases of hormone-dependent mammary- or prostate carcinomas.

i) Operative cancer therapy can, in selected cases, well be combined with radio- and chemotherapy.

This summary, written by the Founder and Director of the German Cancer Research Center in Heidelberg 55 years ago is an attempt of an objective analysis of the situation at the time.

In the meantime, the surgical management of breast cancer has changed over the past decades (8). For prostate cancer, the predictive value of stage-, grade- and prostate-specific antigen for recurrence after radical prostatectomy was proven to be dependent on the surgeon`s experience (9).

An overview of surgery in Ontario (Canada) revealed that lung cancer incidence and surgical care vary significantly by health region, income level, and community size (10). These are just examples of the many variables that might affect the outcome of cancer surgery.

Of importance are advances in surgical techniques. In addition to traditional open surgery there exists the laparoscopic surgery. Robot-assisted surgery is claimed to be one of the biggest breakthroughs in surgery since the introduction of anaesthesia (11). It is said to represent the most significant advancement in minimally invasive surgery of this decade. Natural orifice transluminal endoscopic surgery (NOTES) is another new concept that attempts to reduce the impact of surgery on the patient (12). In future one might imagine even regenerative surgery by combining surgery with cell-, biomaterial-, and molecular-based approaches of tissue engineering (13).

Tables 1 and 2 list milestones in the development of standard therapies for cancer, in particular concerning surgery and radiotherapy.

B. RADIOTHERAPY

Radiotherapy (RT) of cancer tries to make use of electromagnetic rays which were shown to be inductors of mutations in cell culture. The wave lengths range from UV to γ rays. The basis for radiotherapy of cancer rests on the observation that cancer cells in cell culture have a higher sensitivity towards toxic effects of radiation than normal cells.

Pioneers of RT were, among others, Wilhelm Conrad Röntgen, the discoverer of X-rays (14) and Marie and Pierre Curie, the discoverers of radioactive decay (15).

i) NATURE

Irradiation is most often performed by Röntgen-or Gamma (X) rays (high energy photons) and by Electron-rays (Beta-rays). Röntgenrays can be very weak (10 kiloelectronvolt (keV)), medium (50-150 keV) or strong (150 - >250 keV). They have the advantage of easy technical production as well as good control and dosage. Alpha-, Beta- and Gamma (x) rays are ionizing rays from radioactive decay. In a broader sense, Gamma (x) rays represent electromagnetic rays with high quantum energy (> 200 keV).

Proton beam therapy is a new modality over conventional gamma radiotherapy because of its dose deposition advantage.

ii) MECHANISMS OF EFFECTS

The main goal of RT is to deprive tumor cells of their reproductive potential. The primary effect of radiation consists of ionization. The details of the ionization induced molecular changes in a living cancer tissue *in situ* are not yet entirely resolved. Apoptosis and mitotic catastrophe are the two major cell deaths induced by radiation. In addition, in the last few years it was shown that inhibition of proliferative capacity of tumor cells following irradiation, can occur, especially with solid tumors, by permanent cell cycle arrest which is called senescence.

In an in vitro assay with human lung carcinoma cells, proton beam (3MeV) was two times more cytotoxic than γ radiation and induced higher and longer cell cycle arrest. At equitoxic doses, the proton-irradiated cells had reduced

cell adhesion and migration ability as compared to γ-irradiated cells. It was also more effective in reducing a population of cancer stem cell-like cells (16).

Direct effects of ionizing irradiation on cancer cells include DNA and chromosomal damage (17), mitotic catastrophe (18), senescence and apoptosis induction (19-21). P53 plays an important role in determining sensitivity to radiotherapy (22,23).

Besides the direct effects of ionizing irradiation on cancer cells, indirect effects exist also. These are mediated in large part by the immune system. Immunogenic forms of tumor cell death, induced by X-rays, include danger signals like membrane expression of heat shock protein 70 (HSP70) or release of adenosine triphosphate (ATP) and high mobility group box 1 protein (HMGB1) (24).

iii) CLINICAL APPLICATION

Röntgentherapy was developed by G Perthes, a surgeon from Tübingen (Germany) who introduced filters to avoid skin damaging rays as a prerequisite for deep tissue effects. Röntgenray effects depend on the dosis, the volume of the target tissue and the time of exposure. The wavelength does not play a role, only the energy dose.

It would go beyond the scope of this review to describe all the technical developments of radiotherapy in detail. It should suffice to state that - similar to surgery – also in radiotherapy there was a development away from rigid schematism, radicalism and ultraradicalism.

iv) CONCEPTS OF RADIOTHERAPY

Table 3 summarizes different concepts of radiotherapy. Teletherapy consists of methods to expose a body from outside to irradiation, while brachytherapy consists of methods to implant the radiation source into the body. Particeltherapy with protons, neutrons or heavy ions allows to achieve better deep tissue effects. Radio-immunotherapy with radionuclide-labeled anti-tumor antibodies improves the specificity and is part of targeted therapies which will be discussed further below.

CA Perez, LW Brady and EC Halperin have described the principles and practice of radiation oncology (25).

a) Effects

RT seems to be particularly suited in cases of metastases in order to improve quality of life. Between 1930-1940, cancer patients with metastases in bone, lung, skin and at other sites were treated by RT. It was concluded in 1940 that irradiation can be recommended for the following indications: lung metastases (seminom, struma maligna), hilus- and pleurametastases (primary tumors of mouth, pharynx and oesophagus), bone metastases (primary tumors of struma, mamma, testicles and ovaries) and finally skin metastases in absence of distant metastases. In contrast, irradiation of benign bone tumors (fibroma, myxoma, chondroma, exostoses, osteomes, osteoblastoms) was not recommended because they do not seem to respond.

Today, RT is used in the treatment of over 50% of cancer patients. It provides an organ-sparing approach for many patients with early-stage cancer. It can improve overall survival alone or in combination with chemotherapy and it is effective in symptom palliation from metastatic disease.

RT seems to match well with surgery. Pre- and post-operative radiation are today in many situations standard of treatment. RT can be applied in an adjuvant situation in combination with R0 surgical resection. It can also be applied as additive in combination with R1/2 resection or in a palliative situation.

With regard to combinations of RT with surgery, Schmieden described in 1934 three main groups: 1. Carcinoma of skin, face, head and lips where RT is superior to surgery, 2. Carcinoma of lung, oesophagus, stomach, intestine, liver, kidney, pancreas, bladder and rectum where surgery is superior to RT and 3. Carcinomas of mamma, larynx, jaw and tongue, as well as carcinomas of glands including genitalia, which require a combination of treatment by surgery and radiotherapy.

Current advances and future directions of cancer RT have recently been summarized (26,27). 2011 has been designated the year of RT in the UK, celebrating a century of advances since Marie Curie won her second Nobel Prize for her research into radium.

In spite of all the efforts for improvements, it remains a fact that RT *in vivo* lacks the evidence for tumor specificity. Side effects are therefore warranted.

b) Side effects

One distinguishes as damage from irradiation early and late effects. To the early effects belongs the acute irradiation syndrome which has been observed after atom bomb explosions in Hiroshima and Nagasaki. There are symptoms in the acute stage of the vegetative nerve system, of the hematopoetic system, of stomach and intestine and of germ glands.

The late effects include gene effects, damage to the hematopoietic system and to the immune system, increase of damages in embryos and late tumor induction.

With regard to radiotherapy of cancer, the damage to skin is the most obvious. Early skin damage includes dermatitis, exfoliative bullosa and alopecia, while late skin damage includes induration, dryness, changes in pigment, teleangiectasia, fibrous changes of subcutaneous tissue and sklerotization of vessels. Lungs may become affected by radiation fibrosis, the small intestine by atrophy of lymphatic tissue and bones by osteoporosis and spontaneous fractures. Germ glands may show reduced germ cell differentiation, gland atrophy and sterility. Gravidy may be affected by aborts and radiation-induced abnormalities. In humans, the "fetal period" is one of particular sensitivity. In this period, radiation can lead to induction of mental retardation, especially if the exposure occurs between 8-15 weeks of gestation.

Cranial irradiation that is widely used for treatment of brain tumors may induce death in human neural stem cells (NSC) and further cause substantial cognitive deficits such as impaired learning and memory. Direct gamma-irradiation of human neuroblastoma cells in culture using clinical doses (2-5 Gy) resulted in low levels of apoptosis in cancer cells. Unexpectedly, this was accompanied by induction of TRAIL/TRAIL-R2 and strong bystander responses in non-targeted NSC (28). Thus, intercellular communication between brain tumor cells and bystander stem cells could be involved in the amplification of cancer pathology in the brain.

Long-term risks of RT or CT for development of cardiovascular disease (CVD) or of congestive heart failure (CHF) were evaluated in a large, population-based cohort comprising 70,230 surgically treated stage I to III breast cancer patients. Radiation therapy regimens used in breast cancer treatment between 1989 and 2005 increased the risk of CVD, and anthracycline-based CT regimens increased the risk of CHF (29).

Another late damage is the Röntgenulcus which can change into a Röntgencarcinoma.

iii) CONCLUSIONS RELATING TO SURGERY AND RADIOTHERAPY

The history of surgery has been summarized by HA Ellis (30) and the history of the radiological sciences by RA Gagliardi and JF Wilson (31).

To conclude the chapter on surgery and RT, it may be worthwile to take breast cancer treatment as an example and to mention the various improvements achieved over time:

1880 Halsted`s mastectomy

1980 Breast-conserving surgery with equivalent survival but better esthetic outcomes

1980 Chemotherapy established for early breast cancer

1990: Combinations of irradiation technology with imaging and computer technologies introduced to direct radiation to more precisely defined target volumes.

1990 External whole breast irradiation introduced with conservative surgery (reduction of recurrencies)

1990 Sentinel node biopsy avoiding axillary dissection (if the sentinel node was disease-free)

2000 3-week regimens equivalent to 6-week regimens thus easing pressure on patients and radiation centers

As stated by S Zurrida and U Veronesi (32), irradiation systems are evolving rapidly but are being implemented without data on long-term morbidity or efficacy, while costs rise steeply.

Radiowaves and modern Magnet Technology have revolutionized modern tissue imaging, as exemplified by Röntgenimages, Computer Tomographies and Magnet Resonance Tomography (MRT). Nobelprizes were given for these three innovations in medicine in 1901, 1979 and 2003. Nowadays, Röntgenimages are transformed into projection radiographies (PR), which can be dealt with in a computer. This technology is being used to detect not only tumors but also tuberculosis or pneumonia.

C. CHEMOTHERAPY

i) PIONEERING WORK FROM SIDNEY FARBER

Historically, the introduction of CT into standard treatment of cancer has a lot to do with the pioneer Sidney Farber. He was born 1903 in Buffalo (NY, USA), one year after Virchows death. Farber experienced the development and success of new antibiotics in the fight against infectious bacterial diseases during and after the second world war. In 1942 the company Merck delivered for the first time Penicillin. New antibiotics became available in 1947 (chloramphenicol), 1948 (tetracyclin) and 1949 (streptomycin). In addition, after the war the medical and state institutions improved their standards of hygiene. All this led to a dramatic decrease of infectious diseases such as typhoid or tuberculosis (1).

Cancer treatment, in contrast, saw no progress since decades. Aggressive surgery was unable to deal with cancer, once this had become a systemic disease. Farber studied medicine at the universities of Buffalo (USA), Heidelberg (Germany) and Freiburg (Germany) and finally at Harvard (USA). In 1947 Farber had become a researcher on cancer where he studied leukemia and leukemic cells in culture. He became aware that folic acid or folate is a vitamin-like substance which is essential for building up DNA in a cell. He saw no proliferation of his leukemic cells in culture in the absence of folate. So he had the idea to develop antagonists to folate. The first antifolate was sent from the Lederle Laboratories in NY to Farber in 1947. In the same year Farber discovered the cytostatic activity of aminopterin, a folic acid

derivative. Although Farber saw remissions of ALL, these were of only short duration. His published results were met with skepticism and critics by the oncological establishment (1).

In 1948 Farber established in Boston the Children`s Cancer Research Foundation. 1974, one year after Farbers death, his Institute was re-named in his honor into Sidney Farber Cancer Institute and 1983 into Dana-Faber Cancer Institute. Farber is considered the founder of modern pathology of children`s diseases (1).

ii) A NEW DOGMA ARISING: AGGRESSIVE CHEMOTHERAPY

In the late 1970s cisplatin became the latest trend in cancer pharmacology. For instance, patients with cancer of the testis were treated by a combination of bleomycin, vinblastine and cisplatin, abbreviated BVP. The yellow fluid was infused into patients by infusion tubes. The heavy side effects (vomiting 12x per day; antiemetica did not exist yet) were considered necessary and had to be tolerated. A new dogma prevailed, namely that of aggressive CT (1).

The National Cancer Institute (NCI) became a fabric of new cytostatic toxins. More than 100 000 molecules were tested per year, while the basic biology of cancer and its metastases was still in the dark. In the middle of the 1970s, a first success was achieved by CT treatment of Burkitt`s lymphoma. It was a high dosed combination therapy of 7 cytostatics. One substance was a molecular derivative of nitrogen mustard, a toxin of the great war.

There was a financial profit for the NCI. Diverse medication mixtures and study plans were generated: ABVD, BEP, C-MOPP, ChlaVIP, CHOP, ACT … Until 1979 the NCI had constructed a network of 20 cancer centers, which they had selected to be involved in executing all those new studies. Clinical boards which were involved in the approval and in the coordination of those huge studies with human subjects were advised to accelerate the process of approval. It was an experimental set-up of gigantic dimension, not with animal tumors first, but with human individuals. Trial and error was the device, most often error prevailed. The patterns were repeated with many different types of cancer (1).

Table 6 lists some of the cytostatic drugs developed over time. They can be grouped according to their type as alkylating agents, alcaloids, antibiotics or

antimetabolites. Many of these still belong to the armentarium of present-day CT. The list also gives examples of such cytostatics and of their cellular target. That a cellular target can be identified distinguishes CT from RT. Often the target has to do with cellular DNA or RNA and their metabolism. Antimetabolites target purin or pyrimidine metabolic enzymes. Alcaloids target the cytoskeleton (ß-tubulin) and mitosis.

iii) EVALUATION OF THERAPY EFFECTS

Objective criteria for evaluation of therapy effects (Table 4) have been defined by the World Health Organization (WHO). Apart from the already mentioned "tumor response" (extent of tumor remission), these include the determination of remission time, of survival and of toxicity. Subjective criteria can be Quality of Life (QoL), relief of tumor-associated pain and general state of health.

So far missing is the determination of Stable Disease (SD) as response parameter of a therapy and the evaluation of the immune system. The determination of soluble tumor-associated markers from serum samples may serve as surrogate marker for follow up and early therapy evaluation.

iv) THERAPEUTIC EFFECTS

CT became established in the 1970s as a curative treatment for adults in advanced Hodgkin`s disease (33,34), non-Hodgkin`s lymphoma (35,36), teratoma of testis (37) and as adjuvant treatment for early breast cancer (38). Osteosarcoma is another type of cancer that responds relatively well to CT. It can be applied pre-operatively, post-operatively or in combination.

High dose CT was applied to adult ALL (39) and in combination with consolidation for AML (40). Improved survival duration was reported for combination CT induction for Multiple Myeloma (41).

Unfortunately, CT can not be considered as a curative treatment procedure for the most common cancers, namely carcinomas. If anything, there may be effects prolonging somewhat OS. In case of metastatic lung cancer, the application of combination CTs improved OS by 2-4 months, in case of colorectal carcinoma by about 6 months. In case of metastasized breast cancer, studies after 1995 reported an improvement of median survival by 3-

9 months. Often CT is given mainly because of some palliative effects. An OS benefit of less than 5% has been achieved in the adjuvant treatment of breast, colon, and head and neck cancers.

Despite the use of new and expensive single and combination drugs to improve response rates and other agents to allow for dose escalation, there has been little impact from the use of newer regimens. For non-Hodgkin`s lymphoma and ovarian cancer, for instance, cyclophosphamide, adriamycin, vincristine, prednisolone (CHOP) and platinum are still the "gold standard" treatment.

In 2004, a literature search for randomized clinical trials reporting a 5-year survival benefit attributable solely to cytotoxic CT was performed in adult malignancies. It included 154 971 cancer patients from USA and 72 903 cancer patients from Australia. The overall contribution of CT was estimated to be only 2.3% in Australia and 2.1% in the USA (42).

Overall, only 13 out of 22 malignancies evaluated showed any improvement in 5-year survival. The improvement was greater than 10% in only three of those 13 malignancies. The five most "chemo-sensitive" cancers, namely testis, Hodgkin`s disease and non-Hodgkin`s lymphoma, cervix and ovary, accounted for 8.4% of the total cancer incidence in Australia in 1998. In this selected group, the improvement of 5-year survival rate due solely to CT was only 14% (42).

iv) SIDE EFFECTS

The summary of the side effects of CT is much longer than that of the therapeutic effects. Tables 7 and 8 list immediate signs of toxicity and Table 9 signs of chronic toxicity. The data are based on the book "Therapiekonzepte Onkologie" (43). The Tables might serve as a guide line for the educated cancer patient.

Table 7 quantifies toxicity according to the WHO classification (grade 0 – 4): 1. From bone marrow and blood are given the values for hemoglobin (Hb) and the numbers of cells (leucocytes, granulocytes, thrombocytes) in the different categories. 2. Toxicity levels in the gastro-intestinal (GI) tract are characterized by the ratio of two liver enzymes (GOT and GPT) and by the

level of bilirubin. 3. Toxicity to the kidney can be followed by increase in urea, creatinine and proteinuria. 4. Fever is another sign caused by CT.

Table 8 lists other signs of toxicity of approved cytostatic drugs. This time only grade 3 and 4 are given. It is appalling to read and therefore I apologize to the reader. How could drugs have become approved with such side effects and how is it possible that they are still in use ? All organs of the body are affected, including such essential ones as heart, lung and brain! Even if it were only a minority of patients which might be affected that severely, it would be those too much.

In addition, there are chronic (long-term) effects of CT, which are listed in Table 9. They include development of drug resistance, carcinogenicity and infertility. The type of drugs are given as well as the mechanisms of drug resistance which can develop by time. Infertility and carcinogenicity were already mentioned also as possible side effects of exposure to radiation.

v) CONCLUSIONS OVER TIME ABOUT CHEMOTHERAPY BY EXPERTS

1963: KH Bauer made the following comments about the state-of-art of CT (translated from german) : "Never were the hopes of great progress in fighting cancer so high as at the beginnings of CT. Never was the disappointment larger. Nevertheless, - in spite of less than 1 promille of cures -, one should not underestimate the value of the symptomatics, the palliative and the basics." And somewhat later he states: " Carcinomas are the type of tumors which respond only poorly to CT. The proliferation rate of their tissue of origin, epithelia, is low. That of carcinoma is also low. So it is possible that normal tissues with physiologically high proliferation rate will be damaged earlier and stronger than the carcinomas themselves. A patient should think twice whether to treat his carcinoma and metastases with cytostatic drugs. The harm may be bigger than the benefit. "

He explains the disaster and disappointment as follows:

1. It was wrong to conclude from the success of CT in the fight of bacterial infections by analogy a similar success of CT with cancer,

2. It was wrong to assume that a temporary effect with one type of cancer will translate into similar effects with other cancers,

3. There was a high discrepancy between the experimental efforts (> 100 000 substances tested, hecatombs of animals sacrified and > 1 000 publications) and the meager results from the clinic.

The 1980s: Between 1984 and 1985, at the peak of aggressive CT there appeared more than 6 000 articles in medical journals about CT treatment of cancer. Not one reported about a new strategy that led to cure of an advanced solid tumor by combination CT. As a result, one could observe an alienation between patients and doctors. When the medical oncologists, who often were arrogant because of their power, told the patients that the side effects of CT were tolerable they only meant that CT would not directly endanger their life (1).

1990: In Germany it was Prof U Abel, an epidemiologist and biometrician at the Tumorzentrum Heidelberg/Mannheim and at the DKFZ, who in 1990 criticized the excessive use of CT in carcinoma patients. Instead of discussing the evidence against this practice, the Heads of the two Institutions (Tumorzentrum and DKFZ) published a statement emphasizing that Dr Abels conclusions represented his private opinion which was not shared by the Heads of these Institutions. This dogmatic position was not untypical for the Directorship of DKFZ, an Institution which should always be commited to evidence-based medicine.

2000: About ten years later, another german epidemiologist, Prof D Hölzel (Munich), statet that in the last 20 years there had been no progress in the treatment of the often metastasized carcinomas of breast, lung, prostate, colon and rectum. This was based on the data from the Munich cancer registry. 5 years after diagnosis of metastases only 5% (lung carcinoma) up to 20% (breast carcinoma) of the patients had survived and this figure had not changed since the 1980s.

D. HORMONE THERAPY

Hormone therapies (HT) slow or stop the growth of hormone-sensitive tumors. These require certain hormones to grow. They act by preventing the body from producing the hormones or by interfering with the action of the hormones. Hormone therapies have been approved for breast and prostate cancer.

i) THE PIONEERING WORK OF CHARLES BRENTON HUGGINS

Breast and prostate are hormone-dependent organs. Carcinoma derived from these organs often go through a phase in which their growth is hormone-dependent. The Canadian surgeon CB Huggins performed in the 1940s and 1950s classical experiments about the physiology of the prostate in dogs. He demonstrated that the secretion of testosterone from the prostate could be ceased either by orchiectomy or by the application of the female hormone estrogen. Huggins and colleagues also showed that dogs with tumors of the prostate could be successfully treated by deprivation of androgens (44). In 1966 he received for his discovery of hormone treatment of prostate cancer the Nobel Prize for Physiology or Medicine.

The situation with metastatic breast cancer seems to be similar to the situation with prostate carcinoma. It has been known since a long time that the removal of the ovaries had a growth inhibitory effect on breast cancer. In 1939 A Loeser and H Ulrich introduced the treatment of breast cancer with the male hormone testosterone (2).

ii) CONCLUSION ABOUT HORMONE THERAPY BY AN EXPERT

KH Bauer summarized the situation of anti-hormonal therapy in 1963 as follows: " There is no doubt that the combined operative (orchi- or ovariectomy) plus hormone therapy of cancers from hormone-dependent organs is the greatest progress since the introduction of radiotherapy. The development proceeded in 4 steps:

1. A change in the hormonal status by orchi- or ovarectomy can have a long lasting growth inhibiting effect on cancers of prostate or breast, respectively.

2. This effect can be further enhanced by the application of hormones from the opposite sex. Since these hormones are endogenous physiological substances, their use for cancer therapy could be considered as an ideal chemotherapy of cancer.

3. Derivatives produced from such hormones by biochemists in laboratories led to products with increased effects and decreased side effects.

4. This pharmacotherapy of certain cancers also led to the production of other hormone preparations, such as cortisone, hydrocortisone etc., which

had effects on cancers from hormone-independent organs or tissues. Other preparations, such as prednisone and prednisonol, had anti-inflammatory functions and could have positive effects against cancer-associated changes in general metabolism, appetite, body weight etc. In this way developed a general pharmacotherapy of cancer (2).”

Hormonal manipulations have long been applied for the treatment of advanced and early-stage breast, prostate, and thyroid cancers. Table 10 lists the main types of present-day HT. It consists of surgery, competitive HT, inhibitory HT and of ablative drug therapy.

iii) TAMOXIFEN

Tamoxifen, a selective Estrogen Receptor (ER) modulator, was introduced into the treatment of breast cancer by VC Jordan in the 1970s (45,46). The drug and its active metabolites bind to ER and thereby compete with endogenous estradiol (example of inhibitory HT). It was approved for adjuvant therapy and for treatment of metastasized ER positive breast cancer. The recent randomized trial ATLAS revealed that continuing tamoxifen treatment to 10 years versus stopping at 5 years produces a further reduction in recurrence and mortality (47).

HT in breast cancer with inhibitors of aromatase or with analogs that inhibit Gonadotrophine Releasing Hormone (GnRH) increases the risk of osteoporosis. Biphosphonates and other bone agents can be given to counteract osteoporosis. In women with clinically evident breast cancer with bone metastases, bisphosphonates (oral and i.v.) and desonumab (s.c.) reduced the risk of developing skeletal-related events (SREs), as well as delaying the time to SREs (48,49).

Side effects of HT are, among others, changes in endometrium (polyps, neoplasia, hyperplasia) because tamoxifen has not only antagonistic but also agonistic effects onto the ER. The relative risk to develop endometrium carcinoma in women treated with tamoxifen is increased by a factor 2-4.

E. SOME AGENTS OF STANDARD THERAPY AND ENVIRONMENT ARE HUMAN CARCINOGENS

Side effects of Standard Therapies can be due not only to toxicity. They can include carcinogenic effects. The International Agency for Research on Cancer (IARC) has produced a list of agents that are known as human carcinogens. Examples are listed in Table 11. They include physical, chemical and biological agents.

1. Physical agents: Exposure to ionizing radiation of various forms has been shown to cause multiple forms of cancer. Additionally, solar radiation, in particular UV radiation, has sufficient energy to cause photochemical damage leading to skin cancer formation. The incidence of skin cancers, such as melanoma, basal cell carcinoma and squamous cell carcinoma has risen dramatically in recent years. UV radiation of 100 – 400 nm range appears to be causative. Targets of solar radiation-induced mutations include p53, p16, and PTCH.

2. Chemical agents: Most carcinogens are categorized as chemical carcinogens. They include organic and inorganic chemicals. Some chemotherapeutic agents are nitrosamines and heterocyclic amines. Following metabolic activation, N-nitrosamines can react with DNA to initiate carcinogenesis.

3. Biological agents. Hormones like estrogen and tamoxifen can be considered as carcinogens in hormone-dependent tissues. Other biological carcinogens can be viruses such as Epstein-Barr virus, Hepatitis B and C virus, Human Papillomavirus and Human T-cell lymphotropic virus. Helicobacter pylori is an example of a bacterium which can produce carcinogenic effects leading to stomach cancer.

F. LESS MAY BE MORE

There is at present a discussion going on at cancer conferences whether cancer treatment has to be so aggressive. There are still physicians who believe in many operations, high dose radiation and high dose CT. But there are more and more others who doubt such radical concepts and try to reduce the aggressiveness.

For instance, in early-stage breast cancer neo-adjuvant CT, if successful, could make operations superfluous. Also, increasing the precision in adjuvant therapy could reduce the percentage of patients that need to be treated. The 70-gene signature test (MammaPrint) has been shown to improve prediction of clinical outcome in women with early-stage breast cancer. A randomized, phase 3 study enrolled 6693 women with early-stage breast cancer and determined their genomic risk and their clinical risk. Women with high clinical risk and low genomic risk of recurrence based on Mammaprint received no CT. The study revealed that their 5-year rate of survival without distant metastasis was only 1,5 percentage points lower than the rate with CT. It was concluded that approximately 46% of women with breast cancer who are at high clinical risk might not require CT (50).

Key points:

1. **In the first half of the 19th century dogma rather than science dominated cancer therapy. Examples are radical, super-radical and ultra-radical and aggressive chemotherapy.**

2. **The introduction of radiotherapy and its combination with surgery was an important step forward.**

3. **The introduction of chemotherapy as an adjuvant treatment modality to surgery, however, has not fulfilled the original hopes. In spite of the long list of negative side effects, chemotherapy is still part of standard therapy, even in carcinomas where its effectivity is disputable.**

4. **Hormone therapy of cancers from hormone-dependent organs can be considered as a significant progress in cancer treatment.**

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Table 1 Main steps in the development of cancer surgery

1800-1900	**Increases in aggressiveness of cancer operations**
1890	**WS Halsted (USA) introduces the concept of radical mastectomy**
1930	**Radical surgery becomes a dogma worldwide and causes high disability and morbidity over a time period of about 50 years**
1924	**G Keynes (UK) tests combinations of less aggressive surgery with radiation**
1962	**KH Bauer (Germany) publishes a more than 1000 page book about the**

state-of-the art of cancer therapy, including the latest surgical techniques

1981 B Fisher (USA) reports results from a study demonstrating no benefit of radical mastectomy in comparison with simple mastectomy or to tumor resection plus radiation

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Table 2 Milestones in the development of radiotherapy

1895 WC Röntgen (Germany) discovers the Röntgen (X, γ) rays (high energy photons)

1898 M and P Curie (France)* discover radioactivity (from radium and polonium)

1903 E Rutherford (UK) distinguishes between α, ß an γ irradiation

1904 G Perthes (Germany) applies Röntgen rays to cancer patients after introduction of filters

1910 Radiumhemmet Institute is inaugurated in Stockholm, Sweden

1970 Electron rays are being produced by Linear-Accellerators

2009 Particel therapy with neutrons and protons
The Heidelberg Ionray Therapycenter (HIT) with C-ions is inaugurated

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*** Marie Curie received the Nobel prize twice: 1903 the one for Physics together with her husband P Curie and 1911 the one for Chemistry.**

Table 3 Concepts of radiotherapy

Teletherapy:	**- photons of γ rays and electron-rays are used to target tissues in the skin or just beneath** **- energy-rich protons are used to target deeper organs** **- IMRT* is introduced for radiation dose modulation** **- Tomotherapy is introduced for 3-dimensional tissue targeting (rotating system similar to CT)** **- Radio-chemotherapy: combination of RT with cytostatics, e.g. 5-FU or cisplatin, and radioprotectors, e.g. amifostin** **- Radio-surgery: e.g. of small brain tumors; stereotactic targeting, gamma-knife, cyber-knife**
Particeltherapy:	**- protons, neutrons, heavy ions (e.g. C ions)** **- to achieve better deep tissue effects** **- linear energy transfer (LET)**
Brachytherapy:	**- afterloading of iridium-192 (female genital tract application)** **- seed implantation of iodine-125 (prostate application)**
Radio-immunotherapy:	**- combination of radionuclide with anti-tumor antibody, e.g. Ibritumomab-Tiuxetan (anti-CD20 + yttrium isotope)**

*** IMRT Intensity-modified radiotherapy**

Table 4 Concepts for evaluation of therapy effects

A Objective criteria (WHO)
1. Extent of tumor remission
2. Time of remission (or time to progression)
3. Survival (median, progression-free, metastasis-free, overall)
4. Toxicity
B Subjective criteria
1. Quality of life
2. Relief of tumor-associated pain
3. State of health
C So far missing
1. State of the immune system
2. Response of the immune system (suppression vs stimulation)
3. Stable disease as response parameter

Table 5 Concepts of standard therapy of solid tumors, in particular carcinomas, exemplified with breast cancer

Surgery: resection of localized operable stages with curative intent
tumor-. segment- , quadrant- or mastectomy
combined with axilladissection and/or RT and/or CT
Radiotherapy (RT): adjuvant, in combination with R0 resection
additive, in combination with R1/2 resection
palliative
Chemotherapy (CT): neo-adjuvant, pre-operative systemic,
adjuvant systemic
Anti-hormone therapy (HT): adjuvant
combination of adjuvant CT and HT
Biphosphonate-Therapy (BT) against osteolytic bone defects

Table 6 Cellular targets of approved cytostatic drugs

Type of drug	examples	cellular target
Alkylating agents	**cyclophosphamide, melphalan**	**DNA, proteins**
	Ifosfamid, busulfan	**DNA, glutathion**
	dacarbacine (DTIC)	**purin nucleosides**
Alcaloids	**vinca-alcaloid**	**tubulin**

	epipodohyllotoxin	topoisomerase II
	camptothecin	topoisomerase I
Antibiotics	bleomycin	DNA
	anthrachinons	DNA, topoisomerase II
	actinomycin D	RNA polymerase
	mitomycin C	DNA
Antimetabolites	amethopterin (methotrexate)	dihydrofolate acid reductase (DHFR)
	6-mercaptopurin, 6-thioguanin	purin biosynthesis
	5-fluoruracil (5-FU)	thymidylate synthetase
	hydoxy-urea	ribonucleotide reductase

Table 7 Toxicity of approved cytostatic drugs according to WHO classification

Grade	**0**	**1**	**2**	**3**	**4**
Intensity	**none**	**mild**	**moderate**	**severe**	**life-threatening or disabling**
1.Bone marrow[a]					
& blood Hb	**>11,0**	**9,5-10,9**	**8,0-9,4**	**6,5-7,9**	**<6,5**
Leucocytes	**>4,0**	**3,0-3,9**	**2,0-2,9**	**1,0-1,9**	**<1,0**

Granulocytes	**>2,0**	**1,5-1,9**	**1,0-1,4**	**0,5-0,9**	**<0,5**
Thrombocytes	**>100**	**75-99**	**50-74**	**25-49**	**<25**
2. GI Tract					
Liver GOT/GPT	**<1,25xN[b]**	**1,25-2,5xN**	**2,6-5xN**	**5,1-10xN**	**>10xN**
Bilirubin	**“**	**“**	**“**	**“**	**“**
3. Kidney					
Urea	**<1,25xN[b]**	**1,25-2,5xN**	**2,6-5xN**	**5,1-10xN**	**>10xN**
Kreatinin	**“**	**“**	**“**	**“**	**“**
Proteinurie[c]	**none**	**<3 g/l**	**3,1-10 g/l**	**> 10 g/l**	**ne syn**
4. Fever[d]	**none**	**<38° C**	**38-40° C**	**> 40° C**	**pr dec**

a Hb (g/100 ml); cells (x10^9/l); b N = norm value ; c ne syn = nephrotic syndrome; chemotherapy (Mit. C) induced hemolytic-uremic syndrome (c-HUS) with letality between 44 and 82% ; d pr dec = fever caused by therapy and not by the tumor, combined with blood pressure decrease (hypotony);

Table 8 Other signs of toxicity of approved cytostatic drugs according to WHO classification

Grade	3	4
Stomatitis	ulcers	peroral nutrition impossible
Diarrhoe	intolerable	hemorrhagic dehydration
Obstipation	subileus	Ilius
Hematuria	macrohematuria	obstructive uropathy
Lung	dyspnoe	bed stay obligatory

Allergy	**bronchospasms**	**anaphylaxis**
Skin	**ulcerations**	**dermatitis, necrosis**
Hair	**alopecia, reversible**	**alopecia, irreversible**
Infections	**severe**	**severe + hypotonia**
Heartfunction	**dysfunction**	**dysfunction + nonresponsive**
Bleading	**severe**	**circulatory disorder**
Neurotoxicity		
i) central/consciousness	**somnolencia >50%**	**coma**
ii) peripheral	**paresthesia**	**paralysis**
iii) extrapyramidal symptoms	**ataxia > 4 days**	**spasms, coma**

Table 9 Chronic toxicity (long-term effects) of approved cytostatic drugs

1. Drug resistance	**mechanism**
Alkylating drugs	**increase of intracellular glutathion**
	increased glutathion-S-transferase
	increased DNA repair
Vinca-Alkaloids	**increased extracellular transport**
	via P-glycoprotein (mdr 1)
Anthracyclins	**increased expression of P-glycoprotein**
	increased activity of glutathione-S-transferase

	increased DNA repair
Methotrexat	**reduced membrane transport**
	increased concentration of DHFR[a]
5-FU	**reduced membrane transport**
	lack of desoxycytidinkinase
	increased activity of deaminases
	increased intracellular pool of dCTP
2. Carcinogenicity/	**chlorambucil, melphalan, cyclophosphamide**
Mutagenicity	**mustargen, methyl-CCNU, busulfan, razoxan**
3. Infertility	**busulfan, chlorambucil, cisplatin, etoposide**
	cyclophosphamide, melphalan, procabazin

a = dihydofolate-reductase

Table 10 Hormone therapy (HT)

Surgery:	**ovarectomy**
	orchiectomy
	adrenalectomy
	hypophysectomy
Competitive HT:	**anti-estrogens**
	anti-androgens

anti-gestagens

Inhibitory HT: aromatase inhibitors

Ablative drug therapy: GnRH antagonists

HT = Hormone therapy; GnRH = Gonadotrophine Releasing Hormone

Table 11 Some agents of standard therapy and environment staged by IARC* as human carcinogens

Chlorambucil	**Mustard gas**
Ciclosporin	**2-Naphthylamine**
Cyclophosphamide	**Radioiodines**
Estrogen	**Silica**
Etoposide	**Solar radiation**
Melphalan	**Tamoxifen**
Gamma (X)-Irradiation	

IARC* International Agency for Research on Cancer

CHAPTER II. BIOLOGICAL THERAPIES

Having gone through standard therapies, the question may be allowed: Where are we now?

A. STATE-OF-THE-ART OF CANCER THERAPY IN 2014

The WHO in its world cancer report 2014, which was presented by the International Agency for Cancer Research (IARC) in London, gives numbers for the year 2012. Worlwide there have been 14 million new cases of cancer and 8,2 million cancer patients who have died in that year. With regard to Europe, there have been 3,4 million new cases and 1.8 million patients who have died. Of the 1,8 million death cases, lung cancer had the highest rate (20%), followed by colorectal cancer (12,2 %), breast carcinoma (7,5%) and stomach cancer (6,1%).

These figures are disappointing and show that standard therapy is far away from having the disease under control. In the past, after repeated disappointments, the answers by medical oncologists have often been to increase the aggressiveness of treatment. This happened in surgery, radiotherapy and chemotherapy. The tumor was the enemy and the host organism had to tolerate the side effects. There was hardly any understanding of the biology of cancer and its metastases nor of the role which the immune system might play.

B. MOLECULAR BASIS FOR BIOLOGICAL THERAPIES

Hormone therapy is a biological/physiological way of treatment, but it can be applied only to a minority of cancers. It seems logical to go from therapies based on physics (RT) and chemistry (CT) to those based on biology. Biological therapies (BT) are characterized by a higher tumor specificity than RT and CT. That is why, in principle, such therapies have less side effects.

i) BIOLOGICAL THERAPIES

These therapies include:

a) Targeted therapies (TT): small molecules as signal-transduction inhibitors, antiangiogenic and vascular-disrupting agents, or as apoptosis modulators (Chapter III),

b) Immunotherapy with monoclonal antibodies (mabs) (Chapter IV),

c) T-cell mediated immunotherapies targeted to Tumor-associated antigens (TAAs) (Chapter IV),

d) Oncolytic Viruses (Chapter V).

Before describing those therapies, it is important to first explain the enormous progress that has been made in the last decades in cancer research. This is true for molecular biology, for the molecular biology of cancer, for cancer invasion and metastasis and for basic and tumor immunology. This is a prerequisite for understanding the rationale of Biological Therapy.

To identify a molecular target in a cancer cell that might be suited for a Targeted Therapy, it is necessary to identify its genetic basis at the level of a DNA sequence and its protein basis at the level of the amino-acid sequence.

This Chapter is primarily based on the excellent textbooks from Robert A Weinberg (The Biology of Cancer) (1) and from John Mendelson (The Molecular Biology Of Cancer) (2) which appeared in 2007 and 2008, respectively.

ii) MILESTONES FROM MOLECULAR BIOLOGY

The last more than 60 years have provided a number of revolutionary technologies in a research area termed molecular biology. Table 12 lists some of these innovations in the years from 1962 to 2000. It starts with the discovery of the double helix as basic structure of DNA and ends with the first complete sequence of the human genome. This is an arbitrary selection of discoveries from different disciplines such as: gene structure and function, protein structure and function, cytogenetics and cell biology.

Driving forces for these discoveries were new molecular technologies such as protein crystallization, DNA hybridization, use of restriction enzymes and ligases, cell cloning techniques, 2D gel electrophoresis, PCR, etc.

iii) MOLECULAR BIOLOGY OF CANCER

In the 19th century it was discovered that all cells of an organism descend from the fertilized egg. This led to the realization that tumors are not foreign to the body but represent growth derived from normal multicellular tissues. Tumors are classified into four major groups according to their origin (epithelial, mesenchymal, hematopoietic, and neuroectodermal). The most common human cancers are of epithelial origin – the carcinomas. There are two categories: squamous cell carcinomas arise from epithelia that form protective cell layers, while adenocarcinomas arise from secretory epithelia.

a) Carcinogens and tumor viruses

Biochemical and genetic markers revealed that human tumors are monoclonal, thus descend from one ancestral cell. In 1975, the Ames test provided support for the epidemiological studies implicating chemical and physical agents (tobacco, coal dust, X-rays) as causes of cancer. The test demonstrated that these agents acted as mutagens. Other agents were discovered which functioned as co-carcinogens or tumor promoters. These were found to promote tumorigenesis through nongenetic (epigenetic) mechanisms.

The DNA and RNA tumor viruses, characterized in the 1970s, provided cancer biologists with another theory of how human tumors could arise. They could be driven by viruses in addition to the effects of carcinogens and their mutagenic potential. Attemps during the 1970s to isolate viruses from human cancer were, however, mostly unsuccessful. Of the 100 and more tumor types encountered in the oncology clinic, only 2 commonly occurring tumor types in the Western world – cervical carcinomas and hepatomas (liver carcinomas) – could clearly be associated with specific viral causative agents.

b) Cellular oncogenes and tumor suppressor genes

The lack of success in identifying tumor viruses in the majority of human cancers left researchers with one main theory of how most human cancers might arise: that carcinogens act as mutagens and function by mutating normal growth-controlling genes into oncogenes.

Table 13 lists milestones in molecular biology of cancer. It demonstrates that growth-controlling genes have indeed been discovered:

1. oncogenes which stimulate growth

2. tumor suppressor genes which inhibit growth

c) Control of the cell cycle

To understand the functioning of oncogenes and tumor suppressor genes it is worth having a look at the cell cycle. The cell cycle is a precisely programmed series of events that enable a cell to duplicate its contents and to generate two daughter cells. It is conceptually divided into four individual phases: G1(Gap 1), S (synthesis), G2(Gap 2) and M (mitosis). Go or quiescence occurs when cells exit the cell cycle due to the absence of growth-promoting signals or the presence of pro-differentiation signals. The series of events from G1 to M are controlled by the machinery that is often termed the cell cycle clock. It seems to operate similarly in all cell types throughout the body.

One period in the cell cycle is particularly important, the Go/G1 transition phase. This is the one period in the life of an actively growing cell, in which the cell is given license to make decisions about its fate. Within the G1 period, a cell is responsive to mitogenic growth factors and to TGF-ß. At a certain point of G1, called the restriction point (R point), a critical decision is made. In most mammalian cells studied, the R point occurs several hours before the G1/S phase transition.

In the following, only a few additional facts will be mentioned. These are important for understanding the functioning of some products of oncogenes or suppressorgens. Cyclins and cyclin-dependent kinases (CDKs) constitute the core components of the cell cycle clock. Post-translational regulation (activation or inhibition) of CDKs occurs through phosphorylation. The decision concerning growth versus quiescence at the R point is governed by the state of phosphorylation of the tumor suppressor gene product retinoblastoma protein (Rb). D cyclins and cyclin E control the degree to which phosphorylated Rb (pRb) is phosphorylated. Hypophosphorylated pRb

blocks passage through the R point, while hyperphosphorylated pRb permits this passage (1,2).

d) Cellular senescence response

Normal cells that have the ability to proliferate (mitotic cells) can be induced to undergo cellular senescence by potentially oncogenic insults. These include DNA damage, dysfunctional telomeres, chromatin perturbations, and the expression of certain oncogenes. The senescence response requires normal functioning of the p53 or pRb tumor-suppressor pathways. This response permanently suppresses cell proliferation, implementing a postmitotic growth arrest.

Cells with mutant p53 or pRb are deficient in undergoing senescence. When faced with potentially oncogenic insults, such cells are at greatly increased risk for malignant transformation.

In cancer cells, a number of mechanisms operate to ensure that cell proliferation is not constrained by pRb. pRb function can be lost by excessive mitogenic signals since these lead to elevated levels of D cyclins, which then initiate pRb inactivation via phosphorylation. pRb can also become changed by mutation, by binding of a viral oncoprotein (e.g. HPV E7) or by the actions of cellular oncoproteins (e.g. Myc) (1,2).

e) Programmed cell death

Research on cell death is one of the fastest growing fields in cancer research. Programmed cell death (PCD) plays essential roles in maintaining the homeostasis (i.e. a physiological balance) in multicellular organisms. These ensure that individual tissues maintain their correct size and proper function. Also, most of the side effects of standard chemotherapy results from the induction of PCD in normally dividing tissues, such as the intestinal epithelium and bone marrow.

In the 1970s, three types of cell death pathways could be distinguished by electron microscopic analyses: apoptosis, autophagy and necrosis. The initiation of PCD is regulated either by cell-intrinsic or cell-extrinsic apoptotic pathways. The intrinsic pathway is activated by intracellular stress. This can be caused by damage to DNA or proteins caused by exposure to irradiation,

reactive oxygen species, or chemotherapeutic drugs. Also, hypoxia and virus infection can cause endoplasmatic reticulum (ER) stress. Extrinsic apoptotic pathways are triggered by ligands binding to the Fas family of death receptors. They can also be triggered by toxic proteins such as perforin and granzyme B released from cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. Intrinsic and extrinsic apoptotic pathways converge on using highly specific and conserved aspartate-specific cysteine proteases termed "caspases". These are the key executioners of the apoptotic response.

The intrinsic pathway is regulated by mitochondrial outer membrane permeabilization (MOMP) which results in the release of cytochrome c. The extrinsic pathway is initiated by the binding of a ligand (FasL) to the Fas receptor, which thereby is trimerized. This results in the recruitment of "Fas associated via death domain" (FADD). This in turn recruits and activates the initiator caspases -8 and -10. These then cleave and activate the effector caspases -3, -6, and -7 which then direct the destruction of the cell. Caspase substrates include cytoskeletal proteins, components of the nuclear membrane, chromatin and DNA (1,2).

In 2002, the Nobel Prize for Physiology or Medicine was granted to S Brenner, HR Horvitz and JE Sulston for their discoveries concerning genetic regulation of organ development and PCD. In 2016, the respective Nobel Prize was awarded to Y Ohsumi for the discovery of mechanisms of autophagy, by which cells cycle their content. Mutations in autophagy have been linked to diseases such as cancer and neurological disorders like Parkinson`s disease.

C. MILESTONES FROM METASTASIS RESEARCH

Tumors are divided into benign or malignant, depending on their aggressiveness. Invasion and metastasis of malignant cells is responsible for most of the failures of standard therapy. Metastases account for the majority of cancer-associated mortality. There is no cure for metastases, and at least 90% of all people dying from any type of cancer are dying due to cancer metastases.

In 1975, there were virtually no insights into the molecular alterations within human cells that lead to the appearance of malignant disease.

One generation later, we possess such knowledge in abundance. When I began my research at the German Cancer Research Center (DKFZ) in 1976 as Head of the Division of Cellular Immunology, I put much effort into establishing an animal model for the study of cancer metastasis. This could later be used to study immunotherapy of metastases. There was at that time hardly any information in the central library of this Institution about cancer metastasis. Some colleagues even questioned whether such complex phenomenon could be studied systematically at all.

Tables 14 and 15 list the development of various different concepts about the biology of cancer metastasis.

The first and most famous is the "Seed and Soil" hypothesis formulated by the pathologist Stephen Paget as early as 1889 (3). Based on examination of postmortem data from 753 patients with breast cancer, he noted that the organ distribution of metastases was nonrandom. He hypothesized that certain tumor cells, the seed, grew preferentially in the microenvironment of select organs, the soil. In a complementary hypothesis, James Ewing proposed in 1928 (4) that the primary factor that determined the patterns of tumor metastasis was the anatomy of vascular and lymphatic drainage from the site of the primary tumor (i.e. anatomical/mechanical hypothesis). For example, the liver is a common site for the hematogenous metastases for tumors arising in the gastrointestinal tract. This is due to the unique venous drainage that takes place through the portal venous system. Ewing`s theory accounts for the migration of prostate cancer cells to the lumbar vertebrae via Batson`s plexus of draining lymph nodes and Paget`s theory helps explain the organ specificity of prostate cancer metastases to bone.

Another widely accepted theory is the progression or clonal selection model proposed originally by P Nowell in 1976 (5). According to this theory, only a small fraction of the tumor cells acquire the metastatic phenotype. This occurs through a series of somatic mutations as a late event in the course of the tumor. The hypothesis was supported by experiments from IJ Fidler and M Kripke in 1977 (6). Using B16 mouse melanoma cells, they demonstrated that the metastatic capability of tumor cells sampled from pulmonary metastases was greater than that of cells from the primary tumor. The existence of cancer of unknown-primary site, however, argues against this

theory. In such patients, the metastases are present at the onset of clinical disease. There is no primary tumor with enough size and number of cells to achieve the required mutational events for the acquisition of the metastatic phenotype.

In 1980, I published a new hypothesis that contrasted considerably with the nearly dogmatic theory of my American colleagues of selection of pre-existing tumor cell variants. Instead, I proposed that signals from the microenvironment of a target organ with which disseminated tumor cells make contact can induce a shift in the tumor cell`s phenotype. Such shifts were proposed to be based on genetic re-programming (7). About 20 years later, such shifts were indeed described, namely for carcinoma cells. When these epithelial tumor cells loose contact to their underlying extra-cellular matrix (ECM) at the basement membrane of blood vessels, they can change their phenotype into mesenchymal-type cells. This process is called epithelial-mesenchymal transition (EMT). After travel through the blood and extravasation, such mesenchymal-type invasive tumor cells, upon contact with the organ microenvironment, can change their phenotype back again into an epithelial-like carcinoma. This process is called mesenchymal-epithelial transition (MET).

In 2002, Jean-Paul Thierry described EMT in detail (8). Interestingly, the cell-biological EMT program was found to involve transcription factors (TFs) which are normally used by cells early in embryogenesis and during wound healing. Signals released by the stromal microenvironment, operating together with genetic and epigenetic alterations of the cancer cell`s genome, are often responsible for inducing expression of EMT-inducing TFs in the cancer cell and thus the EMT.

The invasion-metastasis cascade became eventually distinguishable into different steps: local invasion, intravasation, transport, extravasation, formation of micrometastases and organ colonization. In 1984, my Canadian colleague R Kerbel forwarded another new perspective, namely that not only genetic but also epigenetic mechanisms may be at work during tumor progression and metastasis (9). This hypothesis was based on extensive experiments with mouse tumor lines subjected *in vitro* to treatment with the drug 5-aza-cytidine. This drug interferes with DNA methylation and can cause

de-repression of genes. In accordance with this theory, we had observed at that time period that immune escape variant cells could be generated *in vivo* with a high frequency from ESb lymphoma cells (10). Such tumor variants had lost expression of a specific tumor-associated antigen (TAA) that had been recognizable on the parental line by specific cytotoxic T lymphocytes (CTL). Upon *in vitro* treatment with 5-aza-cytidine, such immune escape variants were found to re-express the TAA (11). This suggested that epigenetic mechanisms may also be at work during tumor immune escape mechanisms, an important step in tumor progression.

Carcinogenesis has been described to occur in three basis steps: Initiation, promotion and progression. Promoting agents act via epigenetic mechanisms to alter gene expression. Typical skin tumor-promoting agents include phorbol esters like TPA, the phosphatase inhibitor okadaic acid, and the organic peroxide, benzoyl peroxide. Crucial to cancer progression of solid tumors are the processes of invasion and metastasis (1,2). These now also seemed to be influenced by epigenetic mechanisms.

This conclusion was later (2011) further supported by the studies of Fang et al (12). Groups of breast tumors were characterized by the presence or absence of coordinate hypermethylation at a large number of genes. This led to the identification of a breast CpG island methylator phenotype (B-CIMP). Presence or absence of B-CIMP loci was associated with low or high metastatic potential.

The sequence of steps in the metastatic cascade is completed only infrequently. The least efficient step appears to be organ colonization. The pathologist L Weiss formulated in 1990 the concept of metastatic inefficiency (13), based on quantitative calculations .

A role of chemokines in the process of cancer metastasis was proposed by PM Murphy at the beginning of the new millennium (14). J Wang et al in 2006 (15) described the pivotal role of the CXCL12 (SDF-1)/CXCR4 axis in bone metastasis as an important example.

In 2003 and 2011 IJ Fidler revisited the seed and soil hypothesis of S Paget. At these times he did not mention anymore the selection of pre-existing tumor cell variants but rather emphasized the role of tumor-stroma

interactions in metastasis to different organs. In this context, he examined clinically relevant examples, namely bone metastases, lung metastases, liver metastases and brain metastases (16,17).

2003 is a year with many new concepts relevant for the biology of cancer metastasis. For instance, CA Klein (18) addressed the problem of tumor dormancy in breast cancer and G Dontu et al (19) the importance of stem cells for the malignant switch. It is also the year in which molecular genetic aspects in cancer cells were demonstrated to be important. P Steeg (20) proposed that metastatic suppressor genes alter signal transduction of cancer cells and W and C Birchmeier (21) described the importance of the gene Met for cancer cell motility. Cell motility is found to be regulated by a series of small G proteins of the Rho family that are activated by cytoplasmic signal-transducing pathways and control the assembly of the actin cytoskeleton.

A genetic predisposition model was proposed in 2003 by Massagué`s group: Y Kang et al. (22) proposed a multigenic genetic predisposition model mediating breast cancer metastasis to bone. Breast cancer cells that overexpress CXCR4, PTHLH, IL11, MMP1 and OPN genes appeared to preferentially metastasize to bone. Later it was shown that breast cancer cells that overexpress COX, EREG and ANGPTL4 exhibit a tropism for lung (23) and breast cancer cells overexpressing ST6GALNAC5, COX2, HBEGF and ANGPTL4 were found to have a particular affinity for colonizing the central nervous sytem (24). Presumably, the gene patterns specific to each of these cell subpopulations may have been obtained through a series of somatic changes.

Genes of relevance for cancer metastasis could become introduced into the cell from outside sources. One such mechanism is cell fusion. JM Pawelek proposed in 2005 that tumor- host cell fusion could be a means by which myeloid traits are being transferred into cancer cells (25). This corroborates our findings from 1984, more than 20 years earlier (26).

A new concept developed in the years 2005 to 2017: That of the pre-metastatic and metastatic niche (27-30) . Associated with this is the understanding that metastasis is not random and chaotic but rather looks like an organized process involving cells, exosomes, micro-RNA (miRNA) and distinct proteins from the host in a coordinated fashion. This is new and leads

to a better understanding of the biology of metastasis. Further details will be presented in Chapters VII and VIII.

Of interest is also another new concept that was developed 2013 by KJ Pienta, RS Taichman and colleagues (31). This concept views and compares cancer metastasis with the diaspora context of ecology. Interesting therapeutic paradigms are based on network disruption. One application would be the use of traps to treat cancer outside their seed and soil system. For glioma cells a reservoir of neurotropic chemokines could be used to attract cancer cells to an area where they could be radiated. For prostate cancer cells, a reservoir of SDF-1 could be temporarily inserted intravenously that attracts the cells to a one-way trap. An ecological trap could also be constructed to expose cancer cells to cells of the immune system, leading to increased antigen presentation, or to disruption of the ability of metastasizing cells to recruit appropriate host cells.

Finally we like to take up the important topic of tumor dormancy or latency, a long time neglected area of cancer research. In 2016, S Malladi et al from Massagués group published in Cell (32) about metastatic latency and immune evasion. Latency competent cancer (LCC) cells were isolated from early stage human lung and breast carcinoma cell lines. They showed stem-cell-like characteristics and expressed SOX2 and SOX9 transcription factors. By actively silencing WNT signaling, LCC cells were shown to enter quiescence and evade innate immunity to remain latent for extended periods.

It is obvious from the development of different concepts and hypotheses during a time period of more than 100 years that metastasis is complex and not yet entirely understood. Cancer metastasis will be further delt with in Chapter VII under the aspect of physiological regulation and cancer associated dysregulation. Chapter VIII will discuss potential new targets for therapeutic intervention.

D. THE METASTASIS RESEARCH SOCIETY

The Metastasis Research Society (MRS) was founded in 1984 by the initiative of the pharmacologist Kurt Hellmann. He had studied since 1970 antimetastatic drug function (33) and discovered the normalization of tumor blood vessels by the drug ICRF 159 (34).

The mission of the MRS is to support research on processes fundamental to metastases. This includes supporting the exchange of information between researchers, clinicians, industry and patients at regular two-year Conferences. MRS members have estimated that only less than 5% of cancer research funds worldwide go to studying metastatic disease. The MRS feels that this is not appropriate because it is metastasis that makes cancer lethal.

The official Journals of the Society are "Clinical and Experimental Metastasis" and "Cancer and Metastasis Reviews".

Table 16 gives an overview of the 17 MRS Conferences held between 1984 and 2016. It is an international endeaver with many engaged people who did pioneering work. The topic of the 2017 Conference in Berlin (Germany) in November is the following: "Seed and soil: In vivo models of metastasis".

Chapter II

Key points:

1. **The second half of the 20th century witnessed a steady progress in molecular and cell biological research which had a great impact on cancer research.**

2. **Environmental carcinogens were identified as mutagens, tumor promoters as co-carcinogens and tumor viruses led to the discovery of oncogens.**

3. **New molecular technologies allowed to study gene structure and function and also protein structure and function, prerequisites for unraveling the function of oncogens and tumor suppressor genes.**

4. **The latter growth controlling genes interfere with the cell cycle, a complex machinery termed the cell cycle clock.**

5. **Progress was also made in understanding the biology of cancer metastasis. The bianual Conferences of the Metastasis Research Society provide a continuous forum for exchange of information, new concepts and ideas.**

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Table 12 Milestones from molecular biology

1962	**F Crick*, J Watson* and M Wilkins* Discovery of the DNA double helix**
1968	**MF Perutz* Crystal structure of hemoglobin**
1969	**JR Gall and ML Pardue In-situ-hybridization of DNA-DNA and RNA-DNA**
1972	**First recombinant DNA molecule constructed (restriction enzymes and ligases)**
1970	**KW Choi and AD Bloom Cloning of human lymphocytes**
1973	**JD Rowley Philadelphia chromosome as result of a reciprocal translocation**
1975	**High resolution 2D gel electrophoresis of proteins**
1977	**R Roberts First description of splice mechanisms**
1980	**D Metcalf and AW Burgess Cloning of hematopoietic cells in soft agar**
1980	**AH Wyllie Characterization of apoptosis by DNA ladder**
1981	**T Cech Description of self-splicing catalytic RNA**
1982	**Insulin as the first drug produced by gene technology**
1987	**First description of PCR for DNA amplification**
1989	**M Cappechi, MJ Evans and O Smithies First gene knock-out mouse strain**
1989	**PH Krammer Discovery of CD95/APO-1/Fas as the first cell death receptor**
2000	**First complete sequence of the human genome**

*** Nobel Laureat**

Table 13 Milestones in molecular biology of cancer

- discovery of viral oncogenes and of cellular proto-oncogenes

- an oncoprotein that functions like a growth factor (v-Sis of *Simian Sarcoma Virus* functioning similar to PDGF)

- discovery of the tumor suppressor gene (TSG) Rb

- importance of cellular signaling upon development of cell-to-cell communication during evolution of metazoans

- deregulation of such signaling central to the formation of cancer

- the Src oncoprotein functioning as a protein kinase: attachment of phosphates to tyrosine residues of proteins

- tyrosine phosphorylation primarily used by mitogenic signaling pathways

- the cell cycle clock having a restriction point (R); phosphorylated Rb (pRb) controlling passage through the R point

- multistep tumorigenesis: a complex process reflected in the long time periods required for most human cancers to develop; changes involving the activation of oncogenes and the inactivation of TSGs

- critical contribution of telomerase to tumorigenesis

- genetic regulation of organ development and programmed cell death (PCD)

- discovery of cancer stem cells: objects of genetic alteration and clonal selection

- mechanistic studies of DNA repair

Table 14 Concepts about the biology of cancer metastasis: Part I

1889	**S Paget**	**The seed and soil hypothesis**
1928	**J Ewing**	**The anatomical/mechanical hypothesis**
1976	**PC Nowell**	**The clonal selection hypothesis of tumor progression**
1977	**IJ Fidler**	**The hypothesis of selection of pre-existing variants from the primary**
1980	**V Schirrmacher**	**The hypothesis that signals from the microenvironment induce shifts in tumor cell phenotypes**
1984	**RS Kerbel**	**The concept of epigenetic mechanisms in tumor progression**
1990	**L Weiss**	**The concept of metastatic inefficiency**
2001	**PM Murphy**	**The concept of chemokines and the molecular basis of metastasis**
2002	**JP Thierry**	**The concept of EMT transitions in metastasis;**
2003	**IJ Fidler**	**The concept of tumor-stroma interactions**
2003	**CA Klein**	**The concept of early tumor cell dissemination and tumor dormancy**
2003	**G Dontu**	**The concept that a malignant switch may start in stem cells**

Table 15 Concepts about the biology of cancer metastasis: Part II

2003	**PS Steeg**	**Metastasis suppressors alter signal transduction of cancer cells**
2003	**W and C Birchmeier**	**Met, metastasis, motility and more**
2003	**J Massagué**	**A multigenic program mediating breast cancer metastasis to bone; A genetic predisposition model**
2005	**JM Pawelek**	**Tumour - myeloid cell fusion as a source of myeloid traits in cancer**
2005	**RN Kaplan**	**The concept of the pre-metastatic niche**
2010	**T Guise**	**The concept of the metastatic niche**

2010-17 Metastasis as an organized process: communication between seed and soil

2013 KJ Pienta The cancer diaspora: Metastasis beyond the seed and soil hypothesis

2016 S Malladi Metastatic latency and immune evasion through autocrine Inhibition of WNT

Table 16 Metastasis Research Society (MRS)

1984	**Foundation in London (UK)**	
	1. MRS Conference in London (UK)	**President K. Hellmann**
1986	**2. MRS Conference in Tieste (Italy)**	**President T. Giraldi**
1988	**3. MRS Conference in Heidelberg (Germany)**	**President V. Schirrmacher**
1990	**4. MRS Conference in Bethesda (USA)**	**President G. Nicolson**
1992	**5. MRS Conference in Paris (France)**	**President M. France-Poupon**
1994	**6. MRS Conference in Washington DC (USA)**	**President L. Liotta**
1996	**7. MRS Conference in Ghent (Belgium)**	**President M. Mareel**
1998	**8. MRS Conference in San Diego (USA)**	**President W. Stetler-Stevenson**
2000	**9. MRS Conference in London (UK)**	**President S. Eccles**
2002	**10. MRS Conference in Chicago (USA)**	**President A. Raz**
2004	**11. MRS Conference in Genoa (Italy)**	**President A. Albini**
2006	**12. MRS Conference in Tokushima (Japan)**	**President S. Sone**
2008	**13. MRS Conference in Vancouver (Canada)**	**President D. Welch**
2010	**14. MRS Conference in Philadelphia (USA),**	**President P. Steeg**
2012	**15. MRS Conference in Brisbane (Australia)**	**President E. Thompson**
2014	**16. MRS Conference in Heidelberg (Germany)**	**President J. Sleeman**
2016	**17. MRS Conference in Chengdu (China)**	**President Y. Kang**

CHAPTER III. TARGETED THERAPIES WITH SMALL MOLECULE INHIBITORS

The advances of the past 60 years described in Chapter II in molecular- and tumor- biology have led to the identification of key molecular pathways that control tumor progression. Characteristic alterations of neoplastic cells were found which include specific translocations, activating mutations or gene amplifications. These discoveries enabled academic research institutions and the pharmaceutical industry to develop new anticancer agents targeting specific molecules considered to be of importance: signal-transduction inhibitors, anti-angiogenic and vascular-disrupting agents or apoptosis modulators.

Tumor directed monoclonal antibodies (mabs) are often added to targeted therapies. Here, these will be delt with in a separate Chapter (Chapter IV, Immunotherapy). Mabs are classical molecules of the immune system and their mode of action is quite different from that of small molecule inhibitors. Therapeutic mabs target specific antigens found at the tumor cell surface (e.g., transmembrane receptors) or extracellular growth factors. In contrast, small molecule inhibitors can penetrate the cell membrane to interact with targets inside the cell. Small molecule inhibitors are usually designed to interfere with the enzymatic activity of the target protein, for instance a tyrosine kinase.

This Chapter is based primarily on the excellent textbook "Targeted Therapies in Oncology" (1).

A. SIGNAL TRANSDUCTION BY GROWTH FACTOR RECEPTORS

Before going into details about small molecule inhibitors, it seems worthwile to summarize basic principles in cells of signal transduction as described in two excellent textbooks (2,3).

The vital clues about oncoprotein functioning came from studies of normal cells and how they regulate their growth and division. Normal cells receive growth-stimulatory signals from their surroundings. These signals are

processed and integrated by complex circuits within the cell. Eventually, a decision is made whether cell growth and division is appropriate or whether the cell should remain quiescent or become senescent (2). Proper tissue architecture depends absolutely on maintaining appropriate proportions of different constituent cell types within a tissue, on the replacement of missing cells, and on discarding extra, unneeded cells.

Signal transduction from the outside of the cell to the DNA of the cell nucleus consists of the following components and devices:

i) ligands,

ii) cell surface receptors,

iii) adapters and enzymes,

iv) signaling cascades and

v) transcription factors.

These signaling processes are part of the larger problem of cell-to-cell communication. Cell-to-cell communication needed to be addressed and solved at the time when the first multicellular animals (metazoan) arose 600 to 700 million years ago. Wounds must be repaired, and attacks by foreign infectious agents must be warded off through the concerted actions of many cells within tissues. This is why cells in a living tissue are constantly communicating. Growth factors are part of this communication network. These are relatively small proteins that are released by some cells, make their way through intercellular space, and eventually impinge on yet other cells, carrying with them specific biological messages (2,3).

One example from wound healing may illustrate this. While the platelets in a wound site are in the process of aggregating as part of the clot formation, they also iniate the wound-healing process. They do this by the release of growth factors, notably platelet-derived growth factor (PDGF). This is a potent stimulator of fibroblasts, which form the connective tissue including the cell layers beneath epithelia. PDGF attracts fibroblasts into the wound site and then, as a mitogen, stimulates their proliferation. This example

mirrors hundreds of similar cell-to-cell communication routes that operate within living tissues to encourage or discourage cell proliferation (2).

Initiation of signal transduction through the interaction of an extracellular ligand with a specific receptor can take several forms. Steroid hormones readily cross cellular membranes and can therefore bind directly to intracellular nuclear hormone receptors, which regulate gene expression. Polypeptide growth factors and cytokines, in contrast, bind the extracellular regions of transmembrane receptors with intrinsic or associated tyrosine kinase activity.

The normal versions of oncogene-encoded proteins often serve as components of the machinery that enables cells to receive and process biochemical signals regulating cell proliferation. The first clues as to how cell-to-cell signaling via growth factors operates came from biochemical analysis of the *v-src* oncogene of *Rouse sarcoma virus* (RSV) and the protein Sarc (Src) that it specifies. Src was found to operate as a protein kinase, an enzyme that removes a high-energy phosphate group from ATP and transfers it to a suitable protein substrate (4). Src was found to be quite different from all other protein kinases that had been known. While the latter attach phosphate groups to the side chains of serine and threonine amino acid residues, Src phosphorylated distinct tyrosine residues of its protein substrates (5). More than 99% of the phosphor-amino acids in normal cells are phosphor-threonine or phosphor-serine. Phosphor-tyrosine constitutes as little as 0.05 to 0.1 % of the cell`s total phosphor-amino acids (2).

Another oncogene*, erbB*, had been discovered in the genome of *Avian erythroblastosis virus* (AEV), a transforming retrovirus that rapidly induces a leukemia of the red blood cell precursors (erythroleukemia). In 1984 it was discovered that the oncogene product ErbB had sequence homology with the epidermal growth factor (EGF) receptor (EGF-R). It was a sensation because now two areas of cell biology became united: A cellular growth factor receptor gene could have been hijacked by a virus to become an oncogene. In 1983 it was found that the amino acid sequence of PDGF was closely related to the oncoprotein Sis encoded by the *v-sis* oncogene of *Simian sarcoma virus* (SSV). Thus, a cellular gene for a growth factor could also have been hijacked by a virus to become an oncogene.

Another example is *Friend leukemia virus* (FLV), whose *gp55 env gene* was found to act as a mimic of the growth factor erythropoietin (EPO). Normally, when oxygen tension in the blood is less than normal, EPO is released from the kidneys and binds to the EPO receptors displayed by cells in the bone marrow that are immediate precursors of erythroblasts. This stimulates them to increase in numbers and to differentiate into erythrocytes (2).

It became more and more clear that growth factors and tyrosine kinase receptors (TK-Rs) are often involved in tumor pathogenesis. Examples are PDGF/PDGF-R , EGF/EGF-R, FGF/FGF-R, HGF(SF)/Met, VEGF/VEGF-R, IGF/IGF-R1 and SCF/Kit (2,3).

The binding of ligand to a TK-R induces its dimerization. This results in transphosphorylation of tyrosine residues located in the cytoplasmic domain of a receptor outside the immediate kinase domain. This phosphorylation causes selective binding sites for the SH2 domains of intracellular targets, including phosphatidyl-inositol-3-kinase (PI3K) and to activation of downstream signaling pathways (2,3,6).

The pleiotropic actions of a protein kinase usually derive from its ability to phosphorylate and thereby modify the functional state of a number of distinct substrate proteins. As an example serves here the Akt/PKB kinase, which is a serine/threonine kinase. This enzyme, by degrading ATP to ADP, can phosphorylate GSK-3ß, HIF-1α and Bad. This event inactivates the antiproliferative actions of GSK-3ß and the pro-apoptotic powers of Bad, and activates the angiogenic (blood vessel-inducing) powers of HIF-1α (5). Three events initiated by one enzyme which promote tumor growth.

B. RATIONAL BASIS OF A TARGETED DRUG EXEMPLIFIED WITH GLEEVEC

The development of molecularly targeted drugs has a rational basis. The first step consists of selecting the target. Defective proteins in cancer cells are attractive targets for drug development. Functional considerations, however, dictate that only a subset of defective proteins are attractive. Their biochemistry is of importance. Pharmaceutical chemists generate and explore a wide array of potential drugs. Drug candidates must then be tested on cell models as an initial measurement of their utility in whole organisms.

Pre-clinical tests involve studies of the drug`s action in laboratory animals. Thereafter, promising candidate drugs are subjected to rigorous and extensive clinical Phase I trials in humans. Phase II and III trials are finally necessary to provide credible indications of clinical efficacy (1-3).

Table 17 summarizes the long way to the first molecularly targeted cancer therapy.

The Bcr-Abl oncoprotein was discovered and validated as an attractive target and finally used as an object of rational drug design. This particular story begins in 1914, when the German cytologist T Boveri proposed that chromosomal defects might cause a cell to proliferate abnormally, resulting in the formation of some kind of tumor (7). 56 years later, in 1960, two cytologists working in Philadelphia noted that an abnormal, unusually small chromosome (22q-) was characteristically present in the great majority of cells of chronic myelogenous leukemia (CML). Since that time, 22q- had been called the Philadelphia chromosome (Ph). Another 12 years later, a researcher in Chicago demonstrated that a reciprocal translocation between chromosomes 9 and 22 was responsible for creating the Ph chromosome. We now know that this particular translocation is present in more than 95% of cases of CML.

In 1982, molecular biologists discovered that the gene *abl*, the human homolog of the mouse *c-abl proto-oncogene*, participates directly in these chromosomal translocations. The q34 region of chromosome 9 carrying most of the *abl* gene is transferred to the q11 region of chromosome 22, replacing a larger segment of chromosome 22 that is translocated reciprocally to chromosome 9, making it to 9q+. The net result consists in a fusion of the 5`-portion of the *abl* gene with a 3`-proximal portion of a gene termed "*breakpoint cluster region (BCR)*" which normally resides at 22q11. Depending on the precise location of the breakpoint in BCR, three distinct Bcr-Abl fusion proteins may be formed which are found in acute lymphoblastic leukemia (ALL), CML and chronic neurophilic leukemia (CNL).

Within two years of its discovery, the Bcr-Abl fusion protein was found to function as a constitutively activated tyrosine kinase. It functions in this respect like the Abl oncoprotein of *Abelson mouse leukemia virus*. In the early 1990s, a research program was begun to develop low-molecular weight

antagonists of the Bcr-Abl tyrosine kinase. A drug emerged, imatinib mesylate (Gleevec) which was able to bind the catalytic cleft within the ATP binding pocket of the Bcr-Abl tyrosine kinase. This drug, when used at therapeutic concentrations, appears to target only 4 of the 90 or so human tyrosine kinases.

In 1996, Gleevec had been found to inhibit the growth of CML cells *in vitro* while having no effect on normal bone marrow cells. The initial clinical trials, begun in 1998, revealed remissions from disease in all of the 31 treated CML patients, with only minimal side effects registered, even when taken daily for many years. Four years later, 6000 patients had already been entered into Gleevec clinical trials. Treatment of early-stage CML led to a hematological response in 90% of cases: PCR analysis revealed an extraordinary decline in the levels of Bcr-Abl mRNA in blood cells. About 60% of the patients who had already progressed to blast crisis responded to Gleevec, but they generally relapsed after a period of some months.

Gleevec was shown to have the capacity to inhibit also two other kinases: that of platelet-derived growth factor receptor (PDGFR) and that of KIT, which is the receptor for stem cell factor (SCF). Patients suffering from another myeloproliferative disease (hyper-eosinophilic syndrome) also showed a complete response to Gleevec. Furthermore, almost 70% of patients suffering from gastrointestinal stromal cancers (GISTs) responded with clear regressions of their tumors (8).

The successful development of the small molecule inhibitor (SMI) Gleevec paved the way for the development of many other highly targeted compounds (9). Such SMIs include EGF receptor antagonists for treating a wide variety of tumor types, proteasome inhibitors and inhibitors of mTOR, a master regulator of cell physiology.

With regard to the names of therapeutic targeting small molecules, the following formula might be helpful: Name = prefix + substem + stem. The stem –ib stands for small molecule with inhibitory properties. The substem -tinib stands for tyrosine kinase inhibitor. The substem -zomib stands for proteasome inhibitor. The substem -ciclib stands for cyclin-dependent kinase inhibitor and the substem -parib for poly ADP-ribose polymerase inhibitor.

Further information about targeted cancer therapies can be obtained from Blay et al. (9).

C. SIGNALING PATHWAYS AS TARGETS FOR SMALL MOLECULE INHIBITORS

Signal transduction inhibitors block the activities of molecules that participate in signal transduction, the process by which a cell responds to signals from its environment. During this process, the signal is relayed within the cell through a series of biochemical reactions that ultimately produce the appropriate response of the cell. In some cancers, the malignant cells are stimulated from within the cell to divide continuously without being prompted to do so by external growth factors. Signal transduction inhibitors interfere with this inappropriate signaling.

Table 18 lists 5 important targeted pathways for SMIs.

i) PATHWAY PI3K-AKT-mTOR

The "phosphatidyl-inositol 3-kinase" (PI3K) – "Akt serine/threonine kinase" (AKT) – "mammalian target of rapamycin complex" (mTORC) signal axis (PI3K-AKT-mTOR) is critically important for normal and cancerous cell functions (10). The pathway is activated by cell surface receptor stimulation and sends signals to downstream effector molecules that control cell cycle proliferation, growth, survival, protein synthesis, and glucose metabolism. Aberrant activation of the pathway is one of the most frequent occurrences in human cancer and plays an important role in multiple aspects of tumorigenesis (1).

The tumor suppressor gene product "phosphatase and tensin homolog" (PTEN) acts as a lipid phosphatase that regulates major signal transduction pathways and effectively inhibits PI3K-mediated signaling. Genetic mutations of PTEN with functional link to mTOR signaling have been described for cancer of prostate, breast, lung, bladder, kidney, ovary, endometrium, thyroid, brain and for melanoma (1).

mTor, a serine-threonine kinase, is a major biological switch, coordinating an adequate response to changes in energy uptake (amino acids, glucose), growth signals (hormones, growth factors) and environmental stress. mTor kinase is highly conserved through evolution from yeast to man and controls

autophagy. mTor hyperactivation has been detected in several human cancers. mTor exists in two different complexes in cells, mTorC1 and mTorC2 which could both be targeted by potential anticancer agents. Rapamycin is a selective and allosteric inhibitor of mTor1 but not of mTor2. ATP-competitive inhibitors of mTor which act on both complexes, such as OSI-027, could result in a better biological response and have entered clinical trials (11).

In cells, rapamycin and analogs such as everolimus suppress geroconversion of cells from quiescence to senescence. Dual mTorC1/C2 inhibitors such as everolimus or AZD8055 were superior to rapamycin in suppressing hypertrophy, senescent morphology, Oil Red O staining and increasing so-called "chronological life span (CLS)". It was suggested that at doses lower than anti-cancer concentrations, pan-mTor inhibitors can be developed as anti-aging drugs (12).

mTor is constitutively activated in head and neck aquamous cell carcinoma (HNSCC). The pan-mTor inhibitor AZD8055 induced *in vitro* dramatic cell death in Hep-2 HNSCC cells through autophagy and increased *in vivo* the survival of Hep-2 transplanted mice through a significant reduction of tumor growth, without apparent toxicity. Its anti-tumor activity was more potent than that of rapamycin (13).

AZD8055 showed excellent selectivity (approximately 1,000-fold) against all class I PI3K isoforms and other members of the PI3K-like kinase family. There was no significant activity against a panel of other 260 kinases (14). The drug is in Phase I clinical trials.

Another pan-mTor inhibitor, temsirolimus, was found to target multiple hallmarks of cancer (15) and to impede growth of murine mesothelioma *in vivo*. It stimulated tumor cell apoptosis, inhibited tumor angiogenesis, enhanced tumor lymphocyte abundance and blocked pro-tumor myeloid cell recruitment (16).

Hepatocellular carcinoma (HCC) is one of the most common lethal human malignancies worldwide. Its advanced status is frequently resistant to conventional chemotherapeutic agents and radiation. The dual PI3K/mTor inhibitor NVP-BGT226 was shown to have a potent effect on HCC cell lines. This was true *in vitro* under normoxic and hypoxic conditions (17).

ii) PATHWAY BRAF AND MEK

The "mitogen-activated protein kinase" (MAPK) pathway is an intracellular signaling pathway regulating cell cycle and other cellular functions. This pathway is commonly aberrant in human tumors. Its activated kinase cascade drives a serial phosphorylation of the MEK and ERK kinases that leads to cell proliferation and survival. With respect to cell proliferation, ERK1/2 is specifically important in the expression of cyclin D1 to promote progression through the G1 phase of the cell cycle.

A paradigmatic example of this activation is melanoma, where deregulation of the MAPK pathway is evident in over 90% of the cases. In about 50% of cases, this is due to the $BRAF^{V600}$ mutation. This mutation is present in approximately 7% of all cancers in general, which makes them the most prevalent single-nucleodide point mutation in a protein kinase in cancer (1).

Vemurafenib and dafratenib selectively bind to the ATP-binding site of BRAF-V600E kinase. Trametinib is a small molecule inhibitor of mitogen-activated extracellular signal-regulated kinase (MEK). Interestingly, trametinib was shown to be able to modulate cancer multidrug resistance by targeting the ABCB1 transporter (18).

Inhibition of BRAF with vemurafenib was reported to improve survival in patients with the most common BRAF(V600E) mutation and in patients with the less common BRAF(V600K) mutation (19). Dabrafenib, similar to vemurafenib, showed superior clinical outcome when compared to dacarbazine in patients with BRAF(V600E)-positive advanced melanoma (20). Combining dabrafenib with the MEK inhibitor trametinib further improved overall survival in this population of melanoma patients (21,22).

iii) PATHWAY KIT

The identification of specific genetic alterations in the KIT gene (translocations, deletions, point mutations, amplifications) enables to distinguish specific groups within tumor types (gastrointestinal stromal tumor (GIST), melanoma, thymic carcinoma). The nature of these driver mutations in tyrosine kinases enables to guide the administration of inhibitors of KIT, such as imatinib, sunitinib and others in a clinical setting. The KIT protein was

among the first described as activated through gene mutations and, as a driver, in specific tumor types in humans (1).

Some human cancers produce as many as three distinct growth factors (e.g. tumor growth factor α (TGF-α), stem cell factor (SCF), insulin-like growth factor (IGF)) and at the same time express the receptors for these ligands, thereby establishing three autocrine signaling loops simultaneously. In one study of small cell lung cancer (SCLC) patients, those whose tumors expressed Kit, the receptor for SCF, survived for an average of only 71 days after diagnosis while those whose tumors lacked Kit expression survived on average 288 days (2).

Table 18 lists among the KIT SMIs imatinib which was the first successful SMI. Its long way of development has been described above under B. Other similar drugs developed thereafter are also listed.

iv) PATHWAY ALK

Anaplastic lymphoma kinase (ALK) was originally identified in 1994 as a tyrosine kinase activated by chromosomal translocation in an uncommon T cell lymphoma called anaplastic large-cell lymphoma. The translocation which occurred in this disease with a frequency of 50-75%, resulted in the fusion gene product NPM1-ALK. Physiological activation of ALK occurs through binding of membrane-bound ALK with its putative ligands midkine or pleiotropin. This results in homodimerization and activation of the ALK kinase by transphosphorylation.

In certain tumor cells, ALK gene rearrangements result in a fusion protein that is aberrantly expressed and subject to ligand-independent dimerization and constitutive activation of ALK. Also, mutations in the ALK kinase domain result in constitutive activation of the ALK kinase activity (1). In these situations, ALK apparently functions as an oncogene. This seems true for tumor types, such as non-small cell lung cancer (NSCLC), non-Hodgkin`s lymphoma or neuroblastoma (23). The downstream effectors of ALK include the Ras/MAPK/ERK, PI3K/AKT, and JAK3/STAT3 pathways which play important roles in cell survival and proliferation (1-3).

The targeting of ALK in lung cancer by crizotinib has been reviewed recently (24). The review starts with the discovery of the EML4-ALK fusion oncogene

and culminates in the recent validation of ALK as a therapeutic target in patients with ALK-rearranged NSCLC.

The emergence of ALK as a new therapeutic target in NSCLC and beyond has been one of the success stories of modern oncology. Spectacular has been the short timeline from the identification of ALK gene rearrangements in NSCLC (2007) to FDA approval of crizotinib for this indication (2011).

However, there are a number of challenges that scientists, clinicians, and patients alike will face in the years to come. Elucidating mechanisms of acquired resistance to ALK inhibitors and developing strategies that may overcome these will be of paramount importance to achieve long-term disease control in respective targeted patients (1).

v) PATHWAY MET

MET was discovered in 1984 (25) and subsequently found to be a RTK located at chromosome 7q21-q31 (26). MET and its associated physiological ligand hepatocyte growth factor/scatter factor (HGF/SF) have become attractive targets in several types of cancer. HGF/SF, the sole ligand of MET, is secreted by mesenchymal cells, particularly fibroblasts and smooth muscle cells (27), but it can also be secreted by tumor cells. In normal cells, HGF-induced MET activation is under tight regulation by paracrine ligand delivery.

MET overexpression correlates with poor prognosis in several solid tumors (28). Activation of the MET pathway plays a primary role in cancer cell survival, growth and migration (29,30). MET in association with translocated-promoter region (TPR) was found to be a potent oncogene in the early 1990s. Mechanisms of MET activation include i) binding to its ligand HGF with associated paracrine/autocrine activation, ii) activating mutations, including those causing constitutive kinase activity, iii) MET gene overexpression/amplification and iv) decreased degradation (1).

MET pathway inhibitors include zivatinib, cabozantinib, crizotinib and foretinib (Table 18). Tivatinib induces G2/M arrest and apoptosis by disrupting tubulin polymerization in hepatocellular carcinoma (31). It affects the apoptotic and proliferative machinery downstream of c-MET (32). The multiple tyrosine kinase (MET, RET, VEGFR2) inhibitor cabozantinib has been approved in the USA for treatment of patients with advanced renal cell

carcinoma who have received prior antiangiogenic therapy (33). Foretinib is a potent inhibitor of oncogenic ROS1 fusion proteins that may be useful in cases of resistance to crizotinib (34). It blocks proliferation, induces anoikis, and impairs ovarian cancer metastasis (35).

The epidermal growth factor receptor (HER) family of tyrosine kinase (TK) receptors will be dealt with under mabs in the next chapter. Other signaling pathways targeted by SMIs exist as well but can not be discussed here. SMIs have been developed to inhibit the Fibroblast Growth Factor Receptor (FGFR) pathway, the Apoptosis pathways or the Androgen pathways. Other SMIs target stem cells, histone deacetylase, DNA repair, or mitosis. Angiogenesis inhibitors block the growth of new blood vessels to tumors. Such blood supply is necessary for tumors to grow beyond a certain size because blood provides oxygen and nutrients that tumors need for continued growth. Some targeted therapies that inhibit angiogenesis interfere with the action of vascular endothelial growth factor (VEGF), a substance that stimulates new blood vessel formation.

Targeted therapies imply in their simplest version a therapy with a specific molecular target. Any therapy that works must have a molecular target. In some cases, the target is discovered first, while in others (e.g. aspirin), the drug is discovered before the target. One of the best SMI drugs, imatinib, has more than one molecular target. A targeted therapy should attack a biologically important process (not necessarily a single molecule), one central to a hallmark of cancer (15). Such therapy should be applied to a selected targeted population of patients. There is immense potential for improving efficacy and diminishing toxicity through application of genomic, proteomic, and pharmacogenomics technologies.

D. EFFECTS AND SIDE EFFECTS OF SIMs

Strengths and weaknesses of Targeted Therapies in Oncology, based on (1), are summarized in Tables 19 and 20.

i) Anti-tumor activity of PI3K-AKT-mTor inhibitors has been modest, even in patients with tumors harboring mutations in this pathway. Cabozantinib, an oral inhibitor of Met, VEGFR, and AXL, was found superior to the mTor inhibitor everolimus in advanced renal cell carcinoma (36).

ii) With BRAF and MEK inhibitors there has been significant anti-tumor activity in metastatic melanoma.

iii) Tumors with KIT mutations on exon 9 or 11 were generally responsive to KIT inhibitors.

iv) There has been continuous progress in treating advanced ALK-positive NSCLC. Although crizotinib shrinks tumors in a large proportion of such patients, most experience a relapse within the first year of treatment. A next-generation ALK inhibitor, alectinib, has recently shown encouraging results in advanced NSCLC, including those with brain metastases (37). In an early-stage clinical trial, 48% of patients responded to this drug, with a median duration of response of 13,5 months.

v) MET inhibitors showed substantial promise in clinical trials.

vi) Midostaurin, a multi-kinase FLT3 inhibitor, was found to be effective in AML with FLT3 mutations when combined with chemotherapy (38).

vii) Palbociclib, a cyclin-dependent kinase 4 (CDK4) and CDK6 inhibitor was found effective in hormone resistant metastatic breast cancer (39).

In spite of the enormous efforts of pharmaceutical companies to develop these new types of therapeutics, there are a number of weaknesses and challenges that need to be solved. The challenges from the side of tumor cells include:

i) feedback loops and cross talk that can compensate for targeted inhibition,

(ii) selection for mutations leading to resistance and

iii) selection of kinase switch variants.

The challenges from the side of the tumor-bearing host include

i) normal tissue toxicity including the immune system and the blood clotting system,

ii) the appearance of cutaneous squamous carcinoma,

iii) identification of suitable patients,

iv) the potentiation of adverse events in case of combination with CT.

Table 21 lists possible side effects. The most frequent ones relate to the skin. In addition, there are common and serious side effects. Many side effects are similar to those of standard chemotherapeutic drugs. Thus, TTs have limitations and side effects.

Resistance can occur in at least two ways: the target itself can change through mutation so that the TT no longer interacts well with it, and/or the tumor finds a new pathway to achieve tumor growth that does not depend on the target. Perhaps, TTs work best in combination. A recent study revealed that using two drugs that target different parts of the cell signaling pathway that is altered in melanoma by the BRAF V600E mutation slowed the development of resistance and disease progression to a greater extent than using just one targeted therapy (22).

The idea to use multiple targets to avoid resistance development seems rational. Since SMIs have unwanted side-effects, the use of multiple SMIs would, however, also create multiple side-effects. It may be unpredictable in which way the drug effects will interact.

The situation is quite different with immunotherapy (Chapter IV) which has much less side effects. For instance, in immunotherapy, multiple tumor-associated antigens (TAAs) can be targeted at the same time without any problem.

Scientists had expected that TTs would be less toxic than traditional CT drugs because cancer cells are more dependent on the selected targets than are normal cells. However, as an enormous number of clinical studies has revealed, TTs with SMIs can have substantial side effects. The most common are diarrhea and liver problems, such as hepatitis and elevated liver enzymes.

Additional side effects are summarized in Table 21. Changes in the skin are rather frequent and not only found by drugs interfering with epithelial growth factor receptor (EGF) mediated signaling. It appears that signaling pathways are not that tumor-specific as one had thought or would have liked. Problems may be related not only to lack of tumor-specificity but also may have to do with delivery, dosing and timing.

In 2013, 40 drugs were dropped from the global oncology pipeline (40). 12 drugs failed in Phase III development. None of the pivotal trials incorporated

molecular biomarkers for patient stratification. The largest number of drug terminations (50%) occurred in Phase I development.

It is obvious from the many new TT drugs developed in recent years by the big pharma companies that there exists a competition to obtain part of the market "cake" of the health system. Generally, competition is good and can lead to the best possible solution. However, it may narrow at the same time the view for development of drugs which derive from other areas such as immunology.

Table 22 lists examples of cancer types for which the indicated SMIs have been approved by the FDA. The cancer types include carcinomas, sarcomas and lymphomas. Accelerated by the National Cancer Act of 1971 and then by a responsive research infrastructure and increasingly innovative regulatory environment, cancer research today delivers new treatments to patients faster then ever. In just one year`s time, the US FDA has approved 20 therapies for more than a dozen different types of cancer.

Chapter III

Key points:

1. Advances in molecular and tumor biology enabled the identification of key molecular pathways that control tumor progression.

2. Targeted therapies aim at blocking signal transduction through such pathways. Small molecule inhibitors (SMIs) serve this purpose and can be given orally.

3. Imatinib mesylate (Gleevec) is an example of a successful small molecule inhibitor that functions as an antagonist of the Bcr-Abl tyrosine kinase active in CML and other leukemias. Its development involved many steps and lasted for several decades.

4. A whole plethora of SMIs have been developed by pharma companies and were approved by FDA not only for the treatment of leukemias but also for the treatment of sarcomas and carcinomas.

5. There are still a number of weaknesses and challenges of SMIs that need to be solved in the future.

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Table 17 The long way to the first molecularly targeted therapy

1914	**T Boveri Discovery of chromosomal defects in cancer cells**
1960	**Discovery of the Philadelphia chromosome (22q-) characteristic for CML**
1972	**Discovery of the reciprocal chromosomal translocation 9/22 in 95% of cases of CML**
1982	**Discovery that the gene Abl participates in the 9/22 translocation by fusion with the gene Bcr at chromosome 22q11**
1984	**Discovery of the Bcr-Abl fusion protein and its functioning as a constitutively activated tyrosine kinase**
1990	**Start of a research program to develop low-molecular weight antagonists of the Bcr-Abl tyrosine kinase**
1996	**Identification of a drug emerged which was able to bind the catalytic cleft within the ATP binding pocket of the Bcr-Abl enzyme**
1998	**Positive results from testing this drug, imatinib mesylate (Gleevec) on CML cells in vitro and from initial clinical trials in CML patients**
2002	**Positive results from further 6000 CML patients in Gleevec clinical trials**

Table 18 Targeted therapy of signaling pathways with small molecule inhibitors (SMIs)

Targeted Pathway	examples
i) Pathway PI3K-AKT-mTor	buparlisib, pictilisib, idelalisib, BEZ 235, NVP-BGT226, XL765, GDC-0980, SF1 126, MK-2206, GSK690693, AZD8055, OSI-027, everolimus, temsirolimus
ii) Pathway BRAF and MEK	vemurafenib, dabrafenib, trametinib
iii) Pathway KIT	imatinib, sunitinib, nilotinib, masetinib, dasatinib, ponatinib, regorafenib, sorafenib, pazopanib, dovitinib, motesanib, valatinib
iv) Pathway ALK	crizotinib, HSP90 inhibitors
v) Pathway MET	tivatinib, cabozantinib, crizotinib, foretinib

Table 19 Targeted therapies with SMIs : strength and weaknesses

Part I

i) PI3K-AKT-mTor inhibitors: - anti-tumor activity modest, even in patients with tumors harboring mutations in PI3K pathway;

Challenges: - the pathway is complex; feedback loops and cross talk can compensate for targeted inhibition;

- biomarkers to select the patient population most likely to respond have not yet been identified;

- agents to date may be unable to sufficiently inhibit this pathway due to normal tissue toxicity;

ii) BRAF and MEK inhibitors: - significant anti-tumor activity in metastatic melanoma;

Challenges: - toxicities: skin changes, low-grade cuSCC[a], headache, nausea, fatigue, and vomiting;

- the appearance of cuSCC[a] demands a better understanding of the biology;

iii) KIT inhibitors: - tumors with KIT mutations on exon 9 or exon 11 are generally responsive to tyrosine kinase inhibitors (TKIs);

Challenges: - therapeutic pressure selects for mutations at other exons;

- antibodies to KIT may represent a future alternative;

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a = cutaneous squamous carcinoma

Table 20 Targeted therapies with SMIs : strength and weaknesses

Part II

iv) ALK inhibitors:	**- a new therapeutic target in NSCLC**
	- ALK gene rearrangements in NSCLC (2007)
	- FDA approval of crizotinib for NSCLC (2011)
	- FDA approval of alectinib for NSCLC (2015)
Challenges:	**- identification of suitable patients**
	- how to overcome acquired resistance to achieve long-term disease control
v) MET inhibitors:	**- substantial promise in clinical trials**
Challenges:	**- tumor cells may undergo a kinase switch when exposed for instance to erlotinib**
vi) FLT3 inhibitors	**- midostaurin, a multi-kinase inhibitor, effective in AML with FLT3 mutations in combination with chemotherapy**
Challenges:	**- side effects in immune system and blood clotting**
vii) CD4/6 inhibitors	**- palbociclib effective in hormone resistant metastatic breast cancer**
Challenges :	**- severe adverse events substantially higher than with hormone therapy**

Table 21 Possible side effects from targeted small molecule inhibitory drugs

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Skin:

- **Changes in how the skin feels**
- **Increase of photosensitivity**
- **Rash (scalp, face, neck, chest, upper back)**
- **Dry skin**
- **Itching**
- **Red, sore cuticles (the areas around the nails)**
- **Hand-foot syndrome (HFS), painful**
- **Changes in hair growth**
- **Changes in hair or skin color**
- **Changes in and around the eyes**

Common and serious side effects:

- **High blood pressure**
- **Bleeding or blood clotting problems**
- **Slow wound healing**
- **Heart damage**
- **Swelling**

Other side effects are similar to those of standard chemotherapy drugs

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Table 22 Examples of cancer types for which targeted small molecule inhibitors have been approved by the FDA

Brain cancer : everolimus (Afinitor)

Breast cancer: everolimus (Afinitor), tamoxifen (Nolvadex), toremifene (Fareston), fulvestran (Faslodex), anastozole (Arimidex), exemestane (Aromasin), lapatinib (Tykerb), letrozole (Femara), emtansine (Kadcyla)

Colorectal cancer: ziv-aflibercept (Zaltrap), regorafenib (Stivarga)

Gastrointestinal stromal tumor: imatinib mesylate (Gleevec), sunitinib (Sutent) regorafenib (Stivarga)

Kidney cancer: sorafenib (Nexavar), sunitinib (Sutent), pazopanib (Votrient),

Liver cancer: sorafenib (Nexavar), regorafenib (Stivarga)

Lung cancer: crizotinib (Xalkori), erlotinib (Tarceval), gefitinib (Iressa)

Pancreatic cancer: erlotinib (Tarceva), everolimus (Afinitor), sunitinib (Sutent)

Prostate cancer: cabazitaxel (Jevtana), enzalutamide (Xtandi), abiraterone acetate (Zytiga)

Skin cancer: vismodegib (Erivedge), sonidegib (Odomzo), vemurafenib (Zelboraf)

Leukemia: tretinoin (Vesanoid), imatinib mesylate (Gleevec), dasatinib (Sprycel)

Lymphoma: vorinostat (Zolinza), romidepsin (Istodax), bexarotene (Targretin)

Multiple Myeloma: bortezomib (Velcade), carfilzomib (Kyprolis), panobinostat (Farydak)

CHAPTER IV. IMMUNOTHERAPY

A. AUTO-BIOGRAPHICAL NOTES

With this Chapter we move from biochemistry to immunology. It may be appropriate at this step of transition to introduce myself. A short CV is shown in Table 23. In addition, this review includes several auto-biographical notes. These are separated from the main text as numbered BOXES. The first two boxes relate to my decision in 1962 to study biochemistry and in 1969 to do my PhD thesis in immunology. The text of the BOXES can be found in Chapter 10.

BOX 1 1962 Biochemistry

BOX 2 1969 Immunology

B. MILESTONES FROM IMMUNOLOGY

Chapter IV is based on a number of excellent textbooks in immunology, some of which have reached already the 6th to 12th Edition (1-6).

The German Association of Immunology (DGfI), founded in 1967, celebrates this year (2017) its 50th year of existence. As part of the International Union of Immunological Societies (IUIS), the DGfI organized in 1989 the 7th International Congress of Immunology in Berlin.

Immunology as a science has gone through a period of active development which was strongly influenced by new techniques from molecular biology. Vice versa, progress in molecular biology has also been influenced by new techniques from immunology. Immunological methods helped to purify proteins and to identify specific complementary DNA (cDNA) clones. Monoclonal antibody technology has helped to identify proteins and their location in cells, has transformed many fields of medicine and ranges even into fields from agriculture to the food science industry.

Before mentioning the various milestones in the field of immunology, it may be worth quoting some statements from 2017 of the "Annual Report on Progress Against Cancer From the American Society of Clinical Oncology" (ASCO) (7).

"A hundred years in the making, cancer immunotherapy is now a standard treatment option for people with a growing number of different cancers. In 2016 alone, the FDA approved immunotherapies for advanced forms of lung, kidney, bladder, and head and neck cancers, as well as Hodgkin lymphoma (HL). For some people with these advanced-stage cancers, the advent of immunotherapy is truly life changing. It often offers the only chance to live longer and better. And many believe that this first wave of success with cancer immunotherapy is just the beginning."

Another interesting quotation from this ASCO special article concerns cancer by the numbers: " The good news is that, for most people, a diagnosis of cancer is not as grim as it used to be. Today, 68% of adults and 81% of children with cancer will be alive at least 5 years after diagnosis. This is a big improvement from the 1970s, when only 50% of adults and 62% of children were surviving 5 years. "

"New approvals" by the FDA from November 1, 2015, to October 31, 2016 included three monoclonal antibodies (mabs) : Daratumumab , Necitumumab and Atezolizumab. "New uses" included 4 mabs: Nivolumab, Obinutuzumab, Pembrolizumab and Atezolizumab.

Since 2011, the FDA approved 15 immunotherapies in oncology. This led the ASCO to chose "Immunotherapy 2.0" as its cancer advance of the year. It is the second time in a row that immunotherapy was selected.

C. MONOCLONAL ANTIBODIES

This part of description of state-of-the art of immunotherapy relates to B cells and their antibody products. While the success of therapeutic mabs is encouraging, it is important to consider the historic development that made all this possible.

Table 24 lists milestones from immunology research relating to B cells and antibodies. It is a most prestigious list of Nobel Laureats.

The study of antibodies began in 1890 when E von Behring and S Kitasato described antibody activity against diphtheria and tetanus toxins. They proposed the theory of humoral immunity, suggesting that a mediator in serum (the liquid, noncellular component recovered from coagulated blood) could react with a foreign antigen. Their ideas influenced P Ehrlich who eventually formulated his side-chain theory for antibody and antigen interaction in 1897. He hypothesized that receptors ("side-chains") on the surface of cells could bind specifically to toxins – in a "lock-and-key" interaction – and that this binding reaction would trigger the production of antibodies.

In the 1920s, M Heidelberger and O Avery described that antigens could be precipitated by antibodies. The next major advance was in 1940, when L Pauling confirmed the lock-and-key theory and showed that the interactions between antibodies and antigens depend more on their shape then on their chemical composition. In the 1960s, G Edelman discovered that antibodies are composed of disulfide bond-linked heavy and light chains. Around the same time, RR Porter characterized different regions of immunoglobulin G (IgG), namely the antibody-binding region Fab and the antibody tail region Fc. Together, these scientists deduced the structure and complete amino acid sequence of IgG, for which they were jointly awarded in 1972 the Nobel Prize in Physiology or Medicine.

BOX 3 1972 Cellular Cytotoxicity

In 1984 a further Nobel Prize was awarded for antibodies, this time given to NK Jerne for immune regulatory theories and to G Köhler and C Milstein for the invention of the monoclonal antibody production technology.

In 1987, S Tonegawa obtained the Nobel Prize for providing evidence for somatic gene rearrangements of immunoglobulin genes coding for variable and constant regions. In 1976, Tonegawa had used Southern blot analysis of restriction enzyme digested DNA from lymphoid and nonlymphoid cells. He showed that the immunoglobulin (Ig) variable (*v)* and constant (*c*) genes are distant from each other in the germline genome. In contrast, in DNA from an

antibody-producing plasmacytoma cell the two genes localized closely together. They concluded that somatic gene rearrangement, a process entirely new at the time, must have been responsible for this phenomenon (8).

Today we know that the antibody repertoire of an individual is generated through somatic recombination events from a limited set of germline gene segments. The human *heavy chain v region gene* is generated by joining of *vH, D and J gene* segments. The *light chain v region gene* (κ or λ) is generated by joining of *vL and J gene segments*. Still further diversification of the antibody repertoire results from somatic mutation events targeted to the variable regions. Somatic mutation and selection by antigen allows for further affinity maturation of antibodies.

The potential heavy chain gene repertoire can be calculated from the equation: 50 VH x 27 DH x 6 JH = 8.1 x 10^3. The light chain gene repertoire consists of about 365 chain combinations. If we consider that each heavy chain protein could pair with each light chain protein, then the diversity of the Ig antibody protein repertoire is very large, in the order of 3x10^6 possible combinations.

Since this review addresses not only experts in the field but also lay people, some basic information will be given about the structure and function of antibodies. Antibodies are immunoglobulin proteins (Igs), secreted by B lymphocytes (B-cells) of the adaptive immune system, mostly by differentiated B-cells called plasma cells. An immunoglobulin G antibody is a Y-shaped protein consisting of heavy and light chains. Such antibodies are used by the immune system to neutralize pathogens such as bacteria and viruses. Each tip of the Y recognizes a unique structure of the harmful agent, called an antigen. The antigen-binding sites of an antibody (2 for IgG and 5 for immunoglobulin M (IgM)) can be considered as a lock for a fitting antigen as a key. The ability of an antibody to communicate with other components of the immune system is mediated via its tail (Fc) region. The production of antibodies is the main function of the humoral adaptive immune system.

With regard to function, the main categories of action of antibodies consist of

i) neutralization, to render an attack by bacteria or viruses ineffective,

ii) agglutination, in which antibodies glue together foreign cells into clumps that are attractive targets for phagocytosis,

iii) precipitation, by which antibodies glue together serum-soluble antigens, forcing them to precipitate out of solution in clumps that are attractive targets for phagocytosis and

iv) complement activation (fixation) which leads to lysis of foreign cells or inflammation by chemotactically attracted inflammatory cells.

Medical applications of mabs include disease diagnosis and disease therapy. For example, in biochemical assays for disease diagnosis, a titer of antibodies directed against Epstein-Barr virus (EBV) or Lyme disease is estimated from the blood. If those antibodies are not present, either the person is not infected or the infection occurred a very long time ago, and the B cells generating these specific antibodies have naturally decayed.

D. TARGETED MONOCLONAL ANTIBODY THERAPIES

This paragraph, like that on small molecule inhibitors, is based on (9).

Targeted monoclonal antibody (mab) therapy is employed to treat diseases such as rheumatoid arthritis, multiple sclerosis, psoriasis and many forms of cancer.

The formula for generic naming of mabs consists of the following:

Name = prefix + subsystem(s) + stem. The prefix is variable. The target of the subsystem is: -ci(r)- for circulary system, - li(m)- for immune system and –t(u)- for tumor. The stem is either -ximab for chimeric human-mouse, -zumab for humanized mouse or –mumab for fully human.

Therapeutic mabs target specific antigens found at the cell surface, such as transmembrane receptors. In some cases, mabs are conjugated to radio-isotopes or toxins to allow specific delivery of these cytotoxic agents to the targeted cancer cell.

i) COMPARISON BETWEEN ANTI-RECEPTOR MABs AND TK SMALL MOLECULE INHIBITORS

Table 25 shows characteristics of anti-receptor mabs and compares these to those of tyrosine kinase SMIs. While the mab targets a receptors extracellular ectodomain, small molecules target the receptors intra-cellular tyrosine kinase domain. The specificity of a mab is somewhat higher than that of a SMI. After binding of a mab to its target, the receptor is internalized and only slowly regenerated.

There is a great difference in pharmacokinetic properties: SMIs have lifetimes in the circulation that are often measured in hours to days, whereas mabs may persist for weeks in the circulation. As a consequence, SMIs have to be applied daily while mabs may be given once a week. The tissue distribution of SMIs is more complete than that of the larger mabs.

Mabs can interact with cells of the immune system via their Fc domain thus generating for instance a mechanism known as antibody-dependent cellular cytotoxicity (ADCC). SMIs lack such properties. With regard to toxicity, SMIs often produce rash, diarrhea and/or pulmonary problems, while mabs can produce rash and allergy.

ii) THE FIRST APPROVED MAB

Mabs against the epidermal growth factor receptor (EGFR) were the first immunotherapeutic reagents to be approved for application to cancer patients. EGFR signals appear to communicate with the oncogene *ras*. A test with an EGFR expressing cancer cell line *in vitro* revealed Ras protein activation within 5 minutes after addition of the ligand EGF (10).

Blocking mabs competitively inhibit the binding of an activating ligand (e.g. EGF or TGFα) to the extracellular domain of EGFR. Such blocking inhibits receptor autophosphorylation and, in contrast to the tyrosine kinase inhibitors (TKIs), induce receptor internalization and degradation. Subsequent downstream signaling events are similar to those described for the TKIs gefinitib and erlotinib.

Trastuzumab (Herceptin), the first approved mab, is directed against HER2 (Table 26). It has activity against HER2+ breast cancer and gastric cancer. HER2 positivity is defined as 3+ on conventional immunohistochemistry (IHC) or on gene amplification by fluorescence *in situ* hybridization (FISH).

The HER family is composed of four transmembrane tyrosine kinase (TK) receptors. They include ErbB1 (HER1), ErbB2 (Her2/neu or HER2), ErbB3 (HER3) and ErbB4 (HER4). The HER kinases have six known ligands: epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin, betacellulin, heparin-binding EGF, and epiregulin (11).

The overexpression of HER family kinases correlates with poor prognosis and decreased survival in several solid tumors (12). Moreover, tumors that overexpress these TKs often produce their own ligands, such as TGF-α, leading to the activation of survival pathways via autocrine loops.

There have been 5 randomized trials demonstrating the benefit of trastuzumab when added to chemotherapy in HER2+ breast cancer. A joint meta-analysis of two of these trials, including 4045 patients, demonstrated a 48% reduction in the risk of recurrence and a 39% reduction in the risk of death (13).

iii) EGF RECEPTOR ANTAGONISTS

Carcinomas are common epithelial-derived tumors and the EGFR is an interesting target not only for mabs but also for development of TKIs. The best-characterized inhibitors of the EGFR TK are the drugs iressa, also known as gefitinib, and tarceva, also known as erlotinib. The two drugs act by blocking the ATP-binding site of the EGF receptor-associated kinase.

In principle, these low-molecular weight compounds should be able to penetrate into all the interstices of a solid tumor, including those where the far larger antibody molecules may have trouble gaining access. In the first clinical trials, gefinitib showed partial responses in 10% of patients with Non-Small-Cell-Lung-Cancer (NSCLC) including stabilization of tumor growth. Later it was found that such responding patients had tumor cells with mutated EGFRs affecting their kinase domain. Unfortunately, virtually all of these successes have been short-lived. Most patients relapsed within 6 to 18 months, having developed a resistance to drug treatment (14).

iv) MABS DIRECTED AGAINST DISTINCT TUMOR TARGETS APPROVED FOR CANCER THERAPY

Table 26 lists examples of therapeutic mabs which have meanwhile been approved for distinct targets and types of cancer. With regard to the targets, there are the various growth factor receptors, such as those of the HER family (HER1 and HER2), the receptor for vascular endothelial growth factor (VEGFR2) and the receptor α chain for PDGF (PDGFRα). Then there is VEGF, the ligand of the growth factor receptor VEGFR. VEGF is neutralized by the anti-angiogenic agent Avastin (Bevacizumab).

A variety of targets are expressed at the cell surface of different types of tumors of the hematopoietic system, such as myelomas, B- or T-cell tumors : CD52, CD38, CD20 and SLAM7. RANKL is a target of giant cell tumors of the blood and GD2 is a target of pediatric neuroblastomas.

FDA-approved indications for therapeutic mabs include carcinomas (e.g. Her2+ breast and gastric cancer, HNSCC, NSCLL, CRC and Ovarian Ca), sarcomas, neuroblastoma, myeloma and B-or T-cell lymphoma.

v) IMMUNE CHECKPOINT INHIBITORY MABS

Undoubtedly the greatest success with clinical application of mabs in cancer patients has been in recent years the use of checkpoint inhibitory mabs (Table 27). This novel class of immunotherapy was first approved in 2011. These mabs are directed against targets of the immune system, in particular against regulatory target molecules on T-cells.

Immune checkpoint receptors are crucial molecules for the fine-tuning of immune responses (15,16). Checkpoint receptors on T cells such as CTLA-4 or PD-1 mediate negative, dampening signals to T cells to avoid the destructive effects of an excessive inflammatory response and autoimmune reactivity. Tumors use several mechanisms to avoid elimination by the immune system. One involves hijacking checkpoint pathways. Checkpoint blockade therapy utilizes mabs to release the brakes from suppressed T cells, allowing them to be activated and to recover their antitumor activity (17,18).

Table 27 lists 4 checkpoint inhibitory mabs, their target of action and the FDA-approved clinical indications. It all started with metastatic melanoma. Single-agent application of anti-CTLA-4 (18,19) and anti-PD1 was surprisingly effective and caused an improvement in OS. Such improvement was never seen before in this disease. Meanwhile an increasing number of clinical

applications appears possible with benefits of an increasing number of patients. Two further mabs are directed against the ligand of the receptor PD-1, namely PD-L1. These mabs have been approved for application in patients with urothelial carcinoma and with NSCLC.

The mabs from Table 27 have shown impressive clinical efficacy in advanced melanoma, metastatic kidney cancer and NSCLC - all malignancies that frequently cause brain metastases. Several clinical trials of checkpoint blockade have also been conducted in hematological malignancies. The results of PD-1 blockade in Hodgkin lymphoma are remarkable (19-21).

Important for the clinician is the following: About 100 trials were evaluated to assess the safety and efficacy of the approved checkpoint inhibitors of Table 25. The results can be summarized as follows: Ipilimumab and nivolumab, but not pembrolizumab, showed an OS advantage over chemotherapy first line in unresectable/metastatic melanoma. A therapy combining ipilimumab and nivolumab revealed a further increase of efficacy in advanced melanoma (22). It had been shown before that such a combination in murine B16 melanoma leads to expansion of tumor-infiltrating lymphocytes (TILs) and to a reduction of regulatory T (Treg) cells and suppressive myeloid cells (23). Nivolumab had an OS advantage versus chemotherapy in second-line NSCLC.

New emerging mabs have great potential for the systemic control of epithelial cancers such as lung cancer. Reported phase I trials of nivolumab, MK-3475, MEDI4736, and MPDL3280A, are demonstrating durable overall radiological response rates in the range of 20-25% in lung cancer.

Atezolizumab, a mab against PD-L1 or CD274 antigen, has been approved by the FDA for a variety of haematological malignancies and solid tumors (24,25). In combination with the anti-VEGF mab bevacizumab this anti-PD-L1 reagent was shown to enhance antigen-specific T-cell migration in metastatic renal cell carcinoma. It also showed durable activity and good tolerability in patients with locally advanced urothelial carcinoma. Durvalumab is a fully human mab that blocks PD-L1 binding to its receptors PD-1 and CD80 (26).

Dual checkpoint blockade strategies, such as those combining anti-CTLA-4, anti-LAG-3, or anti-KIR, are being tested to increase the proportion and durability of tumor responses.

With targeted immunotherapies, new mechanisms of action require adaptations in study design and statistical analysis, as well as the need for refining clinical trial endpoints. In the Brookings Conference on Clinical Cancer Research held in Washington, DC, in November 2013, several intermediate clinical endpoints, including milestone OS, were proposed for the evaluation of cancer immunotherapies. These are introduced to take into account the possibility of delayed treatment effects and to better characterize the clinical activity profile.

Predictive biomarkers are also important to identify patients accurately who will benefit from checkpoint blockade. A first identified biomarker is soluble IL-2 receptor (sCD25) (27). Other biomarkers might include tumor-infiltrating immune cells such as TILs and molecules such as PD-L1 in the tumor microenvironment. Also, gene analysis such as mutational landscape and mismatch repair deficiency, could become useful (28,29).

Certain types of cancer (e.g. colorectal carcinoma (CRC)) do not seem to respond to immune checkpoint blocking antibodies (30). Means to sensitize tumors to checkpoint blocking therapy include immunogenic drugs, immunogenic chemotherapy (31) and gut microbiota (32).

We expect many new patents regarding immune checkpoint inhibitors and patent-related biomarkers. A patient eligible to a treatment with an immune checkpoint inhibitor will have tremendous commercial value. A patent review for the years 2010 – 2015 presented a selection of international patent applications. These included PD-1/PD-L1, CTLA-4, IDO, TIM3, LAG3, TIGIT, BTLA, VISTA, ICOS, KIRs and CD39 (33).

Meanwhile, over a dozen T cell immune checkpoints and an additional dozen or more co-stimulatory receptors have been described. The challenge for the future, therefore, is to identify the most advantageous combinations. This should be based on knowledge of their underlying biology and on preclinical studies in murine tumor models.

vi) SIDE EFFECTS WITH IMMUNE CHECKPOINT INHIBITORY MABS

Clinical effects of therapies have always to be compared with their side effects. This is not different with immunotherapy. The side effects of blocking the immune systems natural inhibitory mechanisms have manifested clinically as diarrhea, rash, and hepatitis. The symptoms of side effects caused by such new reagents have been termed "immune-related adverse events (irAEs)" (34).

Table 28 lists the major effects. Of particular significance are, apart from general fatigue, the endocrine effects : hypophysitis, thyroid disease and adrenal insufficiency. Acute interstitial nephritis is possibly related to the presence of autoreactive clonal T cells. Renal monitoring every 2 weeks for 3-6 months has been recommended (34-37).

Although steroids can be used to treat these irAEs, the associated immunosuppression may compromise the antitumor response.

vii) MABS FOR RESEARCH APPLICATIONS AND IMPROVED DIAGNOSTICS

Because of their high specificity, antibodies have not only contributed to progress in medicine as therapeutics but also in research and diagnostics. Specific antibodies are produced by injecting an antigen into a laboratory animal such as mouse, rat, or rabbit. Serum isolated from blood of these animals then contains polyclonal antibodies, i.e. multiple antibodies that bind to the same antigen.

To obtain antibody that is specific for a single epitope of an antigen, antibody secreting B lymphocytes are isolated from such animals and immortalized by fusing them with a cancer cell line. The fused cells are called hybridomas. These can grow in culture continually and secrete antibody into culture supernatants. Single hybridoma cells are then isolated by dilution cloning to generate cell clones that all produce the same antibody which are called monoclonal antibodies.

It is possible to isolate from such hybridomas or from immune B-cells the variable Ig genes to produce single-chain Fv (scFv) fusion proteins with antibody binding specificity. We used this technology to produce scFv proteins binding to the HN protein of Newcastle disease virus (NDV), and also scFv fusion proteins binding to CD3, CD28 or CD25 of T-cells. Further genetic

engineering allowed to construct from these reagents bispecific and trispecific fusion proteins (see Chapter V, G).

In research, purified mabs are used for many applications, for instance to identify and locate intracellular and extracellular proteins. Mabs are also used in flow cytometry to differentiate cell types by the proteins they express. Different types of cells express different cell surface antigens which are defined by cluster of differentiation (CD) molecules.

Mabs are also used for immunoprecipitation to separate proteins and anything bound to them (co-immunoprecipitation) in a cell lysate. In Western blots, mabs are used to identify proteins separated by electrophoresis. In immunohistochemistry or immunofluorescence, mabs are used to examine protein expression in tissue sections or to locate proteins within cells with the help of fluorescence microscopy.

Of great diagnostic value are also ELISA and ELISPOT techniques, in which proteins can be detected and quantified with the help of differently labeled mabs.

E. T-CELLS AND DCs

Table 29 lists milestones from immunology research relating to T cells and Dendritic cells (DCs).

i) HUMORAL VERSUS CELLULAR ANTI-TUMOR IMMUNITY

As antibodies do not efficiently penetrate tissues, including tumor tissue, their ability to prevent tumor growth remains limited. The mab herceptin, which is directed against breast cancer, and has proven successful in patients, is probably a rather exceptional case. In case of single-cell tumors, such as lymphomas, the situation is different. These tumors are more accessible to antibodies than solid tumors. This explains the clinical efficacy of a variety of mabs such as Rituximab (anti-CD20), which eliminate B-cell lymphoma and B -cells in an Fc receptor dependent fashion.

T cells, in contrast, have the ability to migrate through tissues and to infiltrate solid tumor tissue. The key anti-tumor effector cells are cytotoxic T lymphocytes (CTL), which can induce direct lysis of tumor cells. In addition, T helper cells (Th) locally produce cytokines in the tumor mass, creating a pro-

inflammatory milieu, facilitating elimination of tumor cells by recruitment and activation of CTLs and nonspecific cells such as macrophages or eosinophils.

The polarization of the T cell mediated immune response towards Th 1 (T-T cooperation) or Th2 (T-B cooperation) provides the basis for the dichotomy between humoral and cellular immunity. CD28, an important T cell co-receptor for co-stimulation, was found to mediate adhesion with B-cells by interacting with the activation antigen B7 (CD80/CD86)(38).

The cellular and molecular details of this polarization of immune responses have been elucidated in the last decades. Of importance in this respect are signals which DCs and other innate immune cells receive from membrane-associated Toll-like receptors (TLRs) and from cytoplasmic RIG-I-like receptors (RLRs). Agonists of such receptors often represent foreign viral or bacterial nucleic acids or oligonucleotides. Such agents lead to immunostimulation and are therefore developed for polarization of DCs towards DC1 (inducing Th1 immune responses) and generally usefull for cancer therapy (39).

A specific DC subset, characterized by expression of CD103, plays an important role in anticancer immunosurveillance. It is dependent on the transcription factors Batf3 and Irf8 and produces interleukin-12 (40).

ii) MOLECULAR NATURE OF THE T CELL RECEPTOR

As with B-cells and their antigen-specific receptors (BCRs) which are based on antibodies, it is of interest to follow the history of the discovery of T-cells and that of their antigen-specific receptors (TCRs).

The differential effects of neonatal bursectomy and thymectomy in the chicken on subsequent humoral and cellular responses paved the way for recognition of two separate lymphocyte lineages within the adaptive immunity system, the B-cell and the T-cell lineage. The emergence of a thymus in the teleost (bony fishes), amphibians, reptiles, birds and mammals was associated with major histocompatibility (MHC) molecules, cell-mediated immunity, cytotoxic T cells and allograft rejection.

In the 1970s, the nature of the TCR was for a long time an enigma. While the BCR was known to be a membrane associated Ig molecule with variability in the binding sites due to their antibody nature, T-cells were known only to recognize antigen in a different way.

As a result of my PhD thesis, the carrier-specificity of the secondary immune response to a hapten-carrier complex could be explained by the interaction of carrier-protein specific T- cells and hapten-specific B-cells. This meant in general that more than one antigenic determinant was required for immunogenicity of an antigen (41). Cell-to-cell interaction and communication was at that time unheard of in medical circles and therefore doubted.

A specific function of thymus-derived lymphocytes (T-cells) in the secondary humoral immune response in mice was described in 1969 by R B Taylor (42) and in 1970 by M Raff (43). Gradually it appeared that T cells recognize linear stretches of amino acids while antibodies recognize three-dimensional structures of proteins. Several approaches to identify the TCR via immunochemical studies of the precipitated protein, however, failed.

In contrast to B-cells, T-cells cannot recognize antigen directly. The antigens that T cells recognize are small peptides from proteins that bind to major histocompatibility (MHC) molecules. MHC molecules act as receptors for T cell antigens and function in particular on professional antigen-presenting cells (APCs) such as dendritic cells (DCs). Each vertebrate species expresses MHC molecules. These were identified originally through their ability to evoke very powerful transplantation rejection. It was the important discovery by R Zinkernagel and P Doherty, who, in 1974, described the phenomenon of MHC restriction by CTLs (44).

Finally, it was the group of T Mak which solved the problem by gene technology. After a long and often frustrating search, the discovery of the genes encoding the mouse and the human TCR was published in 1984 in Nature (45). It still needed much further molecular structural research to unravel the secrets, how the TCR sees peptide antigen and MHC molecules in three dimensions (46-52).

The antigen receptor of MHC-restricted CD4+ helper T cells and CD8+ CTLs is a heterodimer consisting of two transmembrane polypeptide chains, designated TCR α and TCR ß, covalently linked to each other by a disulfide bridge. The V regions of the TCR α and ß chains contain short stretches of amino acids in which the variability between different TCRs is concentrated. These form the hypervariable or complementarity-determining regions (CDRs). Three CDRs in the α chain are juxtaposed to three similar regions in the ß chain to form the part of the TCR that specifically recognizes peptide-MHC (pMHC) complexes: the V domain. A and ß polypeptide chains contain each one variable (V) and one constant (C) domain.

Each TCR chain, like Ig heavy and light chains, is encoded by multiple gene segments that undergo somatic rearrangements during the maturation of T lymphocytes in the thymus. Associated signaling molecules are CD3 and ζ. The affinity for antigen (Kd) is in the range of 10^{-5} to 10^{-7}. For comparison, the affinity of antibodies for antigen is in the range of 10^{-7} to 10^{-11}.

TCR α and ß chains can be isolated from a T cell clone of defined peptide and MHC specificity. Upon transfection into other T cells, these genes confer both the peptide specificity and the MHC restriction of the original clone. Neither TCR chain alone is adequate for providing specific recognition of p-MHC complexes.

The low affinity of specific antigen binding to the TCR is likely the reason why adhesion molecules are needed to stabilize the binding of T cells to APCs during cognate interactions, thus allowing biological responses to be initiated. T cells and APCs interact through pairs of accessory molecules: MHC II-CD4, MHC I-CD8, VCAM-1 – VLA-4, ICAM-1 – LFA-1, LFA-3 – CD2, B7-CD28, B7-CTLA-4. Interactions of B7-CD28 are stimulatory while interactions of B7-CTLA-4 are inhibitory.

Activation of T cells requires two signals. One signal only (signal 1) can produce unresponsiveness (anergy) or death via apoptosis. Signal 1 is provided by the low affinity cognate TCR-pMHC interaction between T cells and APCs. Signal 2 is mediated through ligation of CD28 and B7 (CD80/CD86) adhesion molecules between T cells and APCs .

iii) COGNATE INTERACTION BETWEEN T CELLS AND APCs AND SIGNALING THROUGH THE TCR REQUIRES THE FORMATION OF AN IMMUNOLOGICAL SYNAPSE

Signaling through the TCR complex appears to require prolonged or repeated engagement of p-MHC complexes. This is promoted by stable adhesion between T cells and APCs. The TCR and accessory molecules in the T cell plasma membrane move coordinately with their ligands in the APC membrane to form a transient supramolecular structure called the immunological synapse (47). The formation of this synapse regulates TCR-mediated signal transduction. CD4 and CD8 molecules are T-cell proteins that bind to nonpolymorphic regions of MHC molecules and facilitate signaling by the TCR complex during CD4+ or CD8+ T cell activation, respectively.

Synapses have first been detected in the nervous system. There they serve for transmission of electrical signals between cells within the nervous system or between neurons and muscle cells. The adaptive immune system apparently made use of synapses for transmission of chemical signals for information transfer between its cells. In contrast to the neuronal system, the immune system is characterized by cells moving throughout all body tissues and organs for immune surveillance.

BOX 4 1973 Research on viral superantigens in London, England

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BOX 5 1973 MHC restriction of CTL

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iv) COMPARISON BETWEEN SPECIFIC B-CELL AND T-CELL RESPONSES TO ANTIGEN

Both, B- and T-lymphocytes use randomly generated membrane receptors (BCRs and TCRs) for specific recognition of antigens. The receptors are made up of constant and variable gene domains. The high variability of the antigen binding sites is generated by somatic rearrangement of the genes coding for

the variable domains. During B-cell maturation in the bone marrow, a V(D)J recombinase becomes active to produce homologous recombinations in immunoglobulin *v genes*. During T-cell maturation in the thymus, the recombinase activating genes RAG-1 and RAG-2 (52) become active to produce homologous recombinations in the *v gene segments of the TCR* α *and* β chains. These recombinase enzymes and activating genes in lymphocytes of the B- and T-cell lineages allow the creation of a huge repertoire of mature B- or T-cell clones with different antigen receptor specificities. Sophisticated mechanisms of positive and negative selection in the bone marrow (for B-cells) and thymus (for T-cells) make sure that only clones with a correct and functional receptor are allowed to leave the respective lymphoid tissue into the periphery. Cells that react to self - antigens (all antigens from autologous healthy tissues) are eliminated in bone marrow or thymus by negative selection.

Contact of a mature B- or T-cell with a fitting antigen in a secondary lymphoid organ such as the lymph node or spleen leads to its clonal selection and expansion.

BOX 6 1976 Head of Division Cellular Immunology at DKFZ, Heidelberg

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BOX 7 1980 Chamber music in Paris

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v) MOLECULAR NATURE OF A HUMAN TUMOR ASSOCIATED ANTIGEN RECOGNIZED BY A TCR OF A CTL

The existence of tumor-associated antigens (TAAs) was deduced originally from studies of specific immune rejection of transplanted experimental mouse tumor lines in pre-immunized syngeneic mice. While virus-induced tumors express group (virus)-specific tumor rejection antigens, those of chemically-induced tumors express TAAs that are unique for each tumor line. Whether naturally occurring animal tumors also expressed TAAs and whether

this was the case also for human tumors was not known at the time and thus was hotly disputed in the 1970s and 1980s.

To identify the molecular nature of a TAA required the new techniques of gene technology, just like in the case of the TCR. It was the group of T Boon in Brussels (Belgium) who in 1991 described for the first time the molecular nature of a TAA. The antigen was expressed on a human melanoma cell line and could be recognized *in vitro* by specific CTLs. The sophisticated new technology involved multiple gene transfers. Also, multiple CTL assays had to be established, before this group could identify a gene coding for an HLA-A1 restricted peptide that was not expressed in a panel of normal tissues (53). In the following decades many more human TAAs were discovered. They also consist of peptide-MHC complexes.

The major histocompatibility complex (MHC) represents a genetic region encoding molecules involved in antigen presentation to T cells. Class I MHC molecules are present on virtually all nucleated cells. They are encoded by H-2K, -D and –L loci in mice and by HLA-A, -B and –C in man. Class II MHC molecules are expressed on antigen-presenting cells (primarily dendritic cells, macrophages and B cells). They are encoded by H-2A and –E in mice and by HLA-DR, -DQ and –DP in man.

The MHC is highly polymorphic. Each gene locus comes in a variety of allelic forms. Allelic differences in MHC are associated with the most intense graft rejection within a species.

vi) HUMAN TUMOR-ASSOCIATED ANTIGENS

More than 100 tumor-associated T-cell antigens have meanwhile been characterized. With regard to their identification, characterization and clinical applications we refer to a recent book (54) and to Chapters 53 and 54 of the textbook "The Molecular Basis of Cancer" (55).

Only a short summary can be given here: Every tumor may contain a few hundreds of mutations in coding regions of the genome. In addition, deletions, amplifications, and chromosomal rearrangements can result in new genetic sequences. The vast majority of these mutations occur in intracellular proteins. Therefore, such "neoantigens" would not be readily recognized and targeted by antibodies. However, thanks to the MHC presentation system for

T-cell recognition, peptides derived from all cellular proteins and fitting into respective MHC peptide binding grooves, are transported to the cell surface. There they can be recognized by T-cells with TCR specificity for such p-MHC complexes. It has been estimated that roughly one third of the mutations identified from genome sequencing of breast and colon cancers are capable of binding to common HLA allels.

1. One category of human TAAs arises from common oncogene/tumor suppressor gene mutation. Such mutations can be individually specific or can be shared. Antigen-specific immunotherapies targeting such TAAs must therefore either be patient-specific or focused on those common mutations. Examples of the latter are the mutations Kras G12A (colon and pancreatic cancer), Braf V599E (melanoma) and P53 G249T (hepatoma).

2. Cancer-testis antigens represent examples of widely shared tumor antigens whose expression is restricted to tumors. Many of these epigenetically altered genes are expressed selectively in the testis of males. The most commonly explored antigens in human vaccine trials are Mage3 and NY-ESO-1. They demonstrate a broad tumor distribution. A major drawback of such antigen targets is that none of these appears necessary for tumor growth or survival.

3. Other human TAAs are also upregulated via epigenetic mechanisms, for example CEA (gastrointestinal cancers), WT-1 (Wilm`s tumor, leukemias, lymphomas), Mesothelin (pancreatic, ovarian cancer or mesothelioma) and Her2/Neu (Breast, ovarian cancer).

4. Tissue-specific antigens expressed by tumors represent another category of shared TAAs. They have been popular targets of cancer vaccination. Examples are Tyrosinase (melanoma), MART1/Melan A (melanoma), gp100 (melanoma), PSA (prostate), PAP (prostate).

5. Another important category of tumor antigens encompasses viral antigens for virus-associated cancers or pre-cancerous lesions. Examples are HPV E6,E7 (cervical cancer) and EBV EBNA-1, LMP1,2 (Hodgkins lymphoma, nasopharyngeal cancer).

vii) T-CELL CO-STIMULATION AND T-CELL TOLERANCE

CD28 is the principal costimulatory receptor for delivering second signals for T-cell activation. CD28 is a receptor on T cells that binds to B7 costimulatory molecules expressed on professional APCs. Cross-linking of CD28 chains in the T-cell membrane delivers signals (signal 2) that are required for full T cell activation, in addition to signals generated by cross-linked TCR chains (signal 1). The delivery of signal 1 only to a naïve T cell upon cognate interaction with an APC, for instance a TAA-expressing tumor cell, is not only insufficient for T cell activation, but it induces T-cell tolerance.

A second receptor for B7, called CTLA-4, is induced after T cell activation and functions to inhibit the T cell response. Some members of the CD28 family, such as CD28 itself and ICOS (inducible costimulatory) provide activating signals to T-cells. The CD2 family of receptors includes proteins such as CD2 and SLAM that provide additional activating signals to T cells. Other T-cell receptors, such as CTLA-4 and PD-1, provide inhibitory signals.

The CD3 and ζ proteins are noncovalently associated with the TCR α,ß heterodimer. When the TCR recognizes antigen, these associated proteins link antigen recognition by the TCR to the biochemical events that lead to T cell activation. These events involve Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) of the CD3 γ,δ, and ε proteins and of the ζ chain. These ITAMS are phosphorylated shortly after antigen recognition by Src family kinases such as Lck or Fyn. Lck associates with the cytoplasmic tail of CD4 and CD8, and Fyn is physically linked to CD3. The phosphor-tyrosines in the ITAMs become docking sites for a tyrosine kinase with tandem Src homology 2 (SH2) domains. This kinase, called ZAP-70, is recruited to the ζ chain and triggers signal transduction pathways that ultimately lead to changes in gene expression in the T-cells.

There also exist within cells of the immune system Immunoreceptor Tyrosine-based inhibition (ITIMs) motifs. These are six-amino acid motifs found in the cytoplasmic tails of various inhibitory receptors, including FcγRIIB on B-cells and Killer cell Ig-like Receptors (KIR) on NK-cells.

CD4-mediated T-cell help in the activation of CD8+ T-cells and B-cells, through linked-recognition of antigenic determinants, is a long-standing

concept fundamental to our understanding of immunity (presence of help) versus tolerance (lack of help). The important question is how to overcome immune tolerance to tumor cells (with shared TAAs as self antigens) without causing unwanted autoimmune pathology. The answer requires detailed understanding of central and peripheral tolerance mechanisms.

Progress in the field of T-cell tolerance has been made in the last 2 decades among others by B Kyewsky and L Klein. In 2006 they described a central role for central tolerance (57). The *autoimmune regulator gene aire* was found to regulate the expression of tissue-specific antigens (TSAs) in medullary thymic epithelial cells (mTECs). These play a critical role in the negative selection of autoreactive T cells and in the generation of regulatory T cells. *Aire* was initially identified as the gene causing multiorgan system autoimmunity in humans. Deletion of this gene in mice also resulted in organ-specific autoimmunity.

Regulatory T cells (Tregs) are crucial mediators of self-tolerance in the periphery. They differentiate in the thymus, where interactions with thymus-resident antigen-presenting cells, an instructive cytokine milieu, and stimulation of the TCR lead to selection into the Treg lineage and the induction of Foxp3 gene expression. Once mature, Treg cells leave the thymus and migrate into the periphery (58).

Apart from central tolerance there are also tolerance mechanisms in the periphery. For instance, CD4+ T cell tolerance has been identified to corrupt cancer immunotherapy (59). Also, it has been described, how to help helper T -cells (and B-cells) to become intolerant of tumors: The authors demonstrate that provision of linked foreign helper epitopes, such as influenza hemagglutinin, substantially enhances both CD8+ T-cell and B-cell responses to tumor self-antigens without causing any overt autoimmune pathology (60).

In 2011, RM Steinman, B Beutler and J Hoffmann were granted the Nobel Prize for Physiology or Medicine for their discovery of DCs, Toll-like receptors (TLRs) and innate immunity, respectively. How important these three topics are for the understanding of immune mechanisms and for designing new immunotherapeutic concepts to fight cancer will become clear in the following chapters. Their work provides a rational basis for the concept of

multimodal immunotherapy developed at the IOZK in Cologne (Germany) (see below).

To finish with Table 29, the successful introduction of the immune checkpoint therapy into the clinic in recent years by J Allison and colleagues has already been delt with in the chapter about mabs.

F. MILESTONES OF CANCER IMMUNOTHERAPY

Table 30 lists milestones of cancer immunotherapy, starting from 1890 with Coley`s Toxin and ending 2016 with the FDA approval of T-VEC. It shows periods of quiescence as well as periods of rapid activity. The list involves discoveries from innate immunity and adaptive immunity, from B-cell and from T-cell immunity.

Since we focus here on T cells, we will summarize first developments in adoptive T-cell therapy. These will be divided into therapies with native T cells and those involving gene-modified cells such as CAR T cells.

i) ADOPTIVE T-CELL THERAPY

The field of adoptive T-cell therapy (ACT) has emerged from principles of basic immunology to paradigm-shifting clinical immunotherapy. Several adoptive T-cell therapy strategies have provided clinical benefit to cancer patients (61,62,63). T-cell therapy may rely on T lymphocytes harvested directly from the patient (autologous approach) or from healthy donors (allogeneic approach).

a) allogeneic GvL

In hematological malignancies, allogeneic T cells, infused with allogeneic hematopoietic stem cell transplantation (HSCT), represents the treatment of choice. A consistent fraction of donor-derived T cells can recognize either patient-specific HLA molecules (in the case of haploidentical, or half-matched, transplantation) or patient-specific so-called minor histocompatibility antigens (in the case of fully HLA-matched donors). Donor lymphocyte infusion (DLI) following HSCT can promot graft-versus-leukemia (GvL) effects and disease-free survival. These effects are largely due to the immunological recognition of the tumor by allogeneic T cells (64).

With regard to solid tumors, the usefulness of allogeneic HSCT remains to be fully exploited. Early studies had to be halted because of overt toxicity to the recipient, and limited anti-tumor effects. In addition to eliciting potent graft-versus-tumor (GvT) activity, alloreactive T cells create a risk of precipitating life-threatening Graft-versus-Host (GvH) disease. Less intense preconditioning regimens, pharmacological prophylaxis of GvHD with immuno-suppressive drugs, depletion of donor T cells, and/or immunization with tumor-directed vaccines might favor GvT and limit GVHD (65,66).

The specificity and efficacy of allogeneic GvT could be increased by employing tumor-reactive memory T cells (MTCs) rather than naïve T cells. The problem, however, exists, how to generate these from the donor. In man, donor pre-immunization against the tumor of the host, is not as feasible. This method functioned perfectly well in experimental animals with genetically defined strains. *Ex vivo* stimulation of donor T cells with host tumor lysate-pulsed DCs as APCs would be an alternative. Below, we provide examples of allogeneic GvL activity of MTCs from the bone marrow of pre-immunized donor mice. Also, we will elude to experiments with MTCs from the bone marrow of cancer patients.

MTCs have many properties that are superior to naïve T cells for therapeutic purposes. The forkhead box O (FOXO) transcription factor family – which is central to the integration of growth factor signaling, oxidative stress and inflammation – provides connections between physical well being and the form and magnitude of an immune response (67). FOXO1 has an intrinsic role in establishing the post-effector memory program in T cells that is essential for forming long-lived memory cells capable of immune reactivation (68).

b) autologous TILs

Recent strategies focus on autologous T cells to treat non-hematological tumors. The group of S Rosenberg pioneered the isolation and expansion of TILs. Patient-derived T cells were grown and selected in culture and then infused back for the treatment of advanced melanoma (69). Further studies improved the therapeutic potential of TILs by defined culture conditions, by patient preconditioning strategies, and by the provision of exogenous IL-2. Objective tumor regressions in 50-70% of patients with metastatic melanoma could be observed, with some patients achieving durable complete

regressions beyond 10 years (70). TILs could be recovered from several tumors, including renal carcinoma, ovarian, colorectal and breast cancer. However, technical difficulties in obtaining sufficient numbers of cells have so far limited exploitation of this strategy.

c) Suicide gene adoptive T-cell therapy

Genetic engineering of T cells with "suicide genes" represents one of the first clinical applications of the gene transfer technology in humans. Suicide gene therapy in HSCT aims at providing therapeutic GvT activity of donor T cells while managing unwanted GVHD. This is done by eliminating the alloreactive cells expressing a suicide gene with a specific prodrug (ganciclovir). The prodrug is activated only by the suicide gene product (e.g. HSV-TK) and kills selectively the donor T cells. In different clinical trials, TK suicide gene therapy proved to be safe and feasible, allowing to control acute and chronic GVHD, even in the challenging field of HLA-mismatched transplantation (71).

d) TCR transgenic T cells

A new window of opportunity for cancer immunotherapy has arisen in recent years by advances in gene transfer technology and in cellular immunology. Details can be found in an excellent review (72).

These techniques allow to select the most appropriate target antigen and/or antigen-specific TCR (73). In addition, T cells can be transfected with Chimeric Antigen-specific Receptors (CARs). Such CARs consist of an extracellular antibody binding site fused to an intracellular TCR signal transducing chain (74). TCRs and CARs confer to T cells the ability to recognize cells expressing a given TAA or stromal antigen and to kill such cells via HLA-dependent TCR or HLA-independent CAR mechanisms.

One clinical study involved targeting, by TCR transfer, the cancer germline antigen NY-ESO-1. In 38 patients this approach proved to have clear clinical effects without demonstrable toxicity (75). TCR targeted human central memory T cells were shown to possess superior capacity for adoptive immunotherapy than CD8+ T cells (76). Phase I studies with central memory-derived CAR T-cells demonstrated safety and feasibility following autologous HSCT in patients with B-cell NHL (77).

New techniques in this rapidly developing field are constantly being developed. One new technique allows high-throughput identification of antigen-specific TCRs by TCR gene capture (78). This might open the possibility to exploit a broad, tumor-reactive repertoire of TCRs. Another technique allows enhanced-affinity CDR3 variants to bind to pMHC dimers with enhanced equilibrium kinetics.

Artificial zinc-finger nucleases have been exploited to generate human T cells with disrupted endogenous TCR genes. These cells can then be transduced with a TCR specific for a human TAA without the risk of production of mixed TCR dimers. Such TCR-edited T cells with specificity for the Wilms tumor 1 (WT1) antigen were superior in transgenic TCR expression, because of lack of competition with endogenous TCR. They also showed increased anti-tumor reactivity (79).

Elimination of endogenous α,ß TCR can be introduced to prevent GvH reactivity without compromising CAR-dependent effector functions. This technology might establish a more universally applicable immunotherapy platform for the treatment of B-lineage malignancies (80). Recent gene-editing tools, such as transcription activator-like effector nucleases and clustered regularly interspaced palindromic repeats, provide a platform to delete endogenous TCR and HLA genes. This interesting approach aims at removing alloreactivity and decreasing immunogenicity of third-party T cells (81,82).

e) CAR transgenic T cells

Automated manufacturing of transgenic T cells for clinical application has been made possible by the CliniMACS Prodigy (Miltenyi Biotec) (83).

A chimeric antigen-specific receptor (CAR) consists of several modules: i) a single-chain Fv (scFv) binding domain, ii) a bridge domain and iii) a transmembrane and signal transmitting domain (CD3 zeta). A second generation CAR uses a further costimulatory domain (e.g. CD28) fused to the domain under iii). A third generation CAR uses two costimulatory domains (e.g. CD28 and OX40) attached to CD3 zeta. A CAR of the 4th generation is called TRUCK because it will release a transgenic protein (e.g. a cytokine) upon contact of the CAR with its target (84).

The advantage of CARs over recombinant TCRs is that CARs recognize cell surface molecules independently of HLA expression. CARs are monomeric receptors and should not pose the risk of unexpected specificities.

The promise of this approach is highlighted by the recent success of CAR T cells specific for CD19 antigen, expressed for instance by ALL, CLL, and NHL (85). Complete remissions were seen in patients with refractory lymphoid malignancies for which several lines of therapy had previously failed (86).

Nevertheless, toxicity was often observed: a large proportion of patients experienced an acute cytokine-release syndrome (CRS), likely due to acute release of IL-6 and IFN-γ, in most cases manageable by an Il-6 receptor blocking antibody (Tocilizumab) (87).

The main drawback of CD19-redirected CAR T cells is that these cells cannot discriminate between healthy and transformed CD19+ cells. A suicide gene/prodrug approach, as discussed above, might solve this complication. This can be combined with imaging of the T cells by PET (88).

Another example of innovation are genetically modified T cells expressing T-cell engager (ENG) molecules (BITES). CD19-ENG T cells express secretable CD19-specific BITES redirecting and activating bystander T cells to tumor cells (89).

The CRISPR/Cas9 technique, introduced in 2012, is a simpler and more efficient method of editing genes than previous methods. The acronym stands for "Clustered Regularly Interspaced Short Palindromic Repeats" (90-92).

CAR T cell therapy for solid tumors is a challenge (93). CAR T cells engineered to express heparanase (Hpa) is an attempt in this direction. Such modified T cells were shown to promote tumor infiltration and antitumor activity (94). Another enzyme, catalase, coexpressed by CAR T cells was reported to protect the T cells as well as bystander cells from oxidative stress-induced loss of antitumor activity (95). In a murine tumor model, efficacy of CAR T-cell therapy in large tumors was shown to rely on a combination of antigen-independent stroma destruction and antigen-specific tumor cell targeting (96). The use of biopolymers codelivering engineered T cells and stimulator of

IFN gene (STING) agonists could eliminate heterogeneous tumors in orthotopic mouse models of pancreatic cancer and melanoma (97).

Some efficacy of CEA CAR-T cell therapy was reported for metastatic colorectal cancers (98). Also, regression of glioblastoma was reported after CAR T-cell therapy (99).

CAR T cells are being developed also to target cancer driver gene products. Examples of such driver gene products are

i) epidermal growth factor receptor variant III (EGFRvIII) (100),

ii) CD44v6, the variant of hyaluronate receptor CD44 expressed in AML and MM and

iii) tyrosine kinase receptor gene ROR1 (101).

Apart from $\alpha,ß$ T cells, also other types of cells such as γ,δ T cells, cytokine-induced killer (CIK) cells and NK cells are being explored as carriers for CAR mediated therapy (102,103).

G. MY MAJOR RESEARCH TOPICS 1976 – 2000

It is breath-taking to observe the recent advancements in T cell immunology and clinical translational research. Of course, this is driven, as with the development of mabs, not only by research scientists but also by competition between big pharma companies. Anyway, it is re-assuring to see for someone, like me. I was convinced from the beginning that T cell mediated anti-cancer immunity is of great importance for improvement of cancer therapy.

Let us now go back in time for only a few decades. When I started in Heidelberg in 1976, research on cancer metastasis or on cancer immunotherapy did simply not exist. These topics were considered like a black box. Cell biology was just becoming a so-called "hard-core" science while tumor immunology was considered as "weak" science. Whether or not a research topic into which you may be driven by intuition may one day

become important can often only be judged in retrospective. This is particularly true for areas as complex as applied cancer research.

In new research areas it is important to start with concepts or hypotheses. Whether these then hold true can often be determined not after years (a time period important for public or private investors) but only after decades. Peer-reviewing is a well established procedure in science. The time periods for judgement, however, become increasingly shorter. Here I make a plea to peer-reviewers to have a more historic view: who was right with predictions over time and who not ?

Table 31 lists my major research topics from 1976 to 2017. In this Chapter we will deal with the topics from 1976 to 2000. The next Chapter (V), devoted to oncolytic virustherapy, will deal with my research topics from 2000 onwards.

i) IMMUNE RESPONSES AGAINST METASTASIS

In 1976, a major research question for my Division of "Cellular Immunology" at the German Cancer Research Center in Heidelberg was the following:

Is it possible to generate immune responses against highly aggressive metastasizing tumor cells ? Or could it be that such tumor cells are already selected towards immune resistance ?

There had been a report from syngeneic murine lymphoma that a response to immunotherapy depended on the antigenicity of the tumor. Following injection of a known number of tumor cells, in that report, the mice were treated either by administration of irradiated tumor cells, living *Bacillus Calmette-Guérin* (BCG) vaccine or both (104).

BCG is a vaccine against *tuberculosis* to reduce *tuberculosis*-associated complications in children. A small clinical study in 1976 showed that intravesical (transurethral) BCG instillation can be successful in superficial bladder carcinoma. The treatment of superficial bladder cancer is perhaps the first approved immunotherapy. The treatment of this cancer has three objectives: a) eradication of existing disease, b) prophylaxis against tumor recurrence, and c) prevention of tumor progression (either muscular invasion, metastatic spread, or both). When used with prophylactic intent following

transurethral resection, recurrence rates were lower than those achieved with other agents. In addition, BCG emerged as the consensus drug of choice for treating carcinoma *in situ* of the bladder.

In summary, in the above murine study, the response to BCG alone was small, irradiated cells were more effective and the best results were obtained by a combination of the two components. The least responsive lymphoma line was the least antigenic and the most aggressive.

We obtained two of these lymphoma lines from Prof P Alexander in London (UK) and termed the parental low metastatic line as Eb and a spontaneous high metastatic variant thereof as ESb.

Comparative studies with these related lymphoma lines of low and high metastatic capacity allowed to describe in great detail their differences in tumor invasiveness in vitro and metastasis formation in vivo (105).

BOX 8 1982 Heparanase

Of great importance was the finding that the metastatic variant ESb was not generally immunoresistant. Upon testing a variety of immunization procedures we found a way to reproducibly generate CTLs. These were capable to specifically recognize the ESb line. What was astonishing was that these CTLs did not recognize the parental line Eb. So the metastatic variant had changed the expression of its original TAA (that of Eb cells). The differences in the antigenicity of the two lymphoma lines could also be seen *in vivo*. The tumor-protective immunity generated against one line did only protect against this line and not against the other. Nevertheless, it was of importance that we were able to generate protective immunity *in vivo* even against the high metastatic variant (106).

An unusual but very effective way of inducing protective immunity against the aggressive variant ESb was the inoculation of live proliferation-competent tumor cells into the mouse ear pinna (107). At this site, the induction of the T cell response was so fast that the tumor cells were prevented from growing.

The protective immune response, measurable by rejection of subsequent tumor grafts in the periphery, was long-lasting (> 6 months) and correlated with the induction of a state of tumor dormancy in the bone marrow (BM) (107). Further research revealed that this tumor dormancy state was due to a balance - at a low frequency level - between proliferating tumor cells and tumor-specific controlling CD8+ memory T cells. Persistence of dormant tumor cells in the BM of tumor-cell vaccinated mice was apparently due to control by the immune system and correlated with long-term immunological protection (107).

BOX 9 1982-1988 Honorable Prizes

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ii) BONE MARROW T-CELLS AT THE CENTER STAGE OF IMMUNOLOGICAL MEMORY

a) basic research in mice

Effector T cells, such as CTLs and cytokine-secreting T helper cells were thought, at that time, to be of decisive importance in the fight of the immune system against tumors. Later, tumor-specific memory T cells (MTCs) came into the focus of being perhaps of greater importance for induction and maintenance of long-term protective anti-tumor immunity.

A characteristic feature of MTCs is their long-term survival in the absence of re-exposure to the antigen. Upon disappearance of the source of antigen, the vast majority of effector T lymphocytes are eliminated via apoptosis. A fraction of antigen-responsive cells, however, are retained and these belong to the memory compartment.

Priming of naïve T cells leads to association of the tyrosine kinase Lck with the CD8 co-receptor, thereby enhancing TCR signaling (108). Lck phosphorylates CD3 activation motives (ITAMs). Of significance was the discovery that the association between Lck and CD8 is maintained in MTCs. This explains their enhanced sensitivity to antigen re-exposure. A prerequisite

for this change seems to consist of sustained T cell stimulation via the immunological synapse (109).

Since the above discovery of tumor dormancy in the BM from 1994, we intensified our efforts to understand the phenomenon at least at the cellular level. Active control of proliferating tumor cells in the BM by CD8+ immune T cells was described in 1998 (110).

In 2003, we provided evidence, for the first time, that BM is a priming site for CD8+ T-cell responses to blood-borne antigen (111). Ten years later, 2-photon dynamic imaging revealed indeed cross-presentation of blood-borne antigens to naïve CD8+ T-cells in the BM (112). We extended our studies of the BM microenvironment also to CD4+ T-cell responses. The BM microenvironment was found to facilitate also DC: CD4+ T-cell interactions and maintenance of CD4 memory (113).

Next we investigated the longevity of MTCs from the BM. Longevity of protective anti-tumor immunity could be established in T-cell deficient nude (*nu/nu*) mice following a single transfer of ß-galactosidase (Gal) specific CD8+ T-cells from immunocompetent DBA/2 mice. Upon challenge of naïve nude mice (for control) with live *lacZ gene* (coding for Gal)-transfected ESb tumor cells (ESblacZ), the tumor cells grew out quickly and killed the mice. ESblacZ cells express the dominant T-cell epitope of Gal. Co-transfer of Gal-specific T cells with the tumor cells prevented tumor outgrowth in the nude mice. As a result, we documented long-term persistence, at a high frequency, of Gal-specific T cells in the BM and spleen of these tumor-protected mice (114,115).

The Gal-specific MTCs from the BM could be recruited to the peritoneal cavity and re-activated there by i.p. challenge with irradiated ESblacZ cells to become effector memory T cells (EMT) (114). These *nu/nu* derived EMTs could easily be harvested and transferred together with live ESblacZ cells to secondary *nu/nu* hosts where they protected again against tumor outgrowth. Long-term immune memory and tumor protection could be maintained in this way over four successive transfers over a long period of time (> 8 months). Further studies revealed that the presence of Gal-expressing dormant tumor cells was indispensable to boost specific T-cell frequencies to levels detectable by peptide-MHC multimers. Apparently, there existed in the BM a balance between Gal-expressing dormant tumor cells and Gal-specific T

cells. This possibly provided a selective advantage of Gal-specific T cells over irrelevant clones for homing to and surviving in niches of the BM (115).

Immune MTC transfer studies were also performed in immunocompetent mice. To test the graft-versus-leukemia (GvL) therapeutic potential of MTCs from different compartments (spleen, peritoneal cavity and BM), we selected a GvL animal model of advanced metastasized cancer which we had established in 1995 (116). In 2005, we reported that tumor-immune memory T cells from the BM were superior to MTCs from the other compartments and exerted GvL without graft-versus-host (GvH) reactivity in advanced metastasized cancer (117). The mechanisms of this complete remission of cancer in late-stage disease by radiation and transfer of allogeneic MHC-matched immune T cells have been elucidated over ten years of research and summarized recently (118).

Further details about these results and the complete reversion of cancer-associated dysregulation by the immune system will be given and discussed in Chapter VII.

b) clinically relevant findings

In the year 2000, a very bright medical student, M Feuerer, started to investigate under my supervision the BM in man as an immunological T-cell-response compartment. We were lucky to arrange with the Heidelberg University Hospital of Gynaecology (Head: Prof G Bastert) and with the help of the physician I Diel to obtain samples of BM aspirates from breast cancer patients. These were collected at the hospital to test for the presence of tumor cells while the non-tumor mononuclear cells were of no interest. So, we could obtain part of the samples to study the mononuclear cells. BM derived mononuclear cells from primary operated breast cancer patients (n = 90) were compared by multicolor flow cytometry analysis with those from healthy donors (n = 10) and also with cells from respective blood samples. The most surprising results from all the immunological changes observed in the BM of cancer patients was the significant increase of memory T cells (MTCs) among the CD4+ and CD8+ T cells ($p<0.001$) (119).

In 2001, Feuerer et al. described in an article published in Nature Medicine (120) about therapy of human tumors in NOD/SCID mice with patient-derived

reactivated MTCs from bone marrow. In an analysis of 84 primary-operated breast cancer patients and 11 healthy donors, the BM of most patients was found to contain MTCs with specificity for TAAs. In short-term culture with autologous DCs pre-pulsed with breast cancer lysates, patient`s MTCs from BM (but not from the peripheral blood) could be specifically re-activated to interferon-γ (IFN-γ) producing and cytotoxic effector cells. A single intraperitoneal (i.p.) transfer of re-stimulated BM derived T cells into NOD/SCID mice caused regression of autologous subcutaneous (s.c.) tumor xenotransplants. Tumor regression was associated with infiltration by human T-cells and DCs and with tumor-cell apoptosis and necrosis. Re-activated T cells from the peripheral blood of the same patients showed much lower anti-tumor reactivity.

The BM of breast cancer patients was found to contain, among others, CD8+ T cells specific for peptides derived from breast cancer-associated proteins such as MUC1 and HER2/neu. Most of these had a T-cell central- or effector - memory phenotype. To test their *in vivo* function, BM derived CD45RA+ naïve or CD45RA-CD45R0+ memory T cells were separated, stimulated like before, and then transferred i.p. into NOD/SCID mice bearing autologous breast tumors and normal skin transplants. CD45RA- memory but not CD45RA+ naïve T cells infiltrated autologous tumor but not skin tissue after the transfer. Tumor infiltration included cluster formation in tumor tissue by MTCs with co-transferred DCs. The results demonstrated selective homing of cancer-reactive MTCs to human tumor in vivo and suggested that tumor rejection was based on recognition of TAAs on tumor cells and DCs by autologous specifically activated central- and effector-MTCs (121,122).

Co-culture of MTCs from BM of breast cancer patients with DCs not presenting TAAs (i.e. noncognate co-culture system for specificity control) caused apoptosis of the T cells. Also, transfer of re-activated BM T cells without the DCs from the co-cultures was insufficient to cause tumor regression in vivo. In co-cultures allowing for antigen-specific cognate interactions, the expression on DCs of CD83, MHC class II, CD40 and CD86 molecules was upregulated and the cytokines IL-12 and IFN-α were produced in significantly elevated amounts. These findings suggested that cognate interactions between patient-derived BM derived T-cells and tumor antigen-

presenting BM derived DCs are important for reciprocal cell stimulation, survival and therapeutic activity (123).

c) Influence of adjuvant hormone therapy and chemotherapy on the immune system in the bone marrow of patients with breast cancer

The purpose of this study was to analyse the effect of adjuvant systemic therapy in breast cancer on the immune system in the BM compartment. In 34 patients with breast cancer, BM was aspirated 2 years after primary surgery and adjuvant systemic therapy. The immune system of these patients was compared with that of patients at the time of surgery (n = 90). It was found that the proportion of all T cells was significantly reduced. Chemotherapy apparently had a particularly suppressive effect on naïve CD4 T-cells and, to a lesser extent, on memory CD4 T-cells. Hormone therapy had a significant suppressive effect on both naïve and memory CD8 T-cells. These findings suggest profound and long-lasting negative effects on the BM immune system by present-day standard adjuvant therapy in breast cancer (124).

d) A pilot clinical study of adoptive T-cell therapy with re-activated BM derived cancer-reactive MTCs

Aim of the study was to investigate whether *ex vivo* re-activation of cancer-reactive MTCs from the BM and their adoptive transfer to autologous patients is feasible and increases the frequency of cancer-reactive T cells in the blood. Twelve late-stage metastasized breast cancer patients with a positive pre-test for the presence of cancer-reactive MTCs in their BM were included. They had received standard therapy and therefore reduced T-cell reactivity, as reported above. In all cases, the treatment was feasible and well tolerated.

7 days after the transfer of cells from the re-stimulation cultures, 6 patients (immunological responders) showed by ELISPOT analysis *de novo* TAA-specific IFN-γ secreting T cells in their blood sample. In contrast, 6 other patients (immunological non-responders) showed in their blood sample TAA-induced IL-4 responses (125). Responder patients had received $6.5x10^3$ cells while non-responders had received somewhat lower numbers from their stimulation cultures. This was due to reduced activation of MTCs, to

increased amounts of CD4+CD25^{hi} Treg cells in their BM and to increased TAA-induced IL-10 secretion.

The 6 to 10 million re-activated MTCs from the BM that had been adoptively transferred to responder patients must have extensively expanded *in vivo* to reach the numbers that were detected by ELISPOT 7 days later in the peripheral blood. All these patients had been negative in this assay before the cell transfer.

A follow-up analysis revealed later that immunological responder patients had a significantly higher overall survival compared to the nonresponder patients (58,6 versus 13,6 months) (126).

iii) HYPOTHESIS: INVOLVEMENT OF T-MEMORY STEM CELLS

Several of the reported observations deserve an explanation:

i) MTCs controlling tumor dormancy for long-term and selectively in the BM,

ii) flexibility and dynamics of MTCs,

iii) longevity, and

iv) expansion capacity.

We like to put forward the hypothesis that MTCs from BM contain a fraction of stem cell-like MTCs which, like BM resident hematopoietic stem cells (HSC) divide asymmetrically to provide self-renewal and differentiational capacity. BM can be considered as a central organ for maintenance of immunological B-cell and T-cell memory.

HSC, memory B- and memory T-cells are the only cells of the hematopoietic system that undergo self-renewal for the lifetime of the organism. These 3 cell types were shown to share a transcriptional program of self-renewal. This signature of up- and down-regulated transcripts was not consistently enriched in neuronal or embryonic stem cell populations and, therefore, appears to be restricted to the hematopoietic system (127). Wnt signaling (128) and mTOR signaling (129) seem to be involved in the formation of stem cell-like MTCs.

It is suggested that BM contains not only niches for HSCs but also niches for B- and T-cell MTCs. T-cell niches, rich in IL-7 and IL-15, allow for optimal T cell maintenance, as T cells can survive in the absence of antigen, in an environment rich in these two cytokines (130). Memory CD8+ T cells were found to co-localize with IL-7(+) stromal cells in BM (131). TNF family ligands define BM niches for T-cell memory (132). Several groups described that BM is a major reservoir and site of recruitment for central memory CD8+ T-cells (133). BM thus provides nests for migratory memory T-cells (134). F Di Rosa described T-lymphocyte interaction with stromal, bone and hematopoietic cells in the BM (135) and TC Becker et al. reported that BM is a preferred site for homeostatic proliferation of memory CD8+ T-cells (136).

A human memory T cell subset with stem cell-like properties was described to be a long-lived T cell population with enhanced capacity for self-renewal. These cells had a multipotent ability to derive central memory, effector memory and effector T-cells. Such memory cells contained specificities to multiple viral and self-tumor antigens. Their phenotype was CD45R0(-), CCR7(+), CD45RA(+), CD62L(+), CD27(+), CD28(+) and IL-7Rα(+) similar to naïve T cells. However, they expressed large amounts of CD95, IL-2Rß, CXCR3, and LFA-1, and showed numerous functional attributes distinctive of memory cells (137).

High-troughput sequencing of retroviral vector integration sites (ISs) allowed tracing the fate of more than 1700 individual T cell clones in gene therapy patients. These had received infusions of gene-corrected hematopoietic stem cells or mature lymphocytes. Such in vivo tracking of T-cells unveiled decade-long survival and activity of genetically modified T memory stem cells (138).

It is obvious that stem cell-like memory T-cells are of great relevance for T cell based adoptive immunotherapies. They represent a stable cellular vehicle with memory precursor potential.

FURTHER READING

Cancer immunotherapy has become of such clinical importance that we like to add some recent reviews for the interested reader.

A. Since immunological memory distinguishes immunotherapy from all other cancer therapies, we start with reviews about immunological memory (2004-2016).

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Chapter IV

Key points:

1. It took more than hundred years of development for cancer immunotherapy to become a standard treatment option for patients.

2. Therapeutic monoclonal antibodies, the products of B lymphocytes, can be targeted against defined molecules on tumor cells (e.g. HER2, CD20, VEGFR2). Of particular interest are presently immune checkpoint inhibitory antibodies directed against molecules on immune cells (e.g. CTLA-4, PD-1).

3. Immunotherapy based on T-cell mediated immunity includes active immunization with cancer vaccines (Chapter V) and adoptive cellular therapy with tumor-targeted T cells.

4. Dendritic cells are required for antigen presentation to T cells. They process tumor-associated antigens (TAAs) and present small peptides thereof in association with MHC molecules (pMHC complexes) to T cells. The pMHC

complexes associate like a key and a lock with antigen-specific receptors (TCRs) on CD8+ cytotoxic or CD4+ helper T-cells.

5. Adoptive cellular therapy with T-cells involves semi-allogeneic donor cell transfer to obtain Graft-versus-Leukemia (GvL) effects or autologous immune cells such as Tumor-infiltrating lymphocytes (TILs). Modern gene transfer technologies allow to produce T cells with transfected TAA specific TCRs or with chimeric TAA specific receptors (CARs).

6. This chapter includes auto-biographical notes and examples from the authors research on adoptive cellular immunotherapy.

7. Of particular interest are cancer-reactive memory T cells from bone marrow of cancer patients. They include long-lived stem-like memory T cells.

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Table 23 Curriculum Vitae Prof V Schirrmacher

1962-1967 Diploma Study of Biochemistry, University of Tübingen,

	Germany
1967-1970	PhD Thesis in Immunology, University of Cologne, Germany
1971-1973	Post-Doc at Karolinska Institute, Stockholm, Sweden
1973-1976	Senior Research Fellow , The London Hospital Medical College, London, England
1976-2008	Head of Division at DKFZ, Heidelberg, Germany
2009-present	Head of "Tumor Immunology" at IOZK, Cologne, Germany

Table 24 Milestones from immunology

Part I B cells and antibodies

1908	P Ehrlich* and Il Metschnikow*	Theory of antibody side chains and studies on phagocytic cells
1960	FM Burnet* and PB Medawar*	Acquired immunological tolerance
1972	GM Edelman* and RR Porter*	Chemical structure of antibodies
1984	NK Jerne*, GJF Köhler* and C Milstein*	Principles for production of monoclonal antibodies
1987	T Susumo*	Molecular genetics of antibody variability

* Nobel Laureats

Table 25 Characteristics of anti-receptor antibodies (in comparison to SMIs)

Target : Receptor ectodomain (vs TK domain)

Specificity: Very high (vs less high)

Binding: Receptor internalized, only slowly regenerated (vs rapidly reversible)

Dosing: intravenous, weekly (vs oral daily)

Tissue distribution: less complete than small molecules

Toxicity : rash, allergy (vs rash, diarrhea, pulmonary)

ADCC: possibly (vs not)

Table 26 Therapeutic monoclonal antibodies I : Tumor cell targets

Agent	Target	FDA-approved indication(s)
Trastuzumab (Herceptin)	HER2	Her2+ breast cancer and gastric cancer
Pertuzumab (Perjeta)	HER2	Her2+ breast cancer
Cetuximab (Erbitux)	HER1	CRC (k-ras wt); HNSCC
Bevacizumab (Avastin)	VEGF ligand	CRC; Renal Ca; NSCLL; GBM; Ova Ca
Ramucirumab (Cyramza)	VEGFR2	CRC; gastric Ca; NSCLL
Alemtuzumab (Campath)	CD52	B-cell CLL
Daratumumab (Darzalex)	CD38	Multiple Myeloma
Rituximab (Rituxan, Mabthera)	CD20	Non-Hodgkin`s lymphoma, CLL
Elutuzumab (Empliciti)	SLAM7	Multiple Myeloma
Denosumab (Xgeva)	RANKL	Giant cell tumor of the bone

Dinutuximab (Unituxin)	**GD2**	**Pediatric neuroblastoma**
Olaratumab (Lartruvo)	**PDGFRa**	**Soft tissue sarcoma**

Table 27 Therapeutic monoclonal antibodies II : Targets within the immune system (Checkpoint inhibitors)

Agent	Target	FDA-approved indication(s)
Ipilimumab (Yervoy)	**CTLA-4**	**Melanoma**
Nivolumab (Opdivo)	**PD-1**	**HNSCC, Non-small cell lung cancer (NSCLC), Renal cell carcinoma, Melanoma, Hodgkin Lymph.**
Atezolizumab (Tecentriq)	**PD-L1**	**Urothelial Ca, Non-small cell lung cancer (NSCLC)**
Durvalumab (Imfinzi)	**PD-L1**	**Urothelial carcinoma**

Table 28 Side effects of immune checkpoint inhibiting mabs

IMMUNE-RELATED ADVERSE EVENTS (irAEs)

Skin: rash, rarely bullous pemphigoid (BP)

Liver: hepatitis

Gastrointestinal: diarrhea, vomiting, colitis (all grades and high grade)

Kidney: acute interstitial nephritis

Endocrine: hypophysitis, more rarely thyroid disease, occasionally adrenal insufficiency

Fatigue: Fewer high grade events with anti-PD-1 than with anti-CTLA-4 mabs

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Table 29 Milestones from Immunology

Part II T cells and DCs

1973 R Zinkernagel* and P Doherty*: Discovery of MHC restriction of CTLs; Nobel price in 1996

1984 T Mak*: Cloning of the genes encoding the human T Cell antigen Receptor (TCR)

1991 T Boon: First molecular identification of a human TAA recognized as pMHC I epitope by a cytolytic T lymphocyte (CTL)

2001 M Feuerer: cancer-reactive memory T cells from bone marrow

2006 B Kyewski and L Klein: A central role for central tolerance

2011 RM Steinman*, B Beutler* and J Hoffmann*: Nobel price

for discovery of Dendritic cells (DCs), Toll-like receptors (TLRs) and innate immunity;

2015 JP Allison: Immune checkpoint therapy

*** Nobel Laureats**

Table 30 Milestones of cancer immunotherapy

1890 W Coley Discovery of Coley`s Toxin

1909 P Ehrlich Hypothesis of immune control

1960 BCG showing activity in bladder cancer

1967 Burnet & Thomas Immune surveillance of cancer

1986 HM Golomb Interferon-α for cancer immunotherapy

1991 P Van der Bruggen Cloning of the first human TAA (MAGE-1)

1992 Discovery of Interleukin-2

1996 Discovery of anti-tumoral effect of blockade of CTLA-4

1998 Introduction of Rituximab and Trastuzumab

1998 S Rosenberg Adoptive immunotherapy by T cells

2010 First FDA approved anti-tumor vaccine (Sipuleucel-T)

2011 Clinical application of CAR T cells

2011 Clinical application of anti-CTLA-4 mab

2014 Introduction of bispecific T-cell activating antibody BITE

2015 Clinical application of anti-PD1

2016 Clinical application of anti-PD-L1

2016 Clinical approval of the oncolytic virus T-VEC

2016 First application in humans of CRISPR gene-editing technique for CAR T-cell therapy

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Table 31 Major Research Topics of Prof V Schirrmacher

1976 -2008	**Immune responses against metastases: CTL, Th, memory T cells (MTC) from bone marrow (BM), ADI and GvL studies in early and late-stage metastasis**
1980-2000	**Cancer metastasis research (Eb/ESb mouse tumor model)**
1985-2000	**Active-specific immunotherapy: experimental: ATV-NDV vaccine, post-operative vaccination, Oncolytic virus, DNA vaccination via ear pinna;**
1990-2008	**Active-specific immunotherapy: multiple clinical studies: translational research with ATV-NDV vaccine in cooperation with University Clinics in Heidelberg**
2010-2017	**Development and individual application of the VOL-DC Vaccine and multimodal immunotherapy at IOZK**

ADI Adoptive immunotherapy; ATV-NDV Virus-modified Autologous Tumor Vaccine; CTL Cytotoxic T lymphocyte; GvL Graft-versus-Leukemia; VOL-DC Dendritic cell vaccine pulsed with viral oncolysate

CHAPTER V. ONCOLYTIC VIROTHERAPY AND VIRUS-MODIFIED ANTI-CANCER VACCINES

This Chapter is based on two excellent textbooks, namely "Viral Oncology. Basis Science and Clinical Application" (1) and "Viral Therapy of Cancer" (2). Oncolytic viruses and thereof derived anti-cancer vaccines is the major focus. This includes molecular modification of viruses with therapeutic transgenes, adaptor proteins and bispecific antibodies. For more details we recommend two further textbooks: "Gene Therapy of cancer. Methods and Protocols" (3) and "Molecular Vaccines" (4).

Since this Chapter will not present an overview of Cancer Vaccines in general, we recommend the book "Cancer Vaccines. From Research to Clinical Practice" (5). Since several promising oncolytic viruses in clinical development are RNA viruses belonging to the family of paramyxoviruses, the excellent textbook "The Biology of Paramyxoviruses" (6) is also recommended.

A. MILESTONES FROM VIROLOGY WITH RELEVANCE TO CANCER

Table 32 lists milestones from virology with relevance to cancer.

Research on viruses played an important role in molecular biology, in discovering oncogenes and in identifying oncogenic viruses which are etiological agents in 15%-20% of human cancers. There are six well-established human cancer viruses: *Hepatitic B Virus* (HBV), *Hepatitis C Virus* (HCV), *Human Pappilloma Virus* (HPV), *Human T cell leukemia virus type 1* (HTLV-1), *Epstein-Barr Virus* (EBV), and *Kaposi`s sarcoma herpesvirus* (KSHV).

Several Nobel Prizes for Physiology or Medicine were awarded for research from virology. 1966 the prize was given to FP Rouse who, as early as 1911, had discovered that a malignant tumor growing in chicken could be transferred to another fowl simply by exposing it to a cell-free filtrate. His life-long study on the responsible agent led to the discovery of the retrovirus *Rous Sarcoma Virus* (RSV). M Delbrück, AD Hershey and SE Luria obtained the Nobel Prize in 1969 for elucidating a virus replication cycle and the genetic structure of viruses. D Baltimore, R Dulbecco and HM Temin obtained the Nobel Prize in 1975 for their discovery of tumor virus interaction with host

cell DNA, leading to the description of cellular proto-oncogenes. In 2008, the Nobel Prize for Physiology or Medicine was awarded in part to H zur Hausen for his studies on human *Papillomaviruses* (HPV) and their role in cervical cancer development. The other part was given to two French scientists, FB Sinoussi and L Montagnier, for their discovery of the *human immunodeficiency virus* (HIV) causing AIDS.

i) PREVENTIVE CANCER VACCINES

It is obvious that preventing a disease is more desirable than to become sick and deserve treatment. Viral vaccines that have an effect of prevention on certain types of cancer is a success story.

***Hepatitis B virus* (HBV) was discovered in 1967 by the laboratory of BS Blumberg. An association with primary cancer of the liver (hepatocellular carcinoma (HCC)) was postulated in 1969. There are about 400 million people worldwide who are HBV carriers. Some of them are at risk of developing chronic liver disease and HCC. HCC is the third most common cause of death from cancer in males and the seventh in females. For the WHO, vaccine prevention of HCC was one of the two most important cancer control programs along with smoking cessation projects. In less than two decades after approval of the HBV vaccine, it was in use worldwide and a common and deadly cancer had decreased in incidence.**

There are other infectious agent-related cancers: EBV associated with endemic Burkitt`s lymphoma and nasopharyngeal carcinoma, HTLV-1 associated with adult T-cell leukemia/lymphoma (ATL), Helicobacter pylori associated with mucosa-associated lymphoid tissue lymphoma (MALTOMA) and gastric cancer, HPV associated with cervical and other anogenital cancers.

In 2006, the FDA approved a vaccine (*Gardasil*) against HPV. Another vaccine (*Cervarix*) against HPV was approved in 2007. Both vaccines consist of virus-like particles (VLPs) containing recombinant HPV-derived L1-proteins. The tetravalent *Gardasil* contains L1 proteins from HPV 6,11,16 and 18, while the bivalent *Cervarix* contains L1 proteins from HPV 16 and 18.

The widespread use of HBV and HPV prophylactic vaccines will result in a clear-cut drop in the prevalence of these two viruses in the human population.

ii) ONCOLYTIC VIRUSES

Oncolytic Virus Therapy (OVT) is an emerging biological cancer treatment modality which uses replication-competent viruses to destroy cancer cells. The good news is that such viruses replicate selectively in cancer cells and damage cancerous tissue without causing harm to normal tissues (7). Oncolytic viruses (OVs) and their effects in cancer patients have been observed along the last century. In the 1950s and 1960s there has been an increased attention to such type of viruses and a search to find the most suitable agents for clinical application (8).

Two pioneers in this field at the time were WA Cassel from Atlanta (USA) and J Lindenman from Zürich (Switzerland) (Table 31). Cassel had discovered the particular anti-neoplastic and immune-stimulatory properties of the avian paramyxovirus *Newcastle disease virus* (NDV) (9), while Lindenman had worked mostly with human *Influenza virus*. Both described the phenomenon of viral oncolysis *in vitro* and *in vivo* and discovered the importance of post-oncolytic anti-tumor immunity. Cassel engaged himself thereafter in the development and application of NDV-based oncolysate vaccines for the treatment of early-stage melanoma patients (8). Lindenman discovered type I interferon as an antiviral agent (10). The milestones of Table 32 end with S van Gool from Leuven (Belgium) who in 2015 described in detail in an orthotopic mouse glioma model, NDV induced Immunogenic Cell Death (ICD) and its consequences for induction of a powerful protective anti-tumor immune response (11). S Van Gool joined our Institution IOZK in Cologne (Germany) in 2016.

Examples of oncolytic viruses are listed in Table 33. Some viruses such as *H1 parvovirus, reovirus, NDV, Mumps virus* and *Moloney leukemia virus* (MLV) have a natural preference for cancer cells. Others, like *Measles virus* (MeV), *Adenovirus* (HAdV), *Vesicular Stomatitis Virus* (VSV), *Vaccinia* (VV) and *Herpes Simplex Virus* (HSV) can be engineered to make them cancer-specific.

For further details about the different families of OVs and about safety aspects, we recommend on excellent review (12). SJ Russel reviewed about oncolytic RNA viruses (13) and about the history of oncolytic viruses (14).

More recently, the concept of OV therapy shifted from the oncolytic activity of viruses as being important for the therapeutic effect towards the induced post-oncolytic immune response. So there is an overlap between OV therapy and immunotherapy (15,16). Finally, a recent review deals with T-VEC and other oncolytic viruses which are close to drug approval (17).

B. THE IMPORTANCE OF TYPE I INTERFERONS

The importance of type I IFN, discovered by J Lindenman (1974, Table 31) and its effects on the immune system can hardly be underestimated. Introduction and clinical use of IFNs are one of the major advances in oncology over the past three decades. The 1980s saw the clinical introduction of these highly purified pharmaceuticals as the first products of biotechnology for treatment of cancer. The 1990s were marked by an expansion in clinical use and a better understanding of the molecular events that influence the biological actions.

i) TUMORS RESPONDING TO IFN THERAPY

IFNs are now licensed in more than 50 countries for treatment of various viral, malignant, and immune disorders. In CML, melanoma, renal cell carcinoma, bladder carcinoma, Kaposi sarcoma, hairy cell leukemia, lymphomas, myeloma, polycythemia vera, locally advanced basal cell carcinoma, and essential thrombocythemia, interferons have therapeutic value. For example, the median survival for all patients with CML treated with IFN-α2 has been approximately 6 years, but over 90% of those with complete cytogenetic response were in remission at 10 years (18).

ii) TUMORS NOT RESPONDING TO IFN THERAPY

A majority of human tumors, in particular the carcinomas, do not respond to IFN therapy. Below we will discuss about escape mechanisms by tumor cells and also by viruses. To better understand underlying mechanisms requires understanding of molecular aspects of interferon receptors and signaling, of interferon-stimulated genes (ISGs) and of interferon-regulated proteins and their cellular effects (apoptosis, immunoregulation, angiogenesis inhibition).

IFNs bind to cell surface receptors, which are transmembrane proteins, and trigger signaling by their cytoplasmic domains. Cells of all lineages, except

mature erythrocytes, express receptors for type I and type II IFNs. Type I IFN receptors have two subunits. Both are needed to bind the ligand IFN-α or -ß with high affinity. The critical event in triggering the signaling process for type I and type II IFNs is ligand-driven dimerization of receptors which results in cascades of tyrosine phosphorylation.

There are more than 300 ISG proteins induced and transcriptionally regulated through IFN signaling pathways. They determine anti-tumor and immunoregulatory actions and have anti-viral effects. Suppression of IFNs and their stimulated gene products in and by malignant cells is emerging as an important contributor to the development of human cancer. For example, mutation of a gene in the IFN response pathway, RNAse L, increases prostate cancer risk (19). Epigenetic and genetic silencing of IFN signaling or ISG expression also likely influence tumor development (20).

iii) ONCOLYTIC VIRUSES AND OTHER AGENTS AS INDUCERS OF IFN

Cells of the innate immune system express pattern recognition receptors (PRRs) that detect viral nucleic acids and initiate host antiviral responses. Toll-like receptors (TLRs) are membrane bound while RIG-I-like receptors (RLRs) are present in the cell`s cytoplasm.

NDV, a potent inducer of type I IFN, is an agonist of RIG-I (in man) and MDA-5 (in birds) (21). RGT100 (Rigontec) is a synthetic oligonucleotide and ligand of RIG-I which is being evaluated in Phase I/II clinical studies (22). Examples of TLR agonists in clinical development are Poly-ICLC (Oncovir) (TLR3), Imiquimod (3M Pharmaceuticals) (approved) (TLR7) (17) and the oligonucleotide MGN-1703 (Mologen) (TLR9) (23).

Dendritic cells can function as a link between innate and adaptive immunity systems (24). They are a primary source for production of IFNs. Their maturation is also influenced by IFNs (24). Viral infection or viral oligonucleotides (22) such as double-stranded (ds) or single-stranded (ss) RNA or DNA or viral envelop proteins (25) can trigger the type I IFN response. Type I interferon gene induction involves the interferon regulatory factor family of transcription factors (26). NDV HN protein also activates TRAIL expression on cells and triggers NK cell activation via NKp46 (25,27).

Interferon-regulated proteins exert multiple functions: Some contribute to apoptosis, e.g. TRAIL, FasL, IRF-1, RNAse L, OAS, PKR (28,29). Others contribute to the immune response, e.g. MHC class I and II, LMP-2, LMP-7, TAP, CEA, TAG-72, CCL chemokines, CXC and CXCL chemokines (30, 31). Further induced proteins contribute to inhibition of angiogenesis, e.g. decrease of bFGF, VEGF, and IL-8, CXCL-9, CXCL-10 and CXCL-10 (32,33).

These facts demonstrate the intimate connection between the type I interferon response and physiological regulatory mechanisms in multicellular organisms.

C. IMMUNE ESCAPE MECHANISMS

Tumor cells evolve because they manage to avoid immune detection or destruction. They also manage to resist apoptosis and translational suppression. These are key responses used by normal cells to limit virus infection. Table 34 lists some immune escape mechanisms exerted by tumor cells. The mere fact that tumor cells are selected towards such escape mechanisms is a strong argument in favor of a role of immune surveillance in tumor development. This has been disputed for decades not only within the Institution DKFZ, in which I was engaged to develop immunotherapy strategies but also elsewhere.

Famous are meanwhile the three Es of cancer immunoediting proposed by GP Dunn, LJ Old and RD Schreiber (34). They characterize three basic situations in the battle between the immune system and cancer:

i) elimination,

ii) equilibrium, and

iii) escape.

To avoid attack by the immune system, tumors produce immunosuppressive cytokines (often observed in glioblastoma), recruit inhibitory cells such as regulary T cells (Tregs) or myeloid-derived suppressor cells (MDSCs), upregulate PD-L1, downregulate expression of TAAs and/or MHC molecules or express the enzyme indolamine-2,3-dioxygenase (IDO). This enzyme leads to tryptophane shortage in the tumor environment and to arrest of T cell proliferation (35).

To survive in a perhaps hostile environment, tumor cells also develope escape mechanisms against the growth inhibitory effects of type I interferons (36), against apoptosis inducing signaling (37) and against other mechanisms of control (38). This is the Achilles heel through which OVs have a chance to enter, to develop, replicate and kill their host cells (39).

Viruses also develop immune escape mechanisms. In order to be able to survive in a permissive host, e.g. the species from which the virus derived, OVs had to develop immune escape mechanisms. Many of the proteins developed by OVs in their respective host`s to fight their immune system have meanwhile been identified. Some of these are listed in Table 33.

Human natural viruses with immune suppressive mechanisms are not suited to induce immunogenic cell death mechanisms. It is therefore necessary to produce recombinant virus strains lacking the respective immunosuppressive genes. Often it reqires sophisticated techniques of genetic engineering to develop a recombinant viral vector with all the necessary properties: tumor selectivity, replication competence, therapeutic gene expression, antigenicity, immunogenicity etc.

The cells of the innate immune system of the central nervus system (CNS) also express PRRs (e.g. TLRs, RIG-I, MDA-5) that detect viral nucleic acids and initiate host antiviral responses. However, several emerging viruses (*West Nile Fever, Influenza A, Enterovirus 71, Ebola*) are recognized and internalized by host cell receptors (TLR, MMR, DC-SIGN, CD62 and Scavenger receptor B) and escape immune surveillance (7). Many RNA viruses express viral proteins that inhibit the host cell anti-virus type I interferon response, thus promoting virus replication and encephalitis. Examples are NS1 (*Influenza A*), VP 24 and VP 35 (*Ebola)*, and Glycoprotein (*Rabies*) (7) (Table 33) (7).

The avian virus NDV has the advantage that in non-permissive hosts such as humans certain native strains have

i) a high safety profile (39,40),

ii) tumor selective replication competence,

iii) oncolytic capacity, and

iv) no immune escape mechanism (38).

In contrast, in permissive hosts (birds) the virus developed immune escape mechanisms. These have to do with the viral V protein: It possesses the ability to inhibit IFN-α and this inhibitory function is located in its carboxy-terminal domain (41). The V protein plays an important role in host range restriction (42). In birds but not in man it targets phosphorylated STAT1 to block IFN-I signaling (43). It is of particular significance that immune evasion by the primate virus Ebola uses exactly the same pathways of signaling through RIG-I and type I interferon receptor (IFNRα) as NDV in birds (44). In man, in case of NDV, these signaling circuits lead to immune activation whereas in case of *Ebola* their inhibition via two defined glycoproteins (VP 24 and VP 35) has devastating effects (44).

D. CONCEPTS OF APPLICATION OF ONCOLYTIC VIRUSES

Table 35 lists various concepts of in vivo application of OVs. As single agents, they may be applied intratumorally (if the tumor is non-operable and can be targeted), systemically or locoregionally. There are various barriers to efficient oncolytic virus delivery via the bloodstream:

i) virus neutralization by serum factors such as complement components (46),

ii) sequestration by the mononuclear phagocytic system or insufficient extravasation.

Combining OVs with carrier cells, such as mesenchymal stem cells (47) or activated T cells (48) might be a solution for certain virus applications. Another possibility is to combine OVs with adapter proteins, such as bispecific antibodies or trispecific immunocytokines (see below, Tables 38,39).

NDV (11) and several other OVs induce immunogenic cell death (ICD). Many components released by ICD have a pro-inflammatory effect and support an immune response against released TAAs. It is therefore logic to try to generate ICD under defined conditions *ex vivo* and thereby to create an anti-tumor vaccine.

WA Cassel, J Sinkovicz and others performed in the 1960s and 1970s clinical studies with oncolysate vaccines in melanoma and other cancer patients. Their results have been summarized (8). For all these pioneers it was not easy to perform such studies. The scientific basis was rather weak and skepticism or even opposition by clinicians was omnipresent.

OVs can be combined with tumor cells or TAAs for the purpose of anti-tumor vaccination. Table 34 lists 3 possibilities:

i) oncolysate vaccines (8),

ii) live tumor cell vaccine (ATV-NDV) (49),

iii) oncolysate-pulsed DC vaccine (VOL-DC) (50).

Oncolysate vaccines were developed in the 1960s, ATV-NDV vaccine in the 1990s and VOL-DC vaccine in 2010. Each step of development was based on a different concept of increasing the immunogenicity of the vaccine. First, it was the combination of a virus with a tumor lysate. The second step followed the concept that a live cell vaccine is superior to a cell lysate. Finally, the live tumor cell was replaced by a professional DC as APC presenting TAAs from processed oncolysate.

E. TARGETED THERAPY WITH ONCOLYTIC NDV

Oncolytic NDV has been applied for treatment of Glioblastoma multiforme (GBM). GBM or grade IV glioma is one of the most lethal forms of human brain cancer. The signaling pathways that are responsible for high grade glioma initiation, migration, and invasion are becoming elucidated. The abnormal proliferation and aggressive invasion behavior of GBM is reported to be associated with aberrant Rac1 protein signaling.

It is in this respect of particular interest that NDV has been demonstrated to interact with Rac1 (51). It uses Rac1 upon viral entry, syncytium induction, and actin reorganization of the infected cell as part of the replication process. Ultimately, intracellular stress in the infected glioma cell leads to cell death (52).

Table 36 lists different pathways which NDV can target in human GBM tumor cells:

i) apoptosis pathways,

ii) cell cycle arrest pathways,

iii) Rac1 signalling pathways and

iv) RIG-I and IFNRα signaling pathways.

NDV mediates its oncolytic effect by both intrinsic and extrinsic caspase-dependent pathways of cell death (53). NDV-induced apoptosis is dependent on upregulation of TRAIL and caspase activation, especially in apoptosis-resistant cells (54). This causes opening of mitochondrial permeability transition pores and loss of mitochondrial membrane potential, leading to activation of the apoptosis process (55). MAPK and ER stress pathways also play important roles in NDV-mediated oncolysis (37,53).

Interestingly, NDV can exert oncolytic activity also against hypoxic cancer cells, which is of clinical relevance (56). In addition, tumor selectivity of NDV replication has been attributed to defects of tumor cells in antiviral defense (57,58). Such defects involved the early intra-cellular response initiated by RIG-I as well as the late feedback-loop response to secreted type I IFN initiated by the type I IFNR membrane receptor (36).

Of importance was also the discovery in 2000 (59) that NDV infection induces a B7-1/B7-2-independent costimulatory activity in human melanoma cells. This allowed to break tolerance of a melanoma-specific T helper cell line.

The sum of all these properties make NDV to an interesting biologic agent to overcome immune escape and break therapy resistancies (38).

F. MILESTONES IN MODERN DEVELOPMENT OF OV THERAPY

In 2014, "Frontiers in Immunology" devoted an e-book to the timely Topic: "Harnessing oncolytic virus-mediated antitumor immunity" (60). Topic Editors were P Fournier and V Schirrmacher. Twelve articles by experts from Canada, Germany and the USA were dealing with the following aspects:

i) viral oncolysis and the immune response,

ii) post-oncolytic anti-tumor immune responses,

iii) harnessing OVs with other agents,

iv) delivery of OVs,

v) combining OVs with pharmacological modulators and/or chemotherapy.

We concluded that it was remarkable to what extent the experts in the field were in accord by emphasizing the potential importance of OVs for systemic T cell-mediated anti-tumor immunity.

Table 37 lists milestones in modern development of oncolytic virotherapy. It took about 25 years to develop the first approved OV T-VEC. In 1991, *Herpes Simplex Virus* (HSV) was genetically engineered for the first time to generate a mutant with reduced neurotoxicity. In 1997, an albumin promoter/enhancer was introduced into an HSV vector for targeting hepatoma. In 2001, HSV had been modified with transgenes encoding IL-12 and GM-CSF to improve T cell recruitment and immune stimulation. The first clinical study with intralesional application of T-VEC began in 2009. In 2015 T-VEC was the first approved OV for melanoma immunotherapy.

Barriers affecting efficiency of OV therapy may exist not only in the blood but also within a solid tumor mass. They may prevent optimal virus spread. Such barriers are:

i) extracellular matrix (ECM),

ii) interstitial tissue pressure,

iii) host innate or acquired immune effects.

More efficient spread may be achieved by OVs encoding ECM degrading enzymes or immune combatting proteins. Similar effects may be achieved with antifibrotic drugs or with immunosuppressive drugs, such as low dose cyclophosphamide (61).

The list of Table 37 relates to the OVs *Herpes Simplex Virus* (HSV), *Adenovirus* (AdV), *Measles Virus* (MeV) and *Vaccinia Virus* (VV). Many efforts

have been made to overcome the mentioned barriers and to improve the effectivity of OV therapy (7). These included:

1. Improvements in specificity und tumor selectivity by translational targeting (1991), transcriptional targeting (1997), transductional targeting (2005) or microRNA targeting (2008).

2. Improvements in potency by prodrug activation (1998), immune stimulation (2001), radiovirotherapy (2004) or incorporation of matrix degrading proteins (2006).

3. Improvements of delivery and spread by the addition of immunosuppressive drugs (1999), by the use of cell carriers (2006), by shielding with polymers (2008) and by use of infectious nucleic acid (2011).

For further details see the excellent review by SJ Russel et al. (7).

The year 2015 witnessed two important milestones:

i) approval by the FDA of the first OV (T-VEC) for clinical application,

ii) approval by the German authorities of the VOL-DC vaccine for application by IOZK, Cologne.

The latter includes NDV production according to GMP guidelines. IOZK is the first Institution that has been successful in producing pure high quality oncolytic NDV.

Table 38 lists the progress that has been made in the last decade to produce new recombinant NDV viruses with additional therapeutic genes. The strategies can be categorized as above:

1. Improvements in specificity: targeting TAAs (2008, 2013, 2015).

2. Improvements in potency: introduction of immune stimulatory cytokines such as GM-CSF (2007), IL-2 (2008) and IL-15 (2017) or of the costimulatory ligand ICOSL (2017), combining oncolysis with suppression of angiogenesis (2008), targeting apoptosis pathways via TRAIL (2014), Fas (2015), apoptin (2012) or p53 (2016), introduction of the interferon antagonist NS1 (2009).

3. Improvements in delivery and spread: increasing the activity of the fusion protein (2010, 2013) and of prodrug targeting (2013).

The Table includes the PubMed Library ID numbers (PMID) of the respective original manuscripts.

G. VIRUS-MODIFIED ANTI-CANCER VACCINES:

MY CONTRIBUTION TO ONCOLYTIC VIRUS THERAPY

In our studies, tumor selective replication of NDV was associated with tumor cell defects in antiviral defense (57). Resistance of normal cells and susceptibility of tumor cells to infection by NDV correlated with cellular expression of RIG-I, IRF3, IFN-ß and IRF7 (62). In addition, there was an important role of the cell surface receptor for the type I interferon response, IFNRα (36).

i) THE AUTOLOGOUS VIRUS-MODIFIED LIVE-CELL TUMOR VACCINE ATV-NDV

Our work on the NDV-modified tumor vaccine ATV-NDV began in 1986 (63). In the above mentioned ESb tumor model that we had established to study cancer metastasis, we had observed that post-operative vaccination with virally modified but not with unmodified tumor cells had a prophylactic effect against the outgrowth of metastases. This effect was due to establishment of specific systemic anti-tumor immunity (64).

The modification of tumor cells with a low dose of NDV was found to cause augmentation of the tumor-specific CTL response (65). This effect was a result of CD4+ and CD8+ immune T-cell cooperation (66). Finally, in 1990, we could demonstrate that the potentiation of the tumor-specific CTL response was mediated via induction of interferon-α/ß (67).

BOX 10 1988 Surgeons confronted with immunotherapy

Improvements since 1978

This was about the time period when we started with translational studies, first with human tumor cells and human immune cells *ex vivo*. Later, we developed protocols for the design of an autologous, irradiation-inactivated live tumor cell vaccine similar to the mouse ATV-NDV vaccine (68). Human tumor cell modification by virus infection was found to be an efficient and safe way to produce cancer vaccine with pleiotropic immune stimulatory properties when using NDV (69).

So we tried to convince clinical partners to perform clinical application studies. One of the first colleagues who was interested was the surgeon Prof P Schlag. Ethical committees gave the permit to perform those studies. We had insisted to perform active-specific immunization (ASI) only in the post-operative adjuvant situation, based on our conviction that the patient`s immune system had to be as intact as possible to react to specific anti-tumor vaccination.

BOX 11 1990 – 2008 Clinical studies

Post-operative ASI studies were also performed in primary operated breast cancer patients with the help of Prof G Bastert and Dr T Ahlert. We used a post-operative window of 4 weeks to begin with the vaccinations before start of the standard chemotherapy. The results, published in 1997 (70), revealed that the ATV-NDV seemed to be effective provided that tumor cell number and tumor cell viability of the individually produced vaccine fulfilled defined parameters.

Another clinical study worth mentioning is the post-operative ASI study performed in patients with glioblastoma multiforme (GBM). The study was initiated by my colleague Dr C Herold-Mende in 1995. Many clinicians thought it would be ridiculous to try such procedure in GBM patients because of the problem with the blood-brain barrier. C Herold-Mende had improved the procedure to produce the ATV-NDV vaccine. Instead of using cells from freshly operated cancer specimens with depletion of infiltrated lymphocytes – the standard procedure until this time -, she employed tumor cells from

autologous cell cultures, a difficult procedure that she had managed to perfection.

The objective of the study was to assess feasibility, safety, and clinical benefit. The results were as follows: The median progression-free survival of vaccinated patients (n = 23) was 40 weeks versus 26 weeks in 87 non-vaccinated control patients from the same time period and the same clinic. The median overall survival (OS) was 100 weeks (versus 49 weeks in the control patient group, p < 0.001). In the vaccinated group, immune monitoring revealed significant increases of skin delayed-type hypersensitivity (DTH) reactivity, of numbers of tumor-reactive memory T cells in the blood and in the number of CD8+ tumor-infiltrating T lymphocytes (TILs) in frozen tissue slices from GBM recurrencies. Also, there was one complete remission of non-resectable remaining brain tumor (71).

BOX 12 2006 An unusual single case of GBM immunotherapy

Finally, we were able together with P Schlag to perform a prospectively randomized clinical trial. The study was started in the early 1990s. It investigated the efficiency of ATV-NDV vaccination after liver resection for hepatic metastases of CRC as a tertiary prevention method. 25 of such stage IV CRC patients were vaccinated and compared with a similar number of non-vaccinated control patients.

After an exceptionally long follow-up period of 9-10 years, there was no significant difference between the vaccinated and the control arm. However, when stratified for tumor localization there were significant differences between vaccinated colon and rectum carcinoma patients. A significant benefit of vaccination was only seen in the colon cancer patients. In the control arm, 78,6% of the patients had died, while in the vaccinated arm only 30,8% of the patients had died. The trial results, published in 2009, provide clinical evidence for the value and potential for long-term improvement of overall survival (OS) of the autologous cancer vaccine ATV-NDV (72).

An explanation for the basic mechanism behind this remarkable result was offered in 2014 (49). It suggests that the vaccine was capable of reactivating pre-existing cancer-reactive MTCs and that their persistence over the many years prevented outgrowth of metastases.

BOX 13 2008 Retirement Symposium and Farewell Party

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ii) THE NDV-MODIFIED DENDRITIC CELL VACCINE VOL-DC

After my official retirement at DKFZ (Heidelberg, Germany) in 2008, I was not ready for general retirement. I wanted to continue to work on the topics I was engaged with. Luckily, I found a proper place at the Immunological and Oncological Center (IOZK) in Cologne, Germany. The CEO Dr W Stuecker and clinical colleagues had established this unique Institution in 1985. Since 2005 they work with NDV, autologous tumor cells and patient-derived DCs.

Personalized medicine is a basic concept at IOZK. It includes combinations of anti-cancer vaccination with other treatment modalities, in particular with local hyperthermia and with immune checkpoint inhibitors. The GMP certificate obtained in 2015 includes NDV. This virus was produced worldwide for the first time under the strict guidlines of Good Manufacturing Procedures (GMP).

The vaccine VOL-DC which we developed at IOZK can be considered a second-generation vaccine following the first-generation vaccine ATV-NDV. To use an oncolysate from ATV-NDV and pulse it onto DCs has two advantages:

1. no irradiation step is necessary anymore to inactivate live tumor cells and

2. the replacement of live irradiated but viable tumor cells by DCs produces another type of live cell vaccine which has the capacity of de novo generation of TAA-specific cells from naïve T cells.

iii) MULTIMODAL INDIVIDUAL CANCER IMMUNOTHERAPY

The multimodal individual cancer immunotherapy performed at IOZK consists basically of two steps:

a) First step: immune monitoring and immune modulation/conditioning

Active specific immunization of cancer patients requires an immune system which is competent and not dysregulated. Therefore, before patients at IOZK receive specific vaccinations, their immune system is assessed in depth and modulated if required. Immune modulation/conditioning is done by modulated Electrohyperthermia (mEHT) combined with systemic (i.v.) application of NDV (73).

mEHT is used in such a way that the tissue temperature becomes elevated to 38,5 to 40,5° C. mEHT is applied either locally or systemically, depending on the clinical situation. mEHT is combined with systemic oncolytic virotherapy based on the observations that mEHT can enhance virus tumor targeting (74) and virus replication (75). Viral infection of tumor cells and hyperthermia with a radiofrequency of 13 MHz cause an Endoplasmatic Reticulum (ER) stress response, modify the surface properties of tumor cells and induce Immunogenic tumor Cell Death (ICD) mechanisms (76).

Systemic NDV application can have the following positive effects:

i) induction of IFN-α/ß; this inhibits secretion of Th2 cytokines (IL-4 and IL-5), stimulates Th1 cells and counteracts Treg cells (77),

ii) induction of ICD (76), and

iii) priming of viral oncolysate (VOL)-reactive T helper cells which can be monitored by an in vitro ELISPOT assay (78).

b) Second step: Active-specific immunization with VOL-DC

The second step of this multimodality treatment consists of active-specific autologous anti-tumor vaccination. The VOL-DC vaccine consists of patient-derived DCs combined with viral oncolysate. Viral oncolysate (VOL) serves for

DC programming, polarization and TAA information transfer. We decided to use tumorlysate instead of defined TAAs to produce professional APCs based on a number of pre-studies. These revealed

i) that tumorlysate can serve as a source of TAAs,

ii) that tumorlysate-pulsed DCs as APCs do not induce autoimmune reactivity

iii) that tumorlysate is physiologic and makes it unnecessary to purify TAAs.

To avoid a wrong polarization of T helper cell responses, we decided to introduce so called "danger signals" into the DC vaccine by virus infection. Upon loading of DCs with VOL, they become infected by NDV. The foreign non-capped RNA in the cytoplasm of the infected DC is a Pathogen-Associated Molecular Pattern (PAMP) that stimulates RIG-I receptors, induces a strong type I IFN response and innate immunity (79). Further features of NDV relate to the immune response:

i) up-regulation of MHC I molecules (80),

ii) activation of NK cells (27),

iii) activation of monocytes and macrophages (81,82),

iv) reprogramming and polarization of DCs (83),

v) costimulation of CD4+ (59) and CD8+ (84) T-cells.

IOZK has a GMP facility in which patient-derived tumor cells from operation specimens are propagated in cell culture. Following NDV infection and viral oncolysis, the material is freeze-thawed to become devoid of viable tumor cells and then co-incubated with immature DCs from a short-term culture of a sample of the patient`s white blood cells. After a further maturation step, the vaccine is ready for intradermal application to the patient. The differentiation process from adherent monocytes (CD14++,CD86+,CD209-,CD83-) via semiadherent immature DCs (CD14+,CD86+,CD209++,CD83-) to floating mature DCs (CD14-,CD86++,CD209+,CD83++) is followed by flow cytometry.

Functional tests: A study with memory T cells from breast cancer patients compared the stimulatory capacity of VOL-DCs to that of tumorlysate-pulsed DCs. Stimulation with VOL-DCs showed increased expression of costimulatory molecules and higher IFN-γ ELISPOT responses. Supernatants from co-cultures of MTCs and VOL-DCs contained increased titers of IFN-α and IL-15 (85). Thus, VOL-DCs were superior to tumorlysate-pulsed DCs and potently stimulated cancer-reactive MTCs from cancer patients.

c) Side effects of the multimodal immunotherapy at IOZK

Most side effects were moderate (common cytotoxicity criteria grade 1-2) with eventually short term flu-like symptoms (fever, headaches and chill). There was no negative impact on quality of life.

d) Clinical results from single cases or case-series studies

Since 2005, more than 2000 cancer patients were treated with biological therapies. Among these were more than 70 different types of cancer. In the last two years there has been a steady increase of patients with GBM and also of children with Diffuse Intrinsic Pontine Glioma (DIPG). This increase of patients with GBM and DIPG is due to the recruitment of the Paediatric Neurooncologist S van Gool from Leuven University (Belgium) who joined the IOZK in September 2015.

Children with DIPG have a 5-year survival rate of <1%. The median overall survival of children diagnosed with DIPG is approximately 9 months. It is two early yet to make a general statement about the effect in this fatal disease by the immunotherapy exerted at IOZK although we are optimistic to achieve an improvement.

We can summarize new results from a retrospective case-series study of adult GBM patients treated at IOZK between 2006 and 2010. Median OS of 10 newly diagnosed operated patients was 30 months in comparison to 14.6 months after standard radio/chemotherapy according to the Stupp protocol. The 5-year survival of primary operated GBM in the current series with combinatorial immunotherapy is almost 20% (73).

In addition, we recently published two remarkable single cases. One describes long-term remission of prostate cancer with extensive bone

metastases (86), the other reports long-term survival of a breast cancer patient with extensive liver metastases (78).

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iv) NDV-TARGETED MULTISPECIFIC ADAPTER PROTEINS

Table 39 lists examples of such adapter proteins.

a) Improvement of tumor targeting

To improve the specificity of tumor targeting and to reduce side effects we have developed in the past recombinant fusion proteins which bind with one arm to the hemagglutinin-neuraminidase protein (HN) of NDV. Genetic engineering allowed to clone vL and vH genes from an NDV neutralizing anti-HN mab and to produce from these with the help of a linker a single chain Fv (scFv) binding site (anti-HN). The fusion of a c-DNA coding for human IL-2 enabled the introduction of a new specific second binding site. IL-2 binds with high affinity to the human IL-2 receptor α chain (IL-2Rα). This is expressed on a variety of human HTLV-induced lymphomas. With such cell lines we performed *in vitro* and thereafter *in vivo* proof-of-principle studies of tumor retargeting of NDV. Tumor cell binding occurred through IL-2R and IL-2 of the adapter protein attached to neutralized HN. It was reassuring that this new bridge between the virus and the tumor cell allowed the F protein of NDV to fuse with the tumor cell membrane so that virus infection and replication could follow. The in vivo re-targeting experiments revealed that this approach reduced the liver-toxic side effects of the virus upon high-dose application (87).

b) ATV-NDV vaccine-attached adapter proteins: delivery of 3 T-cell activating signals to produce TAA independent anti-tumor activity

We also produced a tri-specific fusion protein for T cell costimulation. It contained an anti-HN binding site to attach to NDV infected vaccine cells (e.g. ATV-NDV) and two further binding sites: anti-CD28 (for delivery of costimulatory signal 2a) and IL-2 for targeting the IL-2 receptor (CD25, to deliver a costimulatory signal 2b). In addition, we created the following bispecific fusion proteins for T cell activation: anti-HN-anti-CD3 (for delivering

signal 1) and anti-HN-anti-CD28 (for delivering costimulatory signal 2a). Each binding site in the fusion proteins is monovalent and can as such not cause receptor cross-linking and cell activation. This only occurs in the presence of ATV-NDV vaccine cells, where multiple HN molecules in the plasma membrane allow for aggregation of the fusion proteins, for cross-linking with T cells and for T cell receptor or co-receptor aggregation and T cell activation.

The new fusion proteins were first tested *in vitro*. For this, we designed a so-called tumor neutralization assay. It consists of a tumor cell monolayer. On top of this cell culture we added human peripheral blood mononuclear cells (PBMC) or purified T cells and tumor vaccine as stimulatory cells. The tumor vaccine cells consisted of NDV-infected irradiated tumor cells with or without attached above fusion proteins. The T cells could thus become non-specifically activated by the combined signals exerted by NDV, anti-CD3 (1), anti-CD28 (2a) and IL-2 (2b) (88-90).

It took about 3 days of co-culture for full T cell activation and 2 additional days for destruction of the tumor monolayer. This non-specific anti-tumor activity induced by the above signals was mediated by the activated T cells themselves and by soluble factors secreted (91). Upon transfer of the activated T cells to a second tumor monolayer, this was destroyed as well. This procedure could be repeated for a period of about 10 days. The most durable T cell response in this assay required all three activation signals (88).

Such TAA-independent T cell anti-tumor activity (91) may be very useful in cases of tumor immune escape via loss of TAA expression.

c) ATV-NDV-attached anti-CD28 adapter protein causing strong costimulation of TAA-specific anergized T cells in late-stage metastasized colorectal carcinoma patients

In a pilot Phase I dose-escalation clinical study, 14 CRC patients with late-stage disease, which could not be operated anymore with curative intent, were treated with the vaccine ATV-NDV to which different amounts of the adapter protein anti-HN-anti-CD28 were attached. No severe adverse events were observed. With the highest dose of 1 μg purified adapter protein, strong T-cell costimulation occurred which enabled re-activation of possibly already anergized TAA-specific MTCs (92). The study suggests that the three-

component vaccine is safe and can reactivate TAA-specific T cells from patients with advanced-stage cancer.

We are apparently worldwide the first and, so far, only group who has developed bispecific antibodies and trispecific immunocytokines for attachment to a universal anchor molecule of an oncolytic virus. The advantages of this concept for the future have been summarized (93). The following summary includes new strategic thoughts for the future.

1. A viral molecule, such as HN of NDV, can serve as a universal anchor or attachment molecule of an infected tumor cell.

2. A bispecific single chain antibody (bsab) such as anti-HN-anti-CD28 can be added to a ATV-NDV type tumor vaccine in a defined dose (89) to augment T cell costimulatory signals; in this way, T-cell co-stimulatory molecules can be attached to any type of tumor cell infectable by NDV.

3. If the vaccine cells express autologous TAAs and the patient`s immune system is already tolerant (anergic) towards these, the addition to the vaccine of anti-HN-anti-CD28 can overcome this anergy (92). It is possible that an intensification of both signals (1 and 2), could break T cell anergy towards TAAs even more efficiently.

4. A tumor cell without TAAs can be infected by an OV and modified with two adapter proteins to deliver signal 1 and 2. This suffices to activate naïve T cells. Anti-HN-anti-CD3 bsab delivers TCR-complex mediated signal 1 and the addition of anti-HN-anti-CD28 or anti-HN-IL-2-anti-CD28 delivers co-stimulatory signals 2a and 2b (89,90); signal intensity by this modular approach mediated through CD3, CD25 or CD28 can be adapted to the clinical situation by varying the amount of the respective adapter protein.

5. A vaccine modified according to 4. can induce in naïve T cells strong anti-tumor activity that can be quantified in a tumor neutralization assay (TNA) in vitro (88).

6. When IL-2 is incorporated into a bsab construct such as anti-HN-anti-CD28, a trispecific immunocytokine (anti-HN-IL-2-anti-CD28) is being created. The IL-2 in this construct confers costimulatory signals (2b) through CD25 (89,90). Such trispecific immunocytokine modified ATV-NDV stimulates naïve T cells

to destroy tumor cell monolayers upon successive transfers for about 10 days (89).

7. A patient`s T cells , e.g. from PBMC, activated *ex vivo* according to 6. can be re-infused to the patient for adoptive T-cell immunotherapy.

8. Before transfer to the patient, the activated T-cells can be further loaded with oncolytic NDV (48). The loosely attached NDV would be hitchhiking through the patient`s blood on its T cells to be carried into the tumor tissue. Upon arrival there, some of the virus could become released and, in contact with tumor cells, would be capable to infect these. This would intensify the anti-tumor activity exerted through the activated T cells themselves. Such a scenario of tumor attack through activated T cells loaded with oncolytic NDV has already been observed and studied *in vitro* (48).

9. In addition or alternatively, the patient`s bone marrow derived memory T cells could be stimulated in a TAA-specific way by ATV-NDV vaccine without adapter proteins in a short-term memory re-stimulation assay (2-3 days). Such MTCs could similarly be loaded with oncolytic NDV before adoptive transfer to the patient. These re-activated MTCs would target even better to the tumor tissue, release their NDV for tumor cell cross-infection, and attack the tumor via specific CTL activity. Another advantage of this strategy would be the likelihood of co-transfer of stem-like MTCs with their long-lasting memory function (see Chapter IV).

v) MULTIMODAL CANCER THERAPY INVOLVING NDV, AUTOLOGOUS IMMUNE CELLS AND TRI-SPECIFIC ANTIBODIES

a) This new concept has been described recently (94).

The basic idea is that of cancer pre-targeting by on oncolytic virus which is followed by transfer of immune cells which are loaded with tri-specific scFv antibodies. These bind with two binding sites (second and third) to the immune cells and direct the first binding site towards a viral antigen of the OV used for pre-targeting.

Table 40 lists examples from the patent (see below) of potential targets of future tri-specific adapter proteins. The autologous immune cells can be T cells or DCs. The T cells could be isolated from the peripheral blood or from

bone marrow and could contain naïve as well as memory T cells. These cells could be pre-activated or not. The DCs could also be isolated from the peripheral blood or from bone marrow (95) and they also could be pre-activated, loaded with tumor lysate or defined TAAs and polarized towards DC1. The target molecules on these immune cells for the tri-specific adapter proteins are listed in Table 39 and include CD differentiation antigens and growth factor receptors, cytokine receptors and interferon receptors.

Two binding sites of the adapter molecule should bind to the cell surface molecules of the immune cells in order to achieve an affinity and stability sufficient for achieving the virus targeting during the adoptive cell transfer.

b) "Multi-modal cancer therapy using viral hitch-hiking"

This is the title of the US Patent No. US 8,142,791 B2 granted Mar. 27, 2012. It is also the title of the European Patent No. EP 2091972 granted Jan. 13, 2016. The patents belong to W Stücker and V Schirrmacher.

The idea behind the Patent is a multi-step procedure to target T cells and/or DCs to the site of a tumor: 1. Pre-condition the tumor microenvironment, for instance by local hyperthermia, 2. Target the tumor with oncolytic NDV by local or systemic virus application or by virus hitch-hiking, 3. Load the patient`s T cells or DCs with tri-specific adapter proteins in such a way that two binding sites are directed towards the T-cell or DC and that the third site (anti-HN or anti-F) remains free, 4. Re-infuse the loaded cells systemically into the patient so that the virus-specific binding sites can dock to the viral antigens at the virus-infected tumor site.

OVs, tumor cells and adapter proteins allow for a multitude of new clinical applications. The example presented with NDV and NDV-specific adapter proteins can be transferred to other OVs. It should be of advantage to a pharmaceutical company that develops OVs to also develop OV-specific adapter proteins. They can then be used to improve virus to tumor targeting and immune stimulation, in particular stimulation of anergized anti-tumor T cells. They could also serve to improve recruitment of cells to the site of the virus-infected tumor cell, for instance DCs. OV hitchhiking on other cells could improve the efficacy of tumor targeting. This could be followed by targeting *ex vivo* activated immune cells via trispecific adapter proteins.

vi) NDV HN AND F PLASMID DNA FOR IMPROVING ANTI-TUMOR VACCINATION

Table 41 lists the use of plasmid vaccines with genes from an OV to improve innate immunity activation. Plasmids coding for HN or F of NDV served not only this purpose but also the purpose of better understanding structure-function relationships.

The crystallization of the F protein in 2001 has been important for structural studies. Electron microscopic imaging showed the F0`product to consist of club-shaped particles. Trypsin treatment produced disulfide-linked F2 and F1`chains. These showed extensive rosette-like aggregation, indicative of a conformational change (96).

Studies with pHN or pF transfected cells allowed to better understand the function of these two viral spike proteins. HN showed a fusion promoting activity towards F resulting in syncytia formation (97). HN but not F was capable of paracrine activation of an IFN-α response in human PBMC and to induce upregulation of TRAIL (25). HN also was found to determine viral tropism and virulence (98).

pHN plasmids were also tested for their ability to function as molecular adjuvant within an anti-tumor DNA vaccine. We combined these studies with our experience of mouse ear pinna vaccination. Since the ear pinna is rich in DCs we further tried to target the anti-tumor DNA vaccine to DCs via incorporation of a short CD11c promoter sequence which we had just identified (99).

The studies revealed that pHN can indeed serve as a powerful molecular adjuvant in triggering IFN-α and innate anti-tumor immunity (100). Such immune-stimulatory activity in the ear pinna reduced tumor growth and caused changes in the immune cell compartment of the microenvironment of an intradermally growing transplanted mammary carcinoma: a significant increase in NK-cell infiltration and a decrease of infiltration by MDSC suppressor cells (101). The studies highlight the potential adjuvant activity of the HN gene of NDV.

A pHN plasmid was also used for therapeutic targeting of liver cancer. A total of about 38.6 million mortalities occur due to liver cancer annually,

worldwide. Gene therapy is considered a promising option. A recent study, investigated the synergistic effects of the abilities of the HN protein of NDV, the pro-apoptotic factor apoptin from *chicken anemia virus*, VP3 and the interferon-γ inducer interleukin-18 (IL-18) in antagonizing liver cancer in a murine model. The results revealed that the recombinant DNA vaccine containing HN, VP3 and IL-18 genes inhibited cell proliferation and induced autophagy via the mitochondrial pathway *in vivo* and *in vitro* in H22 hepatoma (102).

Chapter V

Key points:

1. Milestones from virology research with relevance to cancer include the identification of tumor-causing viruses (e.g. RSV, HCV, HPV) as well as the discovery of viruses with oncolytic (tumor destroying) potential (e.g. HSV, HAdV, NDV).

2. Tumors and viruses share the ability to develop escape mechanisms from immune recognition and control. Molecular studies revealed the mechanisms behind such escape mechanisms and paved the way for the development of strategies to overcome and break such resistance mechanisms. Genetic modification of oncolytic viruses (OVs) is one way towards such a goal.

3. OV-modified anti-cancer vaccines have been developed starting with oncolysate vaccines, followed later with live infected tumor cells (e.g. ATV-NDV) and continued with oncolysate-pulsed dendritic cells (e.g. VOL-DC). Clinical studies with such vaccines, performed for now over 50 years in case of NDV, have shown promising results in the absence of severe side effects.

4. Bi-specific OV targeted adapter proteins can be used to improve the immunogenicity of OV infected tumor cells. They can bridge immune cells, deliver signals to them and thereby modulate their activity.

5. Tri-specific adapter proteins, binding with one arm to an oncolytic virus (OV) and with the other arms to distinct targets on T cells and/or DCs can

provide bridges between the tumor and its host`s immune cells. Such reagents can improve tumor targeting of immune cells.

5. DNA plasmids may incorporate genes from OVs to improve innate immunity activation.

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Table 32 Milestones from virology with relevance to cancer

1965 WA Cassel: NDV as antineoplastic agent, virol oncolysis and post-oncolytic immunity

1966 FP Rous*: *Rouse Sarcoma Virus* (RSV), a tumor generating virus

1967 BS Blumberg: Discovery of *Hepatitis B virus* (HBV)

1969 M Delbrück*, AD Hershey* and SE Luria*: Virus replication cycle; Genetic structure of viruses

1974 J Lindenmann: Viruses as immunological adjuvants in cancer;

Interferon as antiviral agent

1975 D Baltimore*, R Dulbecco* and HM Temin*: Tumor viruses and their interaction with host cell DNA

1991 RL Martuza: Virus genomes engineered to enhance anti-tumor specificity

2008 H zur Hausen*: Identification of Human *Papillomaviruses* (HPV) and their role in cervical cancer

F Barré-Sinoussi*, L Montagnier*: Identification of *HIV* as causing agent of AIDS

2015 SW van Gool: Immunogenic cell death (ICD) as mechanism of oncolysis induced by NDV

*** Nobel Laureats**

Table 33 Examples of Oncolytic Viruses

Virus family	Virus	Species of origin
Herpesviridae	HSV-1	human
Adenoviridae	HAdV	human
Paramyxoviridae	MeV	human
"	NDV	bird
Rhabdoviridae	VSV	cattle
Picornaviridae	CVA	human

"	PV	human
"	SVV	cow
Poxviridae	VV	cow
Reoviridae	mORV	human
Retroviridae	MuLV	mouse

Table 34 Immune escape mechanisms by Tumors and OVs

Tumors

- Escape from type I interferon , e.g. reduced and/or delayed RIG-I , TLR or IFNRα signaling induced response
- Production of immunosuppressive cytokines, e.g. TGFß, IL-10
- Recruitment of inhibitory cells , e.g. Treg or MDSCs
- Upregulation or secretion of PD-L1 to deliver a negative signal to PD-1^+ TILs
- Downregulation of TAAs and/or MHC molecules
- Constitutive expression of IDO, tryptophane shortage, T cell proliferation arrest

Oncolytic viruses	immune escape mediating proteins:
VSV	matrix protein
Influenza virus	NS1 protein
Paramyxoviruses	V and C proteins

HSV	**γ34.5 protein**
Adenovirus	**E1 and E3 region encoded proteins**

IDO indolamine-2,3-dioxygenase; IFNRα α chain of the type I interferon receptor; IL-10 interleukin 10; MDSC myeloid-derived suppressor cell; MHC major histocompatibility complex; RIG-I retinoic acid-inducible gene I; TAA tumor-associated antigen; TGFß cytokine promoting tissue repair; TLR toll-like receptor; Treg regulatory T cell

Table 35 Concepts of application of OVs

Intratumoral inoculation

Locoregional treatment: intra-nasal, intraportal route, hepatic arterial infusion, intraperitoneal application

Systemic intravenous application

Combining OVs with carrier cells for improving tumor targeting

Combining OVs with bispecific antibodies for improving tumor targeting

For anti-tumor vaccination in combination with TAAs:

- **oncolysate vaccines**
- **live tumor cell infection, ATV-NDV vaccine**
- **oncolysate-pulsed DCs, VOL-DC vaccine**

Combinatorial treatments:

- **systemic OV plus local hyperthermia for immune conditioning**

Table 36 GBM- targeted therapy by oncolytic NDV

Apoptosis pathways
Intrinsic
Extrinsic
Cell cycle arrest pathways
ER stress, transcription inhibition
Interaction with Rac1 to induce syncytium formation
Cellular actin reorganization, denaturation of actin cytoskeleton
Rac1 signalling
Regulation of gene transcription and G1 cell cycle progression
Contributor to cell survival
Key regulator of cell migration and invasion
Activation of matrix metalloproteinases
Dynamic state of the actin cytoskeleton
Lamellipodia formation
RIG-I and IFNRα signaling pathways
Early-phase anti-viral response (IFN-ß, IRF3, IRF7)
Late-phase anti-viral response (JAK1, Tyk2, STAT1, STAT2, IRF9, ISGF3)

Table 37 Milestones in modern development of oncolytic virotherapy

1991 Genetically engineered HSV mutant with reduced neurotoxicity

1997 Targeting HSV for hepatoma using albumin promoter/enhancer

1998 AdV with CD and HSV-Tk for prodrug activation (5-FU+GCV)

1999 Addition of cyclophosphamide to HSV for immune suppression

2001 HSV encoding IL-12 and GM-CSF for T cell recruitment + immune stimulation

2004 MeV encoding NIS which concentrates beta-emitting (radiovirotherapy) and gamma-emitting isotopes (imaging)

2005 MeV with ScFv antibody targeting virus entry and cytopathic effects

2006 Use of cell carriers (CIK cells) to deliver VV to tumor;
AdV with relaxin protein to enhance virus intratumoral spread

2008 Polymer coating and retargeting of AdV for ovarian cancer to enhance viral pharmacokinetics;
MicroRNA targeting to control unwanted toxicity of picornavirus and VSV

2009 Clinical Phase II trial with intralesional HSV in melanoma patients

2011 Delivery of infectious nucleic acid via picornavirus to achieve sustained viremia and tumor regression;
Viremic Threshold: Intravenous delivery of VV in metastatic patients

2015 GMP certificate for IOZK: VOL-DC
T-VEC: First OV approved for melanoma immunotherapy

2017 Review of the efficacy and safety of T-VEC

Table 38 Genetic modification of oncolytic NDV for cancer therapy

		PMID
2007	rNDV-GM-CSF	17914407
2008	rNDV-IL-2	18813797 and 18538434
	rNDV-IL-2 + rNDV-TAA	18714310
	rNDV-Ig(vL)-Ig(vH) (mab against angiogenesis)	18200068
2009	rNDV-NS1 (interferon antagonist)	19209145
2010	rNDV-F3aa	19809404
2012	rNDV-apoptin	21865658
2013	rNDV-cytosine deaminase	24460323
	rNDV-F with PSA cleavable site (prostate Ca specific)	23345509
2014	rNDV-IL2-TRAIL	24971746
2015	rNDV-Fas	25761895
	rNDV-anti-CD147(TAA)	26689432
2016	rNDV-p53	27465066
2017	rNDV-IL-15	28286036
	rNDV-ICOSL	28194010

Table 39 NDV-related multispecific adapter proteins

	Adapter proteins		PMID
2005	anti-HN-IL2 for tumor targeted gene transfer	*in vitro*	16010418
	"	*in vivo*	15645128
	anti-HN-anti-CD28 and anti-HN-anti-CD3	*in vitro*	1575830
2006	NDV-anti-HN-IL2 *in vivo*, reduced liver toxicity		17088973
2011	Targeting IL2 and GM-CSF immunocytokines to the tumor vaccine ATV-NDV		2142118
2013	Bispecific and trispecific adapter proteins for targeting the immune system against cancer: preparing for the future		23329400

Table 40 Examples of targets of tri-specific adapter proteins

First binding site	Second binding site	Third binding site	
		T cells	DCs
NDV HN	IL-2, IL-12, IL-15,	CD2, CD25,	CD1a, CD11c
NDV F	GM-CSF, TNF, IFN-α	CD28, CD107a,	CD40, CD80
		CD122, CD132	CD83, CD86
		CD247, CD3	IFNAR1,
			CD197,

CD205

CD209

For further details see patents US 8,142,791 B1 (2012) and EP 2 091 972 (2016)

Table 41 Oncolytic viral gene plasmid DNA vaccines or vaccine adjuvans

pHN and pF DNA plasmids and NDV subjunit vaccines	PMID
2001 Cloning, expression, and crystallization of F of NDV	11883193
2002 Induction of IFN-α and TRAIL in PBMC by NDV HN but not F	12083832
2004 Syncytia forming activity of F and fusion promoting activity of HN molecules at the cell surface;	15254725
HN of NDV determines tropism and virulence	15047833
2009 Targeting anti-tumor DNA vaccines to DCs via a short CD11c promoter sequence	19616491
2010 HN as a powerful molecular adjuvant	20709006
2011 Triggering innate anti-tumor immunity by pHN application	21172381
2016 Targeting liver cancer with pHN-apoptin-IL18	27900002

CHAPTER VI. COMBINING BIOLOGICAL THERAPIES WITH STANDARD THERAPIES

A. COMBINING OVs WITH PHARMACOLOGICAL MODULATION AND/OR CHEMOTHERAPY

Administration of OVs alone, as monotherapy, rarely induces successful regression of established tumors. Therefore, various strategies have been used to improve therapeutic effects involving OVs. In the previous Chapter, various concepts of combining OVs with immunotherapies have been presented: To use OVs within a tumor vaccine for augmenting their immunogenicity or to combine OVs with hyperthermia for immune conditioning.

In this Chapter, we discuss possibilities of combining OVs with drugs that can cause immunomodulatory systemic effects. This survey is based on two recent reviews (1,2).

Table 42 lists challenges of combinations of OVs with Drugs.

The challenges to be solved are the following: Cancers and OVs use similar pathways to either become increasingly malignant or to support virus replication and spread: These are, for instance, defects in the IFN pathway, apoptotic resistence, immune suppression and angiogenesis or virus spread via leaky tumor vasculature. Combination strategies have to be aware of conflicting mechanisms:

i) apoptosis inducing drugs might reduce virus replication,

ii) anti-angiogenic drugs might reduce virus spreading and

iii) immunosuppressive drugs might increase viral replication but decrease induction of adaptive anti-tumor immune mechanisms.

The challenge thus is, how to keep a right balance between these conflicting mechanisms.

As described in the previous Chapter, OVs are self-amplifying biotherapeutics with tumor selectivity of virus replication and toxicity. OVs exploit cancer-associated cellular defects arising from genetic alterations and epigenetic reprograming (3). Such cellular defects lead to dysfunctional anti-viral responses by tumor cells and immune evasion, increased cell proliferation and metabolism, and leaky tumor vasculature (4).

Most patients enrolled in clinical trials to test the efficacy of OVs suffer from advanced disease and are therefore subjected to some form of chemotherapy. While the evaluation of chemotherapeutic drugs in the context of OV therapy has been fairly empirical, their immunosuppressive effects can inherently support OV activity by increasing OV spread within the tumor and/or increase anti-tumor immune responses.

Table 43 lists examples of combinations of OVs with Drugs.

i) COMBINING OVs WITH CHECKPOINT INHIBITORS

The first drug in the list is ipilimumab, a monoclonal antibody neutralizing negative signals through the T-cell inhibitory receptor CTLA-4. During normal immune responses, T cell checkpoint inhibitors such as CTLA-4 and PD-1 prevent over-reactive T-cell responses. These could otherwise lead to harmful tissue damage. In tumor tissue, tumor-infiltrating lymphocytes (TILs) are often inhibited by negative signals mediated via CTLA-4/CD80(86) and/or PD-1/PD-1L stimulation. As a result, T cell anergy is a major barrier to immune-mediated tumor recognition and rejection. Ipilimumab application stops such negative signals and leads to increases of T-cell immune responses.

The first OV that was successfully tested *in vivo* for combination therapy was NDV. Murine B16 melanomas were first treated by intra-tumoral inoculation of the virus which was followed by ipilimumab treatment. The combination therapy of NDV and anti-CTLA-4 led to nearly 70% cures compared to 20% cures for ipilimumab alone and no effect of OV on its own. Combining localized NDV treatment with systemic CTLA-4 blockade led to rejection of pre-established distant tumors and protection from tumor re-challenge in poorly immunogenic tumor models. The therapeutic effect was associated

with activated CD8+ and CD4+ TILs but not Tregs and was dependent on CD8+ T-cells, NK-cells and type I IFN (5).

Clinical benefit of checkpoint blocking antibodies appears to be limited to subsets of patients with pre-existing lymphocytic infiltrations of their tumors. A successful rational combination would consist of localized oncolytic virotherapy followed by systemic checkpoint blocking immunotherapy. Intra-tumoral application of NDV to B16 melanoma in mice induced lymphocytic infiltrates not only locally, but also in distant non-injected lesions.

Meanwhile, first results of such combinations are coming in from clinical studies. In one study from 2016, the oncolytic virus T-VEC (Talimogene laherparepvec) was combined with ipilimumab in previously untreated, unresectable stage IIIB-IV melanoma. After 18 months, the progression-free survival (PFS) was 50% and OS 67%. The combination appeared to have greater efficacy than either T-VEC or ipilimumab monotherapy. However, grade 3/4 treatment-related adverse events (AEs) were seen in 26,3% of patients. Both agents contributed to the AEs (6).

The authors were satisfied that no dose-limiting toxicities occurred and concluded that this combination showed "a tolerable safety profile" (6).

Defining effective combinations of immune checkpoint blockade and oncolytic virotherapy is important. A period of oncolytic viral replication and directed targeting of the immune response against the tumor were required for the most beneficial effects, with CD8+ and NK, but not CD4+ T-cells mediating the effects (7).

ii) COMBINING OVs WITH DNA ALKYLATING DRUGS

a) Cyclophosphamide (CPA): CPA is a nitrogen mustard alkylating agent that leads to cross-linking of nucleotides. Its active metabolite, phosphoramide mustard, interferes with DNA replication by forming guanine-to-guanine intra-strand and inter-strand crosslinks (8). CPA has been used in combination with several OVs including HSV-1 (9), *adenovirus* (10), *vaccinia virus, reovirus* (11), *measles and vesicular stomatitis virus*, leading to improved anti-tumor activity *in vivo* (1).

Several studies suggest that CPA can be efficacious by preventing immune-mediated viral neutralization. Other studies suggest that CPA can also enhance the generation of anti-tumor immunity by inhibiting Tregs (10,11). The best progression-free survival and OS rates were seen with a combination of low-dose metronomic CPA and intratumoral infection by gene-modified adenovirus Ad-GM-CSF (10).

b) Temozolomide (TMZ, temodal): TMZ is an alkylating agent that leads to alkylation/methylation of DNA. It has demonstrated clinical benefits in patients with GBM (12) and advanced metastatic melanoma (13). At higher doses, TMZ can be myeloablative. Oncolytic HSV (14,15) and AdV (16) have been tested in combination with TMZ. In one study, using Ad5/3-D24-GM-CSF with or without low-dose CPA to reduce Tregs, co-treatment with TMZ increased tumor cell autophagy, anti-tumor immunity, and reduced tumor burden (17).

iii) COMBINING OVs WITH INHIBITION OF DNA REPLICATION

Gemcitabine is a fluorinated deoxycytidine nucleoside analog. Incorporation of this analog into DNA prevents further addition of nucleosides during DNA polymerization and thereby halts DNA replication and cell division. It is thought to promote anti-tumor immune responses by elimination of MDSCs which suppress T-cell responses.

This drug has been shown to increase the anti-tumor activity of *adenovirus, parvovirus, reovirus*, VSV, HSV (18), *vaccinia and myxoma virus*. No such effects occurred in immune-compromised mice, thus corroborating the assumption that a virus-triggered anti-tumor immune response was mediating the combination effect.

A phase I study of the combination of intravenous reovirus and gemcitabine in 16 patients with advanced cancer revealed

i) a decrease of neutralizing anti-reovirus antibodies,

ii) in 80% of evaluable patients a partial response or stable disease, and

iii) a potential interaction between reovirus and gemcitabine in causing liver enzyme rises (19).

iv) COMBINING OVs WITH EPIGENETIC MODULATORS

Many enzymes that are involved in epigenetic regulation are deregulated in cancer. Transformed cells often have defective IFN signaling pathways. It has been estimated that about three quarters of tumor cell lines within the NCI60 panel have defective IFN responses (20). Dysfunctional IFN pathways in cancers are often due to epigenetic silencing including DNA promoter hypermethylation and transcriptionally suppressive histone modifications (1). Manipulation of the cancer epigenome using small molecules has been explored successfully as a treatment modality for cancer. Transcriptional activation of interferon-stimulated genes (ISGs), which are often epigenetically silenced, requires histone deacetylase (HDAC) activity (21).

HDAC inhibitors (HDIs) include, among others, valproic acid (VPA) and trichostatin A (TSA). These have been used in combination with OVs to effectively "reprogram" IFN-responsive tumors to become permissive to OV infection. HDIs enhanced HSV oncolysis in oral squamous carcinoma cells (22) and in gliomas (23). This was attributed to an inhibition of virally induced ISG expression (24). The combination of HSV with HDIs led to prolonged survival in murine tumor models (23,24).

HDIs possibly have additional immunomodulatory properties. Striking effects of HDIs have been observed in the context of a heterologous oncolytic prime-boost strategy (25). It was reported that HDIs caused suppression of primary immune responses, enhancement of secondary immune responses, and abrogation of autoimmunity during tumor immunotherapy (25). In this respective study, mice with syngeneic B16 melanoma brain tumors were first primed with an oncolytic adenovirus expressing a TAA which was overexpressed in B16 and then treated with oncolytic VSV expressing the same TAA. The HDI MS-275 was given along with the VSV boost.

5-AZA-2`-deoxycytidine (5-AZA) is a DNA methyltransferase inhibitor that prevents DNA methylation and allows silenced DNA to regain accessibility to transcription factors. In addition to histone acetylation-mediated gene silencing, ISGs and other genes implicit in the IFN-mediated anti-viral response are often silenced in cancers by DNA hypermethylation at CpG islands in their promoter regions (1). 5-AZA could be successfully combined with oncolytic HSV rQNestin34.5. It de-repressed transcription under control

of the Nestin promoter, allowing viral gene expression, increased viral replication, and HSV-mediated glioma cell killing. An increase in survival was observed in glioma bearing mice when treated with the OV and 5-AZA, compared to either treatment administered alone (26).

As mentioned in Chapter II, 5-AZA had been found by K Bosslet, my first PhD student, to be capable *in vitro* to de-repress expression of a TAA recognized by specific CTLs from a TAA negative tumor immune escape variants (27). In another study (28), 5-AZA had induced with high frequency heritable but phenotypically unstable changes in the tumorigenic and metastatic properties of tumor cells.

v) COMBINING OVs WITH PI3K/AKT/mTOR PATHWAY INHIBITORS

The PI3K pathway is critical to apoptosis/cell survival signaling in response to stress. Genetic mutations in cancers often affect the PI3K pathway resulting in dysfunctional apoptotic responses and pro-survival signaling (29). Various stress signals, including IFN-α, activate PI3K thereby triggering a signaling cascade leading to phosphorylation of Akt. This then activates another kinase which phosphorylates cellular factors involved in cell survival and proliferation, such as NF-κB. The latter is also involved in inducing the type I IFN cascade.

In combination with HSV MG18L, the PI3K inhibitors LY294002, GDC-0941 and BEZ235 acted synergistically to induce apoptosis in glioblastoma stem cells (30). Combination therapy resulted in durable cures in mice bearing GBM tumors, surpassing the efficacy of either therapy administered alone (30).

A master regulator of cellular translation is mammalian target of rapamycin (mTOR). Its position in the cell is downstream of PI3K and Akt signaling. mTOR controls translation of a number of cellular mRNAs and can also impact translation of viral proteins. Evidence suggests that mTOR can control the anti-viral response by regulating translation of IFN and other key mediators of anti-viral responses such as IRF-7 (31). Several OVs, including HSV, VSV, AdV and myxoma virus have been tested in combination with the well-known immunosuppressant rapamycin (32). Reduction of levels of antibodies generated against the viruses was observed (33). In several rodent models of

cancer, improvements of OV activity via combination with rapamycin were reported (32).

vi) COMBINING OVs WITH PROTEASOME INHIBITORS

Bortezomib is a proteasome inhibitor approved to treat multiple myeloma (MM) and mantle cell lymphoma. It binds reversibly the catalytic site of the 26S proteasome with high affinity and specificity (34). Bortezomib may kill cancer cells through ER-stress and activation of the unfolded protein response (UPR) (35). Some studies showed that bortezomib can increase surface expression of HSP90 and HSP60 in cancer cells leading to more effective phagocytosis by DCs (36).

In combination with nTERT-Ad virus, bortezomib enhanced infection-induced ER-stress and activated the UPR and UPR-associated apoptotic cell death *in vitro* (37). *In vivo*, bortezomib focused the immune response towards TAAs by inhibiting immune recognition of the virus. Bortezomib`s efficacy in these subcutaneous hepatocellular carcinoma models was dependent on a functional CD8+ T-cell response (37).

vii) COMBINING OVs WITH IMMUNOMODULATORY DRUGS

A high-throughput screen was performed using oncolytic VSV dM51 in a virus resistant murine breast cancer cell line. One of the molecules identified as "viral sensitizers" was VSe1. It boosted viral replication up to 1000-fold and synergistically with the virus increased tumor cell killing. ISGs typically triggered upon VSV infection remained silenced in cells pre-treated with VSe1. In a murine colon carcinoma model refractory to VSVdM51, VSe1 potentiated OV activity leading to delayed tumor progression, while the virus alone or VSe1 alone had no anti-cancer effects (38).

Triptolide (TPL) is a naturally derived component of the Chinese herb Tripterygium wilfordii. It has been used as an anti-inflammatory remedy with also anti-cancer properties. TPL is a global transcription inhibitor and has multiple effects including the inhibition of RNA polymerase II and the expression of genes involved in apoptosis and NFκB signaling (39). TPL also suppresses IFN signaling downstream of IRF3 (40). The combination of VSV and TPL synergistically improved GBM tumor specific virus replication leading

to prolonged survival and delayed tumor progression compared to either therapy given alone (40).

Sunitinib is a small molecule oral drug with multi-targeted receptor-tyrosine kinase (RTK) inhibitor. It was approved by the FDA in 2006 for the treatment of metastatic renal cell carcinoma (RCC) and of gastrointestinal stromal tumors (GIST). The RTKs targeted by sunitinib include PDGF-R, VEGF-R, KIT (CD117), RET, CSF-1R, and FLT3. Sunitinib was also shown to have off-target effects that block effector proteins of the IFN signaling pathway such as RNaseL and PKR (41). VSV, reovirus and vaccinia virus (VV) have been evaluated in combination with sunitinib (42-44). In the VSV study, sunitinib decreased phosphorylation of the PKR substrate eIF2-α, leading to increased viral titers in vitro. Combination therapy resulted in complete and sustained tumor regression in several immune-deficient and immune-competent mouse tumor models (43).

In conclusion, various drugs have been demonstrated to be able to break immune tolerance and to facilitate OV-mediated immunotherapy of cancer. Ipilimumab can break tumor-induced immune checkpoint control, CPA or gemcitabine can selectively deplete Tregs. Other drugs can affect the cytokine network around the tumor or deplete MDSCs. Successful therapy using OVs will depend on the context (e.g. tumor type, tumor site) and on navigating the delicate balance between the anti-viral reponse and the anti-tumor response.

B. CHEMOTHERAPY-ENHANCED RADIATION THERAPY

Chemotherapy-enhanced radiation therapy (CERT) is a new term derived from trials of the combination of CT and RT for head and neck cancer (45). Multiple trials in patients with disease across sites have meanwhile demonstrated that concurrent chemoradiation therapy can improve local control and therefore disease-free survival, although distant metastatic disease is not generally affected. Improved physical targeting with X-rays and protons in concert with this novel approach of sensitization holds great promise for future improvements in cancer therapy.

C. THE HORMESIS EFFECT

Hormesis has been termed a biological principal, which is of interest not only for toxicologists. It describes a dose-response relationship to stressors with a low dose stimulation and a high dose inhibition. It was shown, for instance, by testing the effect of the carcinogen dioxin on the development of breast cancer in rats: In a low-dose region (0,001 μm/Kg/day), the frequency of tumors was greatly reduced compared to no dioxin or to a dose of 0,1 μm/Kg/day).

Also, when testing the dose-response curve of chemotherapeutics, antibiotics, non-steroidal inhibitors of inflammation (NSAIDs) or toxins it showed a U-curve with a reduction of their toxic side effects at the nadir. Calabrese and Baldwin (46) observed that whole-body irradiation with Röntgen rays in the low dose range (0,5 – 2 Gy) leads to the activation of the immune system.

D. COMBINING LOW DOSE IRRADIATION AND IMMUNOTHERAPY

Another strategy that recently received much attention is the combination of a biological therapy with low dose irradiation. Let us therefore consider first what is known about the molecular basis of radiation therapy. This description is based on EJ Hall (47).

Radiobiology research has resulted in duistinguishing for R`s:

i) repair,

ii) redistribution,

iii) reoxygenation, and

iv) repopulation.

To escape cell death after radiation, tumor cells use DNA repair (i) and repopulation of the tumor, presumably by resistant cells (iv). Conversely, tumor cell kill by radiation is improved through the redistribution of tumor cells, such that a greater proportion is in a more radiosensitive stage of the cell cycle (ii) and through the reoxygenation of previously hypoxic and therefore radioresistant cells (iii).

A fifth R of radiobiology has been suggested, namely molecular regulation. Molecular research revealed that genomic, message, and proteomic effectors in both tumor and stromal cells clearly regulate the other four R`s.

i) DNA repair (48): This includes several distinct mechanisms:

a) Base-excision repair (BER),

b) Nucleotide-excision repair (NER),

c) Recombinational repair , and

d) Mismatch repair.

Molecular regulators of repair are distinct for each type of repair process. Key examples include ATM, Rad51, BRCA, Ku proteins, DNA-PK, and g-phospho-H2AX.

iv) Repopulation (47): Molecular regulators of repopulation involve regulators of stem cell survival/self-renewal such as Wnt, Notch, Sonic Hedgehog and Bmi.

ii) Redistribution (47): In an unsynchronized population of cells as tumors are, cells in late S-phase are most resistant to radiation. After radiation, cells accumulate in the G2/M phase, when they are most sensitive to radiation. Therefore, cells that are not damaged after one dose of radiation may redistribute into a more sensitive G2/M phase where they are more susceptible to the next dose. Key proteins involved in cell cycle regulation include cyclins, cdks, p53 and p21.

iii) Reoxygenation (47): Hypoxic cells are less sensitive to radiation. Oxygen is thought to make the damage caused by free radicals permanent, thereby making the cells more sensitive to radiation: By killing the outermost layer of cells that are closest to the blood supply and therefore the best oxygenated, the inner cells become closer to the blood supply and become more sensitive to the next fraction of radiation. This process is repeated after each fraction of radiation. Candidate molecular regulators involved in this process include HIF-1α, VEGF, NO, and bFGF.

v) Molecular regulation (47): This mode controls all four mechanisms of radiobiology. This involves complex signaling processes that differ by cell

type, cell context, radiation dose, and radiation energy that govern the cellular response to radiation. Molecular regulation distinguishes between low-dose genes which are upregulated upon radiation doses between 1 and 10 cGy and high-dose genes which are upregulated upon radiation doses between 10 and >100 cGy.

Our understanding of the molecular regulation of radiation effects has expanded enormously. An increased understanding of the mechanism of radiation resistance has made targeted cancer therapy possible.

Clinical investigations of one such agent, cetuximab, have shown improved survival resulting from increasing control of advanced cancer of the head and neck (45). This proof-of-principle that the combination of radiation therapy and molecular targeting agents can improve outcome without major toxicity has opened up enormous opportunities for the treatment of cancer patients. Investigations of new targets have hinted at the possibility that we can sensitize tumors and protect normal tissues with a single targeted therapy.

One study showed that low-dose gamma irradiation (LDI) can affect the barrier in the tumor microenvironment preventing efficient T cell infiltration. LDI programs macrophage differentiation to an iNOS+/M1 phenotype that then can orchestrate effective T cell immunotherapy. In a NOD/SCID xenotransplant model of human pancreatic carcinomas, neoadjuvant local LDI caused normalization of aberrant vasculature, efficient recruitment of tumor-specific T cells and T-cell mediated tumor rejection with prolonged survival (49).

In the last decade, several studies have shown that protocols using LDI are effective in providing local tumor control with negligible normal tissue toxicity. LDI stimulates antioxidant capacity, repair of DNA damage, apoptosis and induction of immune responses (50).

Another study combined LDI with sunitinib and anti-tumor vaccination. The cancer vaccine was based on a *Semliki Forest virus* vector encoding the oncoproteins E6 and E7 of human papillomavirus (SFVeE6,7). The trimodal sunitinib, LDI and SFVeE6,7 immunizations enhanced the intratumoral immune compartment by a factor of 10,000 with E7-specific CD8+ T cells. As a

result, the triple treatment strongly enhanced the immunotherapeutic effect, blocking tumor development altogether and leading to 100% tumor-free survival of tumor-bearing mice (51).

In a murine tumor model for multiple myeloma, LDI was combined with PD-1/PD-L1 checkpoint blockade. The bone marrow from untreated myeloma-bearing control mice contained elevated levels of T cells expressing PD-1, 2B4, LAG-3 and TIM-3 proteins. When PD-L1 blockade was combined with blocking antibodies to LAG-3, TIM-3 or CTLA-4, synergistic or additive increases in survival were observed. The increased survival rates correlated with increased frequencies of tumor-reactive CD8 and CD4 T cells (52).

Photodynamic therapy (PDT) is performed with red light and a photosensitizer such as hypericin (Hyp). An interesting study investigated phototoxic and immunological effects of a low dose Hyp-PDT in contrast to the commonly used conditions. It was reported that low dose Hyp-PDT induced complete tumor regression in BALB/c mice bearing CT26 colon carcinoma (53).

E. COMBINING LOW DOSE CHEMOTHERAPY WITH STANDARD THERAPY AND WITH NOVEL THERAPEUTIC STRATEGIES

Standard cytotoxic antiproliferative chemotherapeutic agents are usually administered every 2-3 weeks. Along with acute toxicity and long-term effects of cumulative doses, this strategy potentially allows regrowth of the tumor in the interval period and leads to the emergence of resistant populations of tumor cells.

The administration of chemotherapy at reduced doses given at regular, frequent time intervals, termed "metronomic" chemotherapy (MCT), presents an alternative to standard maximal tolerated dose (MTD) chemotherapy.

The primary target of MCT were originally endothelial cells supporting the tumor vasculature, and not the tumor cells themselves. While anti-

angiogenesis is still an important mechanism of MCT, other mechanisms, including activation of anti-tumor immunity and a decrease in acquired therapeutic resistance, have also been identified.

MCT seems to be capable of selectively eliminating immunosuppressive cells (54). In particular, cyclophosmamide (CPA), paclitaxel, and temozolomide can reduce Treg activity when delivered as metronomic doses (i.e., repetitive, low doses). In the case of CPA, metronomic doses serve to minimize toxicity, inhibit angiogenesis and avoid global immunosuppression which results from administering a single, high dose (55). Metronomic CPA only transiently reduced Treg but induced stable tumor-specific T-cell responses. These correlated with improved clinical outcome in advanced-stage breast cancer patients (56).

Over 15 years ago, low-dose MCT was shown to induce disease control in patients with advanced-stage breast cancer with a lower incidence of adverse events compared with conventional MTD chemotherapy. Good response rates have been seen in heavily pre-treated patients for whome only limited treatment options were available. This holds true for elderly patients with newly diagnosed GBM in which standard CT is often omitted due to fear of side effects. The past over 10 years have seen a marked rise in clinical trials of MCT. It is increasingly combined with conventional therapies (CT or RT), as well as with novel therapeutic strategies, such as targeted small molecules and immunotherapy. A systematic literature analysis of low-dose MCT revealed that the treatment appears to be clinically beneficial and safe in a broad range of tumors (57).

F. COMBINING LOW DOSE WHOLE BODY IRRADIATION WITH IMMUNE CHECKPOINT PROTEIN BLOCKADE FOR MYELOMA

Multiple myeloma (MM) is characterized by the presence of neoplastic plasma cells in the bone marrow. It is generally considered to be an incurable disease. Sublethal whole body irradiation leads to transient lymphodepletion. In a murine MM model, a temporal phenotypic analysis of bone marrow samples revealed an elevated expression in percentages of PD-1, 2B4, LAG-3 and TIM-3 protein expressing T-cells. When PD-L1 blockade was combined

with blocking antibodies to LAG-3, TIM-3 or CTLA4, synergistic or additive increases in survival were observed. Survival rates improved from about 30% to >80%. The increased survival rates correlated with increased frequencies of tumor-reactive CD8 and CD4 T-cells. There was thus a synergistic effect of combining lymphodepleting doses of whole body irradiation with checkpoint protein blockade (58).

G. SUPPORTIVE THERAPIES FROM COMPLEMENTARY MEDICINE

Standard and biological therapies can also be combined with therapies from complementary medicine. Many of these latter approaches are orientated towards normal physiological regulatory systems. Table 43 lists some of these therapies. This paragraph is based on H Heine (59).

It may be worth for lay people to first present some definitions:

i) Metabolic transformation is a term to describe the collective changes in cellular metabolism that arise from cancer-causing mutations and enable cells to grow and proliferate independently of normal physiologic control mechanisms. The Warburg effect, which will be discussed in more detail in Chapter VII, is one component of the metabolic transformation.

ii) Metabolism represents biochemical activities concerned with the handling of organic compounds (sugars, amino acids, nucleotides, lipids) through a variety of enzymatic pathways. Anabolic metabolism is the coordinated metabolic activity that allows the cells to produce macromolecules, such as lipids and proteins. They consume energy. Catabolic metabolism is used to degrade molecules to produce simple constituents and energy. Examples are ß-oxidation of fatty acids and amino acid oxidation. Both processes produce ATP at the expense of intermediates that could have otherwise been used for anabolism.

Differences in metabolism between tumors and normal tissue is exploited for diagnostic and therapeutic benefit. PET is a nuclear medicine imaging modality that allows metabolism to be studied *in vivo* with the use of radioactive tracers. The most commonly used tracer is FDG, a glucose analog that can be transported into cells and phosphorylated by hexokinase, but

cannot be metabolized further. FDG-PET allows to distinguish regions of abnormally high glucose metabolism. It can be used to identify new tumors, to determine regional lymph node involvement or diagnose distant metastases. It is even possible to use this method to evaluate response to therapy.

In the following, we will mention four principles from Table 43.

1. Anti-oxidants, Cox-inhibitors. Oxidative stress is caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS). Such stress can impair pivotal functions of cells in the body. Anti-oxidants and Cox-inhibitors try to counter-act.

2. Basic tissue regulation. This is concerned with the correct transport of metabolites through capillaries, ECM and lymphatics. Mechanisms of homeostasis and homeodynamics regulate for instance the maintenance of a physiological tissue pH. Tumor growth is often associated with tissue acidosis. Edema are a sign of dysregulated water and metabolite transport.

3. Phytotherapy. Carotinoids and flavonoids can be used to influence carcinogen metabolism. Hepatic tissues are enriched with metabolic enzymes specialized in chemical conversion referred to as biotransformation. Biotransformation enzymes are thought to have evolved as natural defenses against environmental toxin exposure. Phase I enzyme reactions include oxidation, reduction, and hydrolysis reactions and, generally, expose functional groups that enable Phase II biotransformation enzymes to proceed. Phase II reactions catalyze for instance glucuronidation, sulfatation, acetylation, methylation, and glutathione conjugation. Phase I biotransformation of carcinogens often results in reactive metabolites capable of covalent modification of cellular macromolecules. Phase II reactions ultimately result in metabolites that are less toxic and more readily excreted.

4. Hyperthermia (HT). This modality is used as a supportive means of other cancer therapies. An increase in tumor tissue temperature can have various effects: i) activation of the immune system (38,5 – 39,5°C), ii) tumor cell toxic effects (>41,5°C), iii) synergistic effects with CT, RT and OV therapy due to changes in blood vessels. Heat can be produced by radiation with micro- or

radiowaves. HT can be applied either locally (e.g. skin tumors) or regionally (deeper in tissues, larger organ areas, e.g. pancreas or colon carcinoma) or, less frequently, as total body HT (in combination with CT in cases of systemic metastatic disease).

5. Chrono-Pharmacology. Circadian rhythm adapts the body to the day-and-night rhythm generated by the earth`s rotation. This is reflected, among others, by plasma cAMP and endorphin level. The Nobelprize for Physiology or Medicine 2017 was awarded to J Hall, M Rosbash and M Young for their research on the circadian clock.

As described in (60), the circadian timing system is composed of molecular clocks. These drive 24-h changes in xenobiotic metabolism and detoxification, cell cycle events, DNA repair, apoptosis, and angiogenesis. The cellular circardian clocks are coordinated by endogenous physiological rhythms, so that they tick in synchrony in the host tissues. Circardian timing can modify 2- to 10-fold the tolerability of anticancer medications in experimental models and in cancer patients.

All biorhythms have an activity phase and a relaxation phase. There are the heart and respiration rhythm (their ratio is usually 4:1), the duodenal rhythm and stomach peristaltic (with a similar ratio of 4:1). The photoentrainment, in which the endogenous clock is synchronized with visuell impressions, has its functional center in the hypothalamus (nucleus suprachiasmatis). Melanopsin and melatonin are mediators of the photoentrainment and the circadian clock. Chronotherapy aims at the optimal time point for the application of a pharmacon.

A few examples may elucidate the supportive effects of such treatments.

1. Anti-oxidants, Cox-inhibitors. The trace element selenium (Se) and selenocysteine-carrying selenoproteins play a pivotal role in the brain. The anti-toxic and cancer-preventive properties of Se in current multimodal brain tumor therapies have been summarized (61). Magnesium (Mg) is the fourth most abundant mineral in the body. It has a co-function in more than 300 enzymatic reactions, many of which are crucial for ATP metabolism (62). Low levels of Mg have been associated with a number of chronic diseases (62).

Intravenous vitamin C (IVC) is a contentious adjunctive cancer therapy. A systematic review (63) revealed that IVC may improve the quality of life and symptom severity of patients with cancer. Well-designed, controlled studies of IVC therapy are, however, missing.

Vitamin B-6 (B-6) has a strong antioxidative effect. B-6 supplementation mediates antioxidant capacity by reducing plasma homocysteine concentration in patients with hepatocellular carcinoma after tumor resection (64).

Coenzyme Q-10 (Q-10) is a widely used alternative medication or dietary supplement as an antioxidant. Although supplementation with Q-10 has been reported to be beneficial in treating hypertension, congestive heart failure, statin myopathy, and problems associated with chemotherapy, its benefit has not been confirmed in randomized clinical studies. Nevertheless, it appears safe in selected clinical situations (65).

L-carnithin as an antioxidant may have neuroprotective effects (66). Alpha-lipoic acid possesses beneficial effects both in the prevention and treatment of diabetes (67). Omega-3 fatty acids have been shown to significantly reduce the risk for sudden death caused by cardiac arrhythmias (68).

Cimetidine has been shown to play an important role in the treatment of cancer and the regulation of the immune system (69). It can alleviate systemic immunosuppression and improve local immune function of colorectal cancer patients in the perioperative period (69). Excess prostaglandin and catecholamine release contributes to postoperative immune-suppression. Treatment combining perioperative COX-2 inhibition and beta-blockade may improve immune competence and reduce risk of tumor metastasis (70).

2. Phytotherapy. *Mistletoe* is amongst the important herbal medicines traditionally used as complementary remedies. The analysed trials suggest that there might be a combination of pharmacological and motivational aspects mediated by mistletoe extract application. These may contribute to the clinical benefit and positive outcome such as improved health-related quality of life (HRQoL) and self-regulation in breast cancer patients (71).

The flavonoid *quercetin* was reported to inhibit pancreatic cancer growth *in vitro* and *in vivo* (72). *Incense* use is an integral part of daily life in large parts of Asia. The results of a study from 2008 indicate that long-term use of *incense* is associated with an increased risk of squamous cell carcinoma of the respiratory tract (73).

Green tea is the most widely consumed beverage besides water and has attained significant attention owing to health benefits against maladies, such as obesity, diabetis mellitus, cardiovascular disorders, and cancer insurgence (74). Cell culture and animal studies elucidated anti-cancer mechanisms of green tea such as induction of apoptosis, altered expression of cell-cycle regulatory proteins, activation of killer caspases, and suppression of nuclear factor kappa-B activation (74).

Sulforaphane is a natural product commonly found in broccoli. Interestingly, this compound was found to inhibit hypoxia-induced HIF-1α and VEGF expression and migration of human colon cancer cells (75). Evidence also suggests that sulforaphane may target the epigenetic alterations observed in specific cancers by HDAC inhibition (76). *Bromelain* and N-acetylcystein are two natural, sulfhydryl-containing compounds with good safety profiles. They have been applied for more than 50 years. Cell cycle arrest, apoptosis and autophagy were induced by these compounds on a panel of gastrointestinal cell lines (77).

3. Chrono-Pharmacology. Gastrointestinal cancer is a disease that affects the population worldwide with high morbidity and mortality. The ability of melatonin to inhibit gastrointestinal cancer is substantial. Its mechanisms of action include inhibition of proliferation, invasion, metastasis, angiogenesis, and promotion of apoptosis and anti-cancer immunity (78).

H. PAIN CONTROL IN CANCER

Pain is an important symptom in cancer patients. A symposium article describes recent findings and trends (78).

30-40% of patients present pain at diagnosis, 40-70% during treatment and 70-90% during the palliative care phase.

Since the 1980s, the guidelines of pain treatment according to the WHO state that analgetics should be readily accessible.

Paracetamol and non-steroidal anti-inflammatory drugs (NSAID) are the standard drugs of the first step of the WHO pain ladder. Weak opioids constitute the second step of the WHO pain ladder. Codein is the standard weak opioid and can be used in combination with paracetamol. Strong opioids are classified at the highest step of the analgesic ladder. Morphine is still the standard drug. More potent ones are hydromorphone, fentanyl, oxycodone and methadone.

The review includes the following tables: 1. Adjuvant drugs used in paib control, 2. Specific receptors, stimuli and effects, 3. Endogenous ligands and receptors in pain modulation, 4. Effects of different opioid receptors, 5. Receptor and pharmacokinetics characteristics of different opioids.

The use of the pain medication according to the WHO pain ladder controls aroud 80% of cancer pain. In future there will be an increase of knowledge of the pathophysiology of pain. The introduction of new drugs and/or the fine-tuning of older medications will hopefully increase the response rate to 100%.

Chapter VI

Key points:

1. This chapter discusses strategies to combine biological with standard therapies to achieve synergistic effects and to increase the efficacy of cancer treatment.

2. Oncolytic viruses may be combined with chemotherapy to increase intra-tumoral virus replication or they may be combined with checkpoint inhibitors to increase systemic anti-tumor immunity.

3. Immunotherapy may profit from combination with low dose irradiation.

4. Standard therapy may profit from combination with low dose (metronomic) chemotherapy.

5. Standard and biological therapies might be further optimized by supporting therapies from complementary medicine.

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Table 42 Challenges of Combination Therapies

Cancer pathways to become increasingly malignant:

- **defects in the IFN pathway**
- **apoptotic resistance**
- **immune suppression**
- **angiogenesis**

OV pathways to support their replication

- **defects in the IFN pathway**
- **apoptotic resistance**
- **immune suppression**
- **angiogenesis (virus spread via leaky vasculature)**

Conflicting mechanisms: apoptosis versus virus replication, anti-angiogenesis versus viral trafficking, antiviral immune responses versus antitumor immune responses.

- **How to balance the need of virus replication in tumor tissue and the associated need to keep down anti-viral immunity with the need to induce innate and adaptive anti-tumor immune mechanisms ?**

- **For example: Low dose CPA may remove immunosuppressive Tregs to improve ICD-induced anti-tumor immunity, however, it may also promote antiviral immune responses leading to early viral clearance (166). High dose CPA may enhance viral oncolysis through widespread immunosuppression but it may also completely abrogate anti-tumor immune responses**

- **The challenge is to obtain a better understanding of the often complex OV-drug interactions.**

Table 43 Examples of combinations of OVs with Drugs

OV	Drug	Mechanism of immunomodulation	PMID
NDV	Ipilimumab	i) systemic	24598590
HSV	"	"	27298410
	Cyclo-P	"	10426310
	Gemcitabine	"	17726607
	Temozolomide	„	16391370
	Valproic acid	ii) specifically anti-viral response	28189010
	Trichostatin A	"	18388912
	5-Azacytidine	"	24056786
	LY294002	"	21505062
	Rapamycin	iii) both	21128236
hTERT-Ad	Bortezomib	i) systemic	20675696
VSV	Sunitinib	ii) specifically anti-viral response	21636578

	Triptolide	"	23985699
Reovirus	Sunitinib	"	20364090
VV	"	"	24474587

Table 44 Combination with therapies from complementary medicine

Type	Substance/means	Target
1. Anti- oxidants, Cox-inhibitors	vitamins, minerals cimetidine, silvestrol	enzymes immune system
2. Basic tissue regulation, metabolites tissue pH, homeostasis, homeodynamics	anabolites, catabolites	transport through capillaries, ECM , lymphatics, tissue acidosis, edema
3. Phytotherapy	carotinoids flavonoids	inhibition of phase I enzymes induction of phase II enzymes
4. Hyperthermia	radiation	tissue temperature
5. Chrono-Pharmacology photoentrainment rhythmic regulation	light melanopsin melatonin	circadian rhythm nucleus suprachiasmatis,

CHAPTER VII. PHYSIOLOGICAL REGULATORY SYSTEMS AND CANCER-ASSOCIATED DYSREGULATION

This Chapter is based on the excellent textbook "Berne & Levy Physology" (1). The description of cancer-associated dysregulation is based on the textbooks "The Molecular Basis of Cancer" (2), "The Biology of Cancer" (3) and on "Lehrbuch der biologischen Medizin" (4).

A. INTRODUCTION

The discipline of physiology deals with the function of the human body as a complex process at multiple levels. The human body consists of billions of cells that are organized into tissues, for instance epithelia, muscle or the nervous system. Then there are the organ systems, for instance the cardiovascular, respiratory, gastrointestinal, renal, endocrine, reproductive or nervous system. The cells in the body must survive and this requires energy supply, maintenance of an appropriate intracellular milieu, and defense against a hostile external environment.

The cells, tissues and organs must be coordinated and regulated. Since cancer can be considered as a dysregulated form of cellular disease, the understanding of basic regulatory systems within the body is important for understanding the dysregulations by cancer and for the design of new strategies targeting such dysregulations. It is within this context that this Chapter is included in this book.

Cancer cells need even more energy than normal cells because of their higher proliferation. They therefore developed additional mechanisms of fuel supply such as tumor-induced angiogenesis and anaerobic glycolysis (see below). At the end of this Chapter we will deal with reversion of cancer-relevant dysregulations such as cachexia and liver metastasis. It may therefore be appropriate to mention some introductory facts about enery storage and consumption:

The liver converts precursors into fuel storage forms (e.g., glucose to glycogen) when food is ingested, and converts storage forms to cellular fuels

during fasting or liver regeneration (e.g., glycogen to glucose and amino acids to glucose). Like the liver, skeletal muscle stores fuel (glycogen and protein) and converts glycogen and protein to fuels (e.g. glucose) or fuel intermediates (e.g. amino acids) during fasting or cachexia.

The gastrointestinal tract digests and absorbs fuel precursors. Adipose tissue stores fuel during food intake (e.g., fatty acids to triglycerides) and releases the fuels during fasting or cachexia. The cardiovascular system delivers the fuel and to and from their storage sites to the cells. The endocrine system maintains the blood levels of the cellular fuels by controlling and regulating their storage and their release from storage (e.g., insulin and glucagons).

Above all, the Nervous sytem monitors oxygen levels and nutrient content in the blood and, in response, modulates the cardiovascular, pulmonary, and endocrine systems and induces feeding and drinking behaviors.

B. MILESTONES FROM PHYSIOLOGY RESEARCH

As with the previous disciplines, this Chapter starts with an overview of milestones since the last century. Thirteen examples of milestones achieved by Nobel Laureats are listed in Table 44 concerning the period from 1900 to 1945. Nine further examples of the period from 1947 to 2012 are listed in Table 45. In the context of the topic of this Chapter, the contributions of A Krogh (1920), O Warburg (1931) and those of AZ Fire and C Mello (2006) will be described in more detail.

C. PHYSIOLOGICAL REGULATORY MECHANISMS

The body is capable of exerting defenses against malignant growth at various levels:

1. Controls imposed on cells by the apoptotic machinery; this triggers the death of cells that are misbehaving or suffering certain types of damage or physiological stress,

2. Controls imposed by the pRb circuit and by the DNA repair apparatus,

3. Loss of contact of epithelial cells with the basal membrane ECM; this may activate a form of apoptosis called anoikis and limits the cell`s capacity to move away from its normal tissue location,

4. Defense mechanisms by the innate and adaptive arm of the immune system.

Cancer can be considered as a disease of dysregulated physiology. Table 46 lists examples of levels of dysregulation by cancer. Colum A shows intra-cellular and colum B extra-cellular levels.

From these only the following will be discussed:

i) the level of DNA (genetics and epigenetics),

ii) the level of RNA (in particular regulation via miRNA),

iii) the level of intra-cellular organelles (in particular mitochondria),

iv) the level of the plasma membrane and glycocalyx

v) the level of ECM

vi) the level of microenvironment in wound healing or cancer

vii) the level of dysregulation of physiological systems for organ metastasis.

Other levels were mentioned already in Chapter VI (Table 43).

In order to understand dysregulatory mechanisms of cancer, it seems appropriate to first summarize normal physiological regulation at these various levels.

i) THE LEVEL OF DNA

a) genetics

Regulatory mechanisms are particularly important to protect normal tissue stem cells and to keep their DNA free from mutations. Such mechanisms include:

1. relatively infrequent replication of stem cell DNA,

2. placement of stem cells in anatomically protected sites,

3. rapid initiation of apoptosis in case of genetic damage,

4. upregulation of drug pumps such as

Mdr-1 (multi-drug resistance protein 1), and

5. assymetric DNA "template strand" allocation.

The major proteins involved with DNA repair include sensory (DNA binding) proteins, enzymes that remove damaged bases, and enzymes that restore the normal DNA sequence. A large number of regulatory enzymes control each DNA repair pathway. Regulatory enzymes, such as helicases, serve to load DNA repair complexes at the site of DNA damage. Other regulatory enzymes, such as topoisomerases, serve to unwind damaged DNA to facilitate DNA repair complex assembly, loading into chromatin, and disassembly. There exist six DNA repair pathways: Base excision repair (BER), Mismatch repair (MMR), Nucleotide excision repair (NER), Homologous recombination (HR), Non-homologous end joining (NHEJ) and Translesional synthesis (TLS).

b) epigenetics

The initiation of DNA methylation, its maintenance, and its role in transcriptional repression (silencing) are all dependent on its interaction with chromatin organization. There has been an explosion of knowledge over the past 15 years in understanding how chromatin functions for packaging of the genome and for direct modulation of gene expression.

A series of proteins, called methyol cytosine binding proteins (MBPs), and the protein complexes in which they reside, can bind to methylated CpG sites to help relay a silencing signal (5). These complexes contain histone deacetylases (HDACs) which catalyze the deacetylation of key amino acid residues. These are highly characteristic of transcriptionally silent regions of DNA. The precise manner in which all of these chromatin components interact to initiate and/or maintain abnormal gene promoter DNA methylation and the attendant silencing of involved genes is not yet known.

When DNA, methylated at CpG residues, is replicated, the newly formed daughter strands initially lack methyl groups on the CpG sites complementary

to those methylated sequences in the parental DNA strands. However, shortly after replication, maintenance methylases add methyl groups to the newly synthesized CpG sites, ensuring the transmission of the methylated state from one cell generation to the next. Such methylation is often associated with the repression of gene transcription. Hence, genes may be inactivated in a heritable fashion without any change in their nucleotide sequence.

ii) THE LEVEL OF miRNA

AZ Fire and C Mello, both from USA, received in 2006 the Nobel Prize for Physiology or Medicine for their discovery of RNA interference (see Table 45). miRNAs are short (21-23 nucleotides in length) noncoding RNAs that regulate post-transcriptionally gene expression by messenger RNA (mRNA) degradation or translation repression.

It has been estimated that miRNAs regulate about 50% of all protein-coding genes. miRNAs play fundamental roles in many biological processes, e.g. carcinogenesis, angiogenesis, programmed cell death, cell proliferation, invasion, migration, and differentiation. The expression of miRNAs is altered in cancers. It could be up- or downregulated. Upregulated miRNAs exert an oncogenic effect, while downregulated miRNAs have tumor suppressor effects. Every tumor has specific miRNA alterations so that these can be used as a tumor-specific signature (6).

iii) THE LEVEL OF MITOCHONDRIA

Regulation of tissue oxygenation (7) includes the respiratory system, the blood circulatory system and the cardiorespiratory system. The respiratory system takes oxygen from the atmosphere and transports it by diffusion from the air in the alveoli to the blood flowing through the pulmonary capillaries. The cardiovascular system then mooves the oxygenated blood from the heart to the microcirculation of the various organs by convection, where oxygen is released from hemoglobin in the red blood cells and moves to the parenchymal cells of each tissue by diffusion.

Oxygen that has diffused into cells is then utilized in the mitochondria to produce ATP, the energy currency of all cells. The mitochondria are able to

produce ATP until the oxygen tension or P_{O2} in their vicinity falls to a critical level of about 1 mm Hg. Thus, in order to meet the energetic needs of cells, it is important to maintain a continuous supply of oxygen to the mitochondria at or above the critical P_{O2} .

Most intracellular ATP is generated by mitochondrial respiration. The inner mitochondrial membrane contains the oxidative phosphorylation system that permits ATP synthesis. Phospholipids environment and especially cardiolipin are crucial for the mitochondrial energy metabolism. Cells need a constant supply of energy. This energy is derived from the hydrolysis of ATP. The cellular ATP supply can be depleted very fast, within less than 1 minute. It therefore must be replenished continuously.

ATP can be generated from the oxidation (burning) of cellular fuels from the blood circulatory system, such as glucose, fatty acids, and ketoacids. The blood levels of these fuels are maintained through the ingestion of precursors, such as carbohydrates, proteins, and fats. The storage form of these fuels are triglycerides (stored in adipose tissue), glycogen (stored in the liver and skeletal muscle), and protein. The maintenance of adequate levels of cellular fuels in the blood is a complex process involving various tissues, organs, and organ systems.

Mitochondria participate in a variety of anabolic pathways, including cholesterol, cardiolipin, heme and nucleotide biosynthesis. These organelles are required for cellular survival but they also play a role in cell death. Mitochondria integrate numerous pro-survival and pro-death signals. Thereby they exert a decisive control over several biochemical cascades. One pathway, called the intrinsic pathway of apoptosis, leads to cell death.

iv) THE LEVEL OF PLASMA MEMBRANE AND GLYCOCALYX

a) the PM

The plasma membrane (PM) and mechanisms of homeostasis are important for the maintenance of a constant volume and composition of the body fluid compartments and their temperature.

The human body is an "open system". The amounts of substances added to or lost from the body can vary widely. Homeostasis occurs through the process of steady-state balance.

Water balance determines the osmolality of the body fluids. Cells within the hypothalamus of the brain monitor body fluid osmolality for deviations from set points (normal range: 280-295 mOsm/kg H_2O). When deviations are sensed, two effector signals are generated. One is neural and relates to the individual`s sensation of thirst. The other is hormonal (antidiuretic hormone: arginine vasopressin), which regulates the amount of water excreted by the kidneys.

Water makes up approximately 60% of the body`s weight. An individual weighing 70 kg would have 42 liter (L) of total body water. 28 L make up the intracellular fluid (ICF) and 14 L the extracellular fluid (ECF). These two fluid compartments are separated by the cells PM. The ECF can be further separated into the 10.5 L of Interstitial fluid and the 3.5 L of Plasma. Here it is the capillary wall that separates these two fluid compartments of the ECF.

The PM separates the intracellular contents from the extracellular environment. Because of the structure and composition of this membrane and because of the presence of specific membrane proteins, the PM is involved in a number of important cellular functions:

- **Selective transport of molecules into and out of the cell, a function carried out by membrane transport proteins.**
- **Recognition of extracellular substances and signaling molecules through cell surface differentiation antigens and receptors.**
- **Cell communication through neurotransmitter and hormone receptors and through signal transduction pathways.**
- **Tissue organization, such as temporary or permanent cell junctions, and interaction with the ECM, with the use of a variety of cell adhesion molecules.**
- **Membrane-dependent enzymatic activity.**
- **Determination of cell shape by linkage of the PM to the cytoskeleton.**

Normal cellular function requires that the ionic composition of the ICF is tightly controlled. Also, the intracellular composition of other electrolytes is held within a narrow range. This is necessary for the establishment of

membrane potential, a cell property especially important for the normal function of excitable cells (e.g., neurons and muscle cells) and for intracellular signaling (e.g. intracellular [Ca++]). Finally, the volume of cells must be maintained because shrinking or swelling of cells can lead to cell stress, cell damage and death.

b) the Glycocalyx

Syndecan is the dominant cell surface Heparan-sulfate-proteoglycan (HSPG). The ectodomains of syndecan and of all other glycocalyx proteoglycans (PGs) contain receptors for ECM components. Tumor cells show dysregulations of cell-surface PG expression. They can therefore more easily change their cell shape. This is important for intra- and extra-vasation. Tumor cells also respond less to cell-contact mediated cytostasis which allows them to grow on top of each other.

Glypican, another cell surface HSPG, is necessary for the development of a normal nervous system. It is particularly important for the visual system (8). HSPGs control the filter properties of basal membranes (e.g. glomerula of the kidney), the binding of acetylcholinesterase in neuro-muscular synapses, they bind protease inhibitors (e.g. antithrombin) and facilitate the attachment of cells to the ECM. bFGF binds to heparan-sulfate side chains of HSPGs and can be removed by plasmin and heparanase. Thereby bFGF retains its biological properties such as acceleration of chemotaxis of cells, wound healing and induction of cell proliferation (9). The side chains of HSPGs can also bind Na+, K+, Ca++ and Mg++ ions in a reversible way and exchange them. This depends on the tissue pH.

c) Epithelial structure and function

Epithelial cells are arranged in sheets and provide the interface between the external world and the body´s internal environment. Depending on their location, epithelial cells serve many important functions:

- Establishing a barrier to microorganisms (lungs, gastrointestinal tract, and skin);
- Prevention of loss of water from the body (skin);
- Maintenance of a constant internal environment (lungs, gastrointestinal tract, kidneys); this function is the result of the ability of epithelial cells to

carry out regulated vectorial transport from one side of the epithelial cell sheet (e.g., the apical side) to the opposite side (e.g., the basolateral side). Adhering junctions, desmosomes, and hemidesmosomes provide mechanical adhesion by linking together the cytoskeleton of adjacent cells or to the underlying connective tissue.

- Tight junctions separate the apical from the basolateral membrane. Gap junctions provide connections between cells and allow exchange of ions and small molecules.

v) THE LEVEL OF THE ECM

Three excellent reviews are recommended with regard to the ECM. 1. "Molecular assembly and mechanical properties of the extracellular matrix: A fibrous protein perspective" (10). 2. "Regulation of cellular functions by extracellular matrix" (11). 3. "Extracellular matrix regulation of stem cell behavior" (12).

The ECM regulates tissue development and homeostasis. Its dysregulation contributes to neoplastic progression and modulates the hallmarks of cancer (13). The ECM serves not only as the scaffold upon which tissues are organized but provides critical biochemical and biomechanical cues that direct cell growth, survival, migration and differentiation. It also modulates vascular development and immune function.

ECM influences each of the hallmarks of cancer defined by Hanahan and Weinberg (14) : sustained proliferation, evasion of growth suppression, death resistance, replicative immortality, induced angiogenesis, initiation of invasion and metastasis. Recently, two further hallmarks have been added: reprogramming of energy metabolism and avoidance of immune destruction (14).

ECM structure: A matrisom has been defined to consist of 3 macromolecules of the ECM and of transitory attached molecules. One type of macromolecule represents a PG with its glucosaminoglycan (GAG) side chains, another type the structural glycoproteins and a third type the net forming glycoproteins. The transitory attached small molecules include water, cytokines, hormones, peptides, neurotransmitters etc. Such structural unit of the ECM can be repeated n-fold (15).

Proteoglycans are the main component of the ECM. They are everywhere in the interstitium, in slime, intra-cellular and extra-cellular in the glycocalyx. They are produced by cells of mesenchymal origin and in the CNS by astrocytes. Mesenchymal cells are associated with blood vessels and form all the kinds of interstitial tissue. Derivatives of the omnipotent mesenchymal stem cell can be fibro-, osteo-, chondro- and myoblasts. Other cell derivatives form the reticulo-histiocytary system, the hematopoetic cells of the bone marrow and the reticular and dendritic cells of lymphatic organs. All these cells exchange information via gap junctions. This allows the transport of small ions and molecules. Lymphocytes can form very distinct contacts with mesenchymal DCs in the form of immunological synapses. There thus exists a network of transfer of information within the interstitial tissues. Like a homeostat, the system is supervised via connections with hormonal and the CNS systems.

The basic scaffold of the molecular sieve of the ECM is made of the electronegatively charged PGs and GAGs. PGs have a 300 nm protein backbone with over 100 sulfated GAG-side chains. The GAG polysaccharides make up 90-95% of the total mass of a proteoglycan molecule (16).

Fluid recirculation is regulated via the ECM as molecular sieve. The concept of Virchow of defining the cell as the smallest functional unit of the body has been extended by concepts from humoral pathology. In their view, the smallest functional unit is the cell with its surrounding milieu. For single cell organisms which originated in the sea, the milieu was the sea water. It is perhaps not fortuitous that the interstitial tissue fluid of multicellular organisms has a composition of salts and osmolarity similar to that of the oceans.

The interstitial tissue fluid does not circulate in a free form between the cells and the blood vessels. It circulates through a network of high molecular weight PGs and GAGs of the ECM which function like a flexible Gel. Their composition must ensure that about 15-18 liter of such fluid (in case of 75 kg body weight) can circulate constantly. This movement has been called the inner circle (17). Only when the inner circle functions normally, can sufficient amounts of metabolites be transported via the PM to the cell and catabolites be removed. The movement of interstitial body fluid is a result of the

hydrophilic power of colloids like albumin in the blood plasma (colloidosmotic pressure), of the hydrostatic pressure of blood capillaries and of the interstitial fluid itself. The capillaries release a protein-free ultrafiltrate (blood water) into the interstitial fluid. The volume of this should correspond to 11-13% of the blood volume (about 4,5 l in case of 75 kg body weight). The interstitial fluid is resorbed back into venous capillaries. Only a small proportion is drained through the lymphatic system.

Each damage of blood capillaries and their permeability endagers the metabolism of cells in their neighbourhood. Cells need a constant supply of energy which requires a constant supply of fuel, as mentioned at the beginning. In case of loss of body fluid, the gel of the ECM can absorb water molecules more strongly than plasma albumin does. This ensures a longer osmotic balance. In case of increased amounts of fluid, the ECM can release the excess into the lymphatic system.

Only when the ECM can no more absorb excessive fluid, this will be released into tissue Edema. If damaged capillaries release proteins into the ECM, this can lead to a complete block of the interstitial fluid circuit with life threatening edema formation. Also in cases of adiposita and asthma, the internal fluid circuit is reduced.

The interstitial fluid circuit is connected through the capillaries with the endocrinium and via the terminal nerve buds with the central nervous system.

A Krogh received his Nobel Prize in 1920 for his work on capillary-motoric regulatory mechanisms. He was interested in the question, how muscles regulate their requirements of oxygen and energy. His studies revealed that the net of blood capillaries of muscles was filled with blood only when the muscle was active. The results from studies of insects and birds led to an explanation of mechanisms of activation and regulation of capillary blood flow.

vi) THE LEVEL OF THE MICROENVIRONMENT IN WOUND HEALING

Cells require effective interactions with the vasculature not only because of oxygen supply but also to acquire nutrients and to shed metabolic waste

products and carbon dioxide. Capillary networks are arranged in tissues so densely that virtually all cells are no more than several cell diameters away from the nearest capillary.

The process of developing this vasculature through angiogenesis can be observed during embryonic development, implantation of the placenta, and during wound healing. Angiogenesis is normally suppressed by physiological inhibitors: In wound healing, the burst of angiogenesis that is required to repair the wound site must be shut down once the newly formed capillaries have reached a density that suffices to support normal tissue function. This shutdown is achieved, among others, by suppressing formation of the HIF-1 transcription factor.

Another factor is thrombospondin-1 (Tsp-1) protein. Tsp-1 associates with a receptor (CD36) that is displayed on endothelial cells and halts their proliferation. TSP-1 can also cause endothelial cells to release FasL. This may then act in an autocrine fashion to trigger the death of cells displaying the Fas receptor.

Transcription of the TSP1 gene is strongly induced by p53, apparently as part of the p53-mediated emergency response. This response leads to a generalized shutdown of cell proliferation and tissue growth. Conversely, the loss of p53 function, which is seen in almost all human tumors, leads to a substantial decrease in Tsp-1 levels. This permits angiogenesis to be induced by cells that normally would have been prevented from doing so by the high Tsp-1 concentrations in the surrounding ECM.

Major physiological regulators that work to promote or inhibit angiogenesis within tissues balance the angiogenic switch. Activators include: VEGF-A, -B, -C, FGF1 (aFGF), FGF2 (bFGF) other FGFs and others. Inhibitors are thrombospondin-1, -2, interferon α,ß, angiostatin, endostatin, collagen IV fragments and others.

D. DYSREGULATORY MECHANISMS OF CANCER

i) GENETIC AND EPIGENETIC DYSREGULATION

Somatic mutations which activate oncogenes or inactivate tumor suppressor genes (TSGs) are relatively rare events in the life of a cell. They occur perhaps at a rate of 10^{-6} per cell generation. This rare mutation frequency and the reqirement of multiple mutations to progress to a malignant tumor, provide a partial explanation for the fact that humans develop relatively few cancers.

Xeroderma pigmentosum (XP) is an inborn cancer susceptibility syndrome attributable to greatly increased mutational frequency. People suffering from XP show abnormally high sensitivity to UV radiation. This evokes squamous cell skin carcinomas and melanomas at exposed sites at a high rate. In skin cells of most humans, the pyrimidine dimers created by UV radiation are quickly excised from the damaged DNA and the initial, wild-type nucleotide sequence is restored. This is achieved by a cohort of DNA repair proteins that are specialized to effect this particular alteration of DNA structure. In patients with XP, one or another essential component of this specialized DNA repair apparatus is absent or defective (18). Altered DNA sequences are transmitted to the progeny of the initially irradiated cell, resulting in large numbers of mutations in their genomes.

XP represents only one of the familial cancer syndromes attributable to defective DNA repair. Another one is the Ataxia telangiectasia syndrome (19). In hereditary nonpolyposis colon cancer (HNPCC), the apparatus that recognizes recently made mistakes in DNA replication (mismatch repair apparatus) is defective (20).

Many familial breast cancers have recently been associated with inheritance of mutant versions of the BRCA1 and BRCA2 genes (21). Recent experiments suggest that both of these genes specify proteins that participate in the repair of double-strand DNA breaks.

UV radiation from the sun causes not only damage to DNA. It also causes production of reactive oxygen species that may interact with DNA to indirectly cause oxidative DNA damage. A benefit of sunlight is vitamin D, which is formed following exposure of 7-dehydrocholesterol in skin cells to UV. Vitamin D compounds have recently been shown to prevent UV-induced cell death and DNA damage in human skin cells (5).

It is likely that the development of most human tumors depends on losses of function of two major classes of cellular genes: TSGs and DNA repair genes. In addition, there is a mechanism of heritability that does not depend on genetic alterations (i.e. alterations of nucleotide sequence in a cell`s genome). This epigenetic mechanism depends on methylation of the cytidine residues present in CpG dinucleotide sequences that are found in proximity to the promoters of various genes. Such methylation often results in major shifts in the configuration of nearby chromatin, and in the shutdown of expression of nearby genes – a process called transcriptional repression.

The mechanisms that control DNA methylation result in the inactivation of genes at higher rates per cell generation than those involving somatic mutations. The obvious conclusion is that the function of TSGs and DNA repair genes is likely lost more frequently through DNA methylation than through mutation (6).

Hence, cancer pathogenesis is a disorder of genes and gene function. Such disorder does not always depend on genetic alterations. It may rather be attributed to a dysregulatory mechanism. The epigenetic mechanism of promoter methylation may contribute as frequently, if not more frequently, to tumor formation than do genetic mechanisms.

The organization of the genome, as mediated by chromatin and DNA methylation, appears to be abnormal in cancer cells of all types (23,24). Individual tumors may actually contain hundreds of genes affected by promoter DNA hypermethylation (25). Genes affected involve TSGs as well as genes involved in cell cycle control (p16, p15), apoptosis (DAP-kinase, ASC/TMS1, HIC1), increased stem/developmental pathway activity (SFRPs), DNA damage repair (MLH1, O6-MGM, GST Pi), cell adhesion (E-cadherin), cell migration (TIMPs), differentiation (GATA-4, GATA-5, TGF-ß receptor) or chromosomal stability (CHFR) (23-26).

Cancer has been described as a dysregulated epigenome allowing cellular growth advantage at the expense of the host (27).

ii) DYSREGULATION VIA miRNAs

The discovery of circulating miRNAs in body fluids has led to their possible use as biomarkers and treatment-response predictors. Evidence was provided that tumor cells communicate via the secretion and delivery of miRNAs packed into tumor-released microvesicles. This has prompted to investigate contributions of miRNA as signaling molecules to the establishment and maintenance of the tumor microenvironment and the metastatic niche (28). Types of body fluids that carry miRNAs include whole blood, serum, plasma, urine, saliva, pancreatic juice and cyst fluid.

The topic of regulation of cancer metastasis by cell-free miRNAs has been delt with in an excellent recent review (29). It includes regulatory aspects of cancer metastasis, biogenesis and function of miRNAs, their importance in cancer and metastasis, the role of exosomes in cancer and metastasis, secretion and uptake of cell-free miRNAs, cell-free miRNAs in metastasis, and miRNA targeting and therapy. Another review discusses a role of miRNAs as regulators of cancer metastasis and EMT (30).

iii) DYSREGULATION OF ENERGY SUPPLY (MITOCHONDRIA)

O Warburg had noticed that cancers "ferment" glucose via pyruvate to lactate thus causing acidification of tissue pH. An unusual concentration of lactate, a product of anaerobic glycolysis, was found even when there was enough oxygen around for aerobic glycolysis. He hypothesized that mitochondria of cancer cells have defects in function. This hypothesis from 1930 seems to have been confirmed in 2008 by American Scientists. They reported cardiolipid and electron chain abnormalities in mouse brain tumor mitochondria. Their findings are interpreted as lipidomic evidence supporting the Warburg theory of cancer (31).

Cardiolipin content was also found to be involved in liver mitochondrial energy wasting associated with cancer-induced cachexia (32). Cancer-induced cachexia describes the progressive skeletal muscle wasting associated with many cancers, leading to shortened survival time in cancer patients.

Respiratory Complex II of the mitochondrial membrane serves as a link between the tricarboxylic acid cycle and the electron transport chain. Complex II dysfunction has been implicated in cancer by a mechanism that likely involves the production of reactive oxygen species (ROS). The presence of cardiolipin was found to be critical for the assembly and enzymatic activity of Complex II, as well as for the prevention of ROS production.

iv) DYSREGULATION AT THE PLASMA MEMBRANE

Neurosurgical procedures, for instance in patients with GBM, and cerebrovascular accidents (strokes) often result in the accumulation of interstitial fluid in the brain (i.e. edema) and swelling of the neurons. Because the brain is enclosed within the skull, edema can raise intracranial pressure and thereby disrupt neuronal function. This dysregulation at the PM of neurons can lead to coma and death. The blood-brain barrier, which separates the cerebrospinal fluid and brain interstitial fluid from blood, can be permeated freely by water but not by most other substances.

Excess fluid in the brain can be removed by imposing an osmotic gradient across the blood-brain barrier. Manitol can be used for this purpose. Manitol is a sugar that does not readily cross the blood-brain barrier and membranes of cells (neurons and other cells). Therefore, mannitol is an effective osmole, and intravenous infusion results in the movement of interstitial fluid out of the brain by osmosis.

v) DYSREGULATION AT THE LEVEL OF THE ECM

Epithelial cells are particularly dependent on signals from the underlying ECM for maintaining their state of differentiation, function and survival. Loss of matrix attachment leads to metabolic stress. This is characterized by reduced nutrient uptake, decreased ATP production, and increased levels of ROS (33). Such loss of contact with the basement membrane ECM can result in anoikis, a special form of apoptosis. Thus, ECM attachment is a key regulator of cellular metabolism. Alterations in metabolism owing to changes or loss of ECM engagement during tumorigenesis may serve important tumor-suppressive functions (33).

Cancers derived from epithelial cells are all carcinomas. These represent the vast majority of human cancers. It is therefore of importance to try to understand their pathology and biology.

B Vogelstein studied in detail the multistep process leading to colorectal carcinoma formation. He could distinguish the following steps: human colonic epithelial cells from normal epithelium change via hyperplastic epithelium, early, intermediate and late stages of adenomas to carcinomas and to malignant carcinomas with invasive and metastatic potential (34). His multistep human tumor progression model associated those histological changes to molecular alterations, such as loss of the APOC gene, DNA hypomethylation, activation of the K-ras oncogene, loss of 18q TSG, loss of p53, etc. (34).

An alternative to this multistep mechanism involves the action of genes that are normally involved in programming certain key steps of embryogenesis. In such steps of embryogenesis, epithelial cells undergo a profound change in their differentiation program and acquire many of the phenotypes of mesenchymal cells, including motility and invasiveness. This transdifferentiation program is termed the "epithelial-mesenchymal transition" (EMT) (35).

Half a dozen transcription factors (TFs) acting during early embryogenesis are capable of programming EMTs. These TFs are for instance Snail, Slug, Twist, Goosecoid, and SIP-1. Each of these is able to act pleiotropically to cause the repression of epithelial genes and the induction of mesenchymal genes. Increasing experimental evidence indicates that carcinoma cells exploit these TFs to execute many of the steps of the invasion-metastasis cascade (36).

Such TFs seem to be induced by signals that the carcinoma cells experience in the tumor microenvironment and that originate in the tumor-associated stroma. For instance, transforming growth factor ß (TGFß) which may be released from the ECM, can impinge on certain cancer cells the expression of several of the mentioned TFs that are capable of programming an EMT (37). JARID2 , an interacting component of the PRC2 complex, catalyzes methylation of lysine 27 of histone H3 (H3K27). The expression of JARID2 was

increased during TGFß-induced EMT of lung and colon cancer cell lines and knockdown of JARID2 inhibited TGFß-induced morphological conversion of the cells associated with EMT (37).

These recent findings greatly simplify our concepts concerning the late stages of malignant progression. The genotypes of certain primary cancer cells allow them, in response to stromal signals, to activate long-dormant cell biological programs : EMTs. Once activated, this program seems to enable a carcinoma cell to complete most of the steps of the invasion-metastasis cascade, except one: organ colonization. This last step of the metastatic cascade appears to involve an adaptation to the novel tissue microenvironment.

Interestingly, carcinoma cells forming a metastasis often recapitulate the histopathological appearance of the primary tumor, including its distinctive epithelial cell sheets and ducts. It seems that signals from the new tissue microenvironment allow the mesenchymal carcinoma cells to revert via a mesenchymal-epithelial transition (MET) to the epithelial phenotype of their progenitors in the primary tumor.

Eribulin mesilate (eribulin), a non-taxane microtubule dynamics inhibitor, was recently shown to suppress experimental metastasis of breast cancer cells by reversing phenotype from EMT to MET states (38). Erubilin treatment of triple negative cancer cells with a mesenchymal phenotype led to decreased expression of several mesenchymal marker genes and to increased expression of epithelial markers. The cells, treated with eribulin for 7 days, showed decreased capacity for *in vitro* migration and invasion. In a xenograft model in vivo, the pretreated cells showed decreased numbers of lung metastases (38). It was concluded that these findings provide a plausible scientific basis for clinical observations of prolonged OS in breast cancer patients treated with eribulin.

While this concept involving EMT at the primary site and MET at a secondary site, can explain the malignant behavior of many carcinomas, it is less clear how tumors of other tissue origins (those of neuroectodermal, mesenchymal or hematopoietic tissues) acquire their aggressive growth and metastatic properties.

vi) DYSREGULATION OF THE TUMOR MICROENVIRONMENT

The tumor microenvironment (TME) contains, apart from ECM, resident stromal cells. These can be fibroblasts, adipocytes, macrophages, mast cells and vascular components. The TME also contains inflammatory cells of the innate and acquired immune systems such as NK cells, macrophages, DCs and tumor-infiltrating lymphocytes (TILs).

a) DCs

The TME imposes often negative effects on DC functions. These can result in inefficient antigen presentation or polarization into immunosuppressive DCs. There is thus increasing interest to use OVs to overcome such immunosuppressive influences in the TME.

A recent review examined how OV-DC interactions can affect DC recruitment, OV delivery, and anti-tumor immunity activation (39). The review includes major chapters about i) Development and function of DC subsets, ii) DCs in viral infections, iii) DCs in the TME and iv) Interaction between DCs and OVs.

b) Tumor-associated macrophages (TAMs, polarization towards M2, secreting ROS and RNS and factors like CSF-1, M-CSF and MMP-9)

Tumor-associated macrophages (TAMs) are recruited to the tumors trough cytokines and chemokines secreted by the cancer cells (40). Unlike macrophages in a normal, healthy tissue or wound-healing environment (41), TAMs are modified in the tumor microenvironment and loose the ability to phagocytose cancer cells or present TAAs to T cells (42).

Macrophages promote both early and late stages of tumor progression. They are found infiltrating the tumor at sites undergoing basement membrane breakdown. This is necessary for carcinoma cell invasion into the surrounding stroma (43). They release cytokines and chemokines that promote invasiveness of the cancer cells. This process is dependent on TNF-α and on matrix metalloproteinases, such as MMP-9 (44).

MMP-9 can degrade collagens of the ECM, in particular collagen types IV, V, XXI and XIV. Other targets of MMP-9 include laminin, chemokines, fibrinogen,

and latent TGFß. Macrophages are also found in hypoxic areas of the tumor (44). This may lead to up-regulation of VEGF (45), one of the proposed mechanisms by which TAMs may promote angiogenesis (46).

Macrophages also facilitate the seeding of cancer cells at the secondary site (47). As one of the key players in inflammatory responses, macrophages are at sites of chronic inflammation where they recruit other cell types (e.g. granulocytes, macrophages and DCs via G-CSF and GM-CSF). They also create a mutagenic environment through the secretion of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (48).

c) Cancer-associated fibroblasts (CAFs, secreting pro-angiogenic factors like FSP1, MMP-13 and TGF-ß1)

Fibroblasts, during wound healing change their phenotype to become "reactive". Carcinoma-associated fibroblasts (CAFs) differ from normal fibroblasts. They have an abnormally high expression of smooth muscle actin and increased expression of proteolytic enzymes and ECM proteins, such as tenascin-C. CAF-like cells may be derived from carcinoma cells that have undergone EMT. CAFs may also be expanded precursor mesenchymal cells, epigenetically changed fibroblasts or mutated fibroblasts.

CAFs stimulate epithelial cancer progression through secreted factors. One is fibroblast secreted protein 1 (FSP1, also called S100A4, metastasin or mts1) (49,50). This is a crucial stromal factor regulating metastasis (51,52). SDF-1α (CXCL12) is another important tumor promoting factor secreted by CAFs (53).

TGFß is a key player in the communication between CAFs and carcinoma cells. When acting on fibroblasts, TGFß normally protects epithelium from developing into carcinomas. TGFß secreted by CAFs, however, acts on epithelium to promote carcinogenesis (54, 55).

In carcinoma progression, CAFs are largely responsible for the desmoplastic response. This stromal response involves changes in the ECM with increased amounts of collagens, fibronectins, proteoglycans, and glycosaminoglycans (56).

There may be cross-talk between the collagen-rich stroma and the infiltrating leukocytes in tumors: Macrophages and DCs become activated and secrete chemokines in response to binding to type I collagen (56). Vice versa, leukocytes produce the ECM protein SPARC, which determines stromal collagen deposition in carcinomas.

d) Vascular endothelial cells and tumor neo-angiogeneis

Like normal tissues, tumors require access to the blood circulation in order to grow and survive. Pathologists noted in the mid 1950`s that cancer cells grew preferentially around blood vessels. Tumor cells that were located more than 0.2 mm away from blood vessels were found to be nongrowing or dying because of hypoxia. The threshold of approximately 0.2 mm represents the distance that oxygen can effectively diffuse through living tissues.

There are two ways by which tumors assemble vasculature. Myofibroblasts in the tumor stroma can release chemotactic signals, such as SDF-1/CXCL12. These chemotactic signals help to recruit circulating endothelial precursor cells into the stroma. This is aided by the release of VEGF, which helps these cells to mature into functional endothelial cells.

VEGF functions as a ligand to VEGF receptor-1 (FLT-1) and VEGF receptor 2 (Flk-1/KDR). Similarly, basic FGF (bFGF), another important angiogenic factor, binds to its own cognate receptor on endothelial cells. Once stimulated by such angiogenic factors, endothelial cells proliferate and construct within their cytoplasms bridges with neighbouring endothelial cells to form the lumen of a capillary. Such capillaries penetrate through existing tissue layers, and move towards the highest localized concentration of angiogenic factors. Similar mechanisms appear to operate during the formation of lymphatic vessels (lymph-angiogenesis).

In normal tissues, there is a systematic covering of capillaries by pericytes. In contrast, there is a chaotic dispersion of pericytes near tumor-associated capillaries. The walls of capillaries in tumors are about ten times more permeable than those of normal capillaries. The leakiness of tumor-associated capillaries leads to the accumulation of substantial amounts of fluid in the parenchymal spaces within a tumor. The ongoing expansion of

cancer cell populations exerts pressure on those few lymphatic vessels that do succeed in forming, causing their collapse.

The resulting defective lymphatic drainage within the cores of solid tumors further exacerbates the elevated accumulation of fluid. Capillary leakage generate high fluid pressure in the nonvascular parts of tumors. This pressure, in turn, greatly complicates the effectiveness of anti-cancer therapeutic drugs.

e) Dysregulation of TILs (recruitment of Tregs, checkpoint inhibition of CTLs)

CD4+CD25+Foxp3 expressing T-cells were found to be overrepresented in melanoma lymph node metastases (57). This may represent a mechanism by which tumors escape the immune system by first generating immunosuppression at the local lymph node site.

Melanoma cells were found to express IL-10, which is capable of inducing Tr-1 cells. Tr-1 cells represent another type of CD4+ regulatory cell type that can induce T-cell anergy and suppression of immune responses. It works primarily via the production of high levels of IL-10 and TGFß (58,59).

PD-L1 (B7-H1) is the ligand of the T cell checkpoint inhibitory receptor PD1. It is capable of inhibiting T-cell function and inducing T-cell "exhaustion". PD-L1 was found to be expressed in 22 of 22 melanoma biopsy samples (60).

f) Hijacking physiological systems for organ metastasis:

By EMT, carcinoma cells can escape regulatory influences from the subendothelial ECM. In an organ environment, such as lung or liver, they can make use of the reversion of EMT. This MET serves to organize the carcinoma cells themselves and their microenvironment to generate carcinoma-derived metastases. Two examples will be given:

1. Example: Signaling across distances via chemokines and their respective cell surface receptors. This is a physiological principle that can be hijacked by cancers. One of the more intriguing aspects of cancer metastasis is the predeliction of certain cancers for specific target tissue sites. This is especially true for breast cancer which attracted the attention of S Paget more than

hundred years ago. It now has become clear that breast cancer cells often express chemokine receptors, such as CXCR4. These receptors become stimulated via their chemokine ligand CXCL12 produced by normal cells from the sites of metastatic spread. Blocking the interaction between CXCR4 on a breast cancer cell line and its ligand CXCL12, produced by lung tissue, exerted a strong anti-metastatic effect (61).

2. Example: Bone metastases. The physiological balance between bone formation and resorption is created by signaling between osteoblasts, which assemble bone, and osteoclasts, which dissolve it. The osteoblasts release RANKL, which acts via the RANK receptor displayed by osteoclast precursors to induce the latter to mature into functional osteoclasts. The osteoblasts may also secrete osteoprotegerin (OPG), which acts as a decoy receptor to ambush RANKL before it can activate osteoclast precursors. Hence, the balance between RANKL and OPG determines the net rate of bone growth versus loss.

Breast cancer cells can dysregulate this physiological process and turn it into a vicious cycle of osteolytic metastases. Release by a breast cancer cell of parathyroid hormone-related peptide (PTHrP) causes osteoblasts to change the mix of signals that they release: They increase RANKL synthesis and decrease OPG synthesis. RANKL induces osteoclast precursors to mature into functional osteoclasts. The latter undertake osteolysis. This causes bone demineralization, exposes the ECM within the bone and results in liberation of TGFß, Ca++, and IGF-1. IGF-1 and Ca++ cause cancer cell proliferation and survival. The additional presence of TGFß induces the cancer cell to release more PTHrP, resulting in a self-sustaining positive-feedback loop that has been termed the "vicious cycle" of osteolytic metastasis (62).

E. REVERSION OF HEPATIC FIBROSIS

Liver fibrosis, a major health problem worldwide, is caused by the excessive accumulation of ECM proteins including collagen that occurs in most types of chronic liver diseases (63). Activated hepatic stellate cells (HSCs), portal fibroblasts, and myofibroblasts of bone marrow origin have been

identified as major collagen-producing cells in the injured liver (64). Liver fibrosis occurs in response to any etiology of chronic liver injury including hepatitis B and C, alcohol consumption, fatty liver disease, cholestasis, and autoimmune hepatitis (64).

Liver fibrosis can be the precursor of liver cirrhosis (65). Defenestration and capillarization of liver sinusoidal endothelial cells are major contributing factors to hepatic dysfunction in liver cirrhosis. Activated Kupffer cells destroy hepatocytes and stimulate the activation of HSCs. By interaction with tumor cells, activated HSCs become involved in development of hepatocellular carcinoma (HCC) (66).

Recent studies revealed that liver fibrosis is reversible (67). Activated HSCs can revert to quiescent HSCs when causative agents are removed (67). In line with this notion, it was shown that Newcastle disease virus represses the activation of human HSCs and reverses the development of CCL4 induced hepatic fibrosis in mice (66). Also, it was shown that overexpression of miR-483 in vivo inhibits mouse liver fibrosis induced by CCl4. This miRNA targets two pro-fibrosis factors, PDGF-ß and TIMP 2 (68).

F. AN EXAMPLE OF REVERSION OF DYSREGULATION IN LATE-STAGE CANCER

We have studied the basic question of reversion of cancer associated dysregulation in a mouse tumor model of advanced metastasized disease. There is no doubt that the immune T-cell mediated effects observed required a very special experimental model. The details of the model and of the immune mechanisms behind it were recently reviewed (69).

The tumor model is based on many years of immunogenetic research about resistance mechanisms in mice against the highly aggressive lymphoma variant ESb. It was possible to transfer the resistance from an immunized donor strain (B10.D2) via immune T-cells to the tumor susceptible host strain (DBA/2) of tumor origin. The immune T-cells were targeting TAAs, minor histocompatibility antigens and viral superantigens (vSAG-7, formerly known

as Mls[a]) expressed by the tumor cells. The results of this Graft-versus-Leukemia (GvL) model system are of basic importance because they suggest that dysregulations, even in advanced stages of cancer, are principally correctable and reversible.

i) REVERSION OF CACHEXIA AND COMPLETE REMISSION OF CANCER IN LATE-STAGE DISEASE

Cachectic DBA/2 mice to be treated were bearing a subcutaneously transplanted syngeneic tumor (ESb-MP lymphoma) of 1,5 cm diameter and had macroscopic liver and kidney metastases. 4 weeks after tumor cell transplantation, they could be successfully treated by a combination of sublethal (5 Gy) irradiation followed by a single intravenous transfer of 20 million anti-tumor immune spleen cells from tumor-immunized resistant allogeneic MHC-matched B10.D2 mice. The animals regained body weight with reversal of cachexia after treatment.

We are just at the beginnings to understand molecular mechanisms of cachexia (70-74).

ii) REVERSION OF TUMOR TISSUE pH FROM ACID TO NEUTRAL AS A FIRST SIGN OF IMMUNOTHERAPY

Longitudinal therapy evaluation was performed by means of high-field nuclear magnetic resonance (NMR). The spectra of phosphor metabolites of primary tumors (PTs) were acquired in 40 min from anesthetized animals using a 14-mm-diameter surface coil placed over the tumor.

The spectra from control animals demonstrated that tumor growth was associated with an increase in phosphor-monoesters (PME) and inorganic phosphate. The average tissue pH determined from the chemical shift of organic phosphate showed an increase of mild acidosis in control tumors with time. Spectra acquired 1 day before and 8 days after adoptive T cell immunotherapy (ADI) demonstrated a dramatic decrease in PME, a decrease in phosphate metabolites (necrosis and cell death) and a return to neutral tissue pH. Changes associated with an effective GvL effect (decrease in PME, increase in tissue pH) were detectable within 2-3 days.

iii) PRIMARY TUMOR TARGETING, ENCAPSULATION, REJECTION FROM THE SKIN, WOUND HEALING, AND SURVIVAL

Immunohistology of frozen tissue sections from PTs revealed details about donor immune cell infiltration and capsule formation. Six days after cell transfer, CD4+ T-cells could be seen in tumors of pre-irradiated animals, either in association with blood vessels or deep in the tumor mass. At this time point, a broad capsule of fibrous tissue between the tumor area and the skin could be seen in which Langerhans cells and dermal DCs were embedded as revealed by ATPase staining.

Three months after ESb-MP tumor inoculation and ADI treatment at day 28, surviving mice still carried scar tissue from the PT. Or they had rejected the PT from the skin showing wound healing underneath. This was followed by hair growth and reconstitution of normal fur (74).

40% of the ADI treated mice survived long-term (more than 4 months). Animals from non-treated controls, irradiation-only or ADI only groups, all died within 35 days.

iv) ERADICATION OF LIVER METASTASES

The therapy effect against established liver macrometastases was evaluated by immunohistochemistry of frozen liver tissue sections. A massive infiltration by donor T cells was seen 6-12 days after ADI, which consisted of CD4+ and CD8+ T-cells. Six days after ADI, large areas of necrosis could be distinguished in metastases from live tumor tissue. Twenty-one days after ADI treatment, neither live tumor tissue nor TILs could be detected. Sites of previous metastases were replaced by scar tissue (74).

The complete eradication of late-stage metastases by ADI could be demonstrated noninvasively in vivo by ^{1}H-NMR micro-imaging using a Bruker AM-300 spectrometer with a 7.0 T vertical magnet (74).

The effective immune rejection of advanced cancer in this GvL model and the subsequent return to normal tissue homeostasis can be explained by several types of cellular interactions:

i) donor CD4+ and CD8+ immune T-T cell interactions,

ii) donor T cell-host macrophage immune cell interactions, and

iii) help of vß6 T cells with specificity for the tumor-associated MMTV-derived viral superantigen vSAG-7 (69).

v) PROGRAMMED CHANGES IN LIVER GLYCOGEN AND LIPID METABOLISM DURING TRANSIENT GRAFT-VERSUS-HOST AND GRAFT-VERSUS-LEUKEMIA REACTIVITY

Glycogen in hepatocytes decreased dramatically 5 days after ADI. This coincided with a high increase of large fat granules. Liver marker enzymes, GOT and GPT, showed peak values also at day 5, coinciding with the loss of glycogen. 8 days after ADI, the livers started to re-express glycogen and to decrease their lipid content. Normalization of both parameters was seen after day 30.

Immune system recovery from irradiation damage and liver regeneration after immune cell mediated liver damage are likely explanations for the reversibility of the metabolic changes and for the lack of GvH disease and mortality in this effective cellular cancer immunotherapy model.

vi) LIVER RECRUITMENT OF BONE MARROW-DERIVED MESENCHYMAL STEM CELLS AND THEIR DIFFERENTIATION INTO ADIPOCYTES

It is likely that at day 5 after ADI, - the peak of GvH/GvL induced stress and toxicity -, the liver induced the recruitment of mesenchymal stem cells (MSCs) from the bone marrow and/or from other adipose tissue. Stromal cell-derived factor-1 (SDF-1) was found to increase the chemokine receptors CXCR4 and CXCR7 in adipose tissue-derived mesenchymal stem cells (75).

Hepatic stellate cells (HeSCs) are liver-resident BM-derived MSCs located in the space of Disse. Upon activation, the star shaped HeSCs can differentiate either into myofibroblasts to produce ECM, or they can differentiate into adipocytes. The lipid droplets at day 5 were observed primarily in HeSCs (formerly designated as Ito cells). The liver regeneration apparently used the lipid as fuel to produce the required energy. This may have involved resident

HeSCs as well as recruitment of MSCs via the SDF-1/CXCR4 axis and their activation and differentiation into adipocytes.

A similar situation was recently described for rats with acute pancreatitis (76). The expression of SDF-1 was significantly increased in the injured pancreas. The levels peaked on days 5-7 and began to decrease on day 10. SDF-1 induced a dose-dependent migration of BMSCs in an *in vitro* transwell migration assay. Furthermore, *in vivo*, the SDF-1/CXCR4 axis facilitated migration of dye-labeled BMSCs and repair of the injured pancreas (76).

It is likely that the Hippo pathway was involved in the described process of liver regeneration. The Hippo pathway plays pivotal and specific roles in organ growth, cellular plasticity, and stem cell biology. These phenomena are important for regeneration. Hippo regulates cell proliferation, apoptosis, and stemness in response to a wide range of extracellular and intracellular signals, including cell-cell contact, cell polarity, mechanical cues, ligands of G-protein-coupled receptors, and cellular energy status (77).

Dysregulation of the Hippo pathway has been observed in a variety of cancers (78). As oncoproteins, YAP and TAZ, two major effectors of the Hippo pathway, are frequently activated or highly expressed in cancer specimens. Therefore, targeting the Hippo pathway, for instance by inhibition of YAP/TAZ activity, has been suggested as a further approach of regulatory anti-cancer therapy (78).

Liver regeneration is a complex and well-orchestrated phenomenon. The process is associated with signaling cascades involving growth factors, cytokines, matrix remodeling, and several feedbacks of stimulation and inhibition of growth related signals (79).

F. TUMORS: WOUNDS THAT DO NOT HEAL ?

In 1986, the pathologist HF Dvorak expressed his conviction that tumors were wounds that would never heal (41). He saw similarities between tumor stroma generation and wound healing but something went wrong in tumors.

With the above described findings we argue against this hypothesis. A single transfer of tumor-reactive T-cells from B10.D2 animals which had rejected transferred ESb-MP tumor cells to 5 Gy pre-irradiated DBA/2 animals with a heavy load of syngeneic ESb-MP tumor caused complete tumor remission in most of the animals. Effector and memory T-cells with specificity for TAAs and minor histocompatibility antigens were supported by a high frequency of vß6 T cells directed against a viral superantigen.

How could such a reversion of cancer-associated dysregulation function? The cancer had already established a large primary tumor in the skin and macroscopic metastases in liver and kidney and the animals were already cachectic. The transferred T-cells had already been pre-immunized against that same tumor and were equipped with high specificity and diversity. We identified the following weaponry: Granzyme B, perforin, TRAIL, NO. Other weapons of the immune system such as superoxides, HOCL, H2O2, FasL, myeloperoxidase, complement and phagocytes are not excluded.

But these weapons must be directed, coordinated and adapted to the local needs. It appears like the fight in the film epos "Star Wars" between two systems: the good against the evil. We do not know the answer but one thing is clear: the immune system gained control over a body nearly destroyed by cancer.

Chapter VII

Key points:

1. **Seven levels were selected to describe mechanisms of physiological regulation and cancer-associated dysregulation.**

2. **The first concerns the level of DNA with six DNA repair mechanisms. It also includes other mechanisms to keep normal tissue stem cell DNA free from mutations. There exist cancer susceptibility syndromes with a**

greatly increased frequency of DNA mutations and defects in DNA repair mechanisms.

3. **Epigenetic mechanisms exerted through methylation of cytidine residues also work at the level of the DNA but affect the configuration of nearby chromatin and the shutdown of nearby genes. Such mechanisms lead to loss of function of two major classes of cellular genes: Tumor suppressor genes (TSGs) and DNA repair genes.**

4. **At the level of RNA, the focus lies on micro-RNAs (miRNA), noncoding small RNAs which regulate about 50% of all protein-coding genes. Every tumor has specific miRNA alterations which can be used as a tumor-specific signature.**

5. **Mitochondria are important for energy supply, cell survival and cell death. Changes of cardiolipin in cancer cell mitochondria lead to energy waste and to anaerobic glycolysis causing acidification of tissue pH, a phenomenon correctly described by O Warburg in 1930.**

6. **The next levels of regulatory importance are the cell`s plasma membrane, the extra-cellular matrix, the microenvironment (wound healing versus cancer) and aspects of organisation of distant organ metastases.**

7. **Two examples of reversion of dysregulation are being presented. One concerns liver fibrosis, the other advanced metastasized cancer. In the first example, reversion is mediated via the oncolytic virus NDV or via a distinct miRNA. In the second example, reversion of cancer and its metastases in late-stage disease, including reversion of cachexia is mediated, via immune T-cell transfer, by the immune system.**

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Table 45 Milestones of Research in Physiology and Medicine Part I

1901	**E von Behring***	**Serum therapy, Diphteria**
1904	**IP Pawlow***	**Physiology of Digestion**
1905	**R Koch***	**Research on Tuberculosis**
1912	**A Carrel***	**Research on Organ Transplantation**
1913	**C Richet***	**Research on Anaphylaxis**

1920	**A Krogh***	**Capillary-motoric regulatory mechanisms**
1922	**OF Meyerhof***	**Relationship between O_2 consumption and lactate production by muscles**
1923	**F Banting***	**Discovery of Insulin**
1930	**K Lansteiner***	**Discovery of the Blood Groups**
1931	**O Warburg***	**Nature and function of oxydative enzymes**
1935	**H Spemann***	**Role of an organisator in embryogenesis**
1936	**HH Dale***	**Chemical signal transmission in neurons**
1945	**A Fleming***	**Discovery of Penicillin**

*** Nobel Laureats**

Table 46 Milestones of Research in Physiology and Medicine Part II

1947	**CF Cori***	**Metabolism of glycogen**
1953	**HA Krebs***	**Discovery of the citric acid cycle**
1959	**S Ochoa* and A Kornberg***	**Mechanism of biological synthesis of RNA and DNA**
1964	**K Bloch* and F Lynen***	**Mechanism and regulation of the metabolism of cholesterin and fatty acids**
1968	**RW Holley*, HG Khorana* and MW Nirenberg***	**Interpretation of the genetic code and its function in protein synthesis**
1991	**E Neher* and B Sakmann***	**Direct demonstration of ion channels in cell membranes and signal transmission**

2001	**L Hartwell*, T Hunt* and P Nurse***	**Control of the cell cycle**
2006	**AZ Fire* and C Mello***	**Discovery of RNA Interference**
2012	**J Gurdon* and S Yamanaka***	**Re-programming of differentiated cells to pluripotent stem cells**

*** Nobel Laureats**

Table 47 Examples of levels of dysregulation in cancer

A Intra-cellular	**B Extra-cellular**
- Genetic	**- Glycocalyx/ECM**
- Epigenetic	**- Tumor microenvironment**
- miRNA	**- Metastatic organ microenvironment**
- Transcriptional networks	**- Innate immunological control**
- Metabolic	**- Adaptive immunological control**
- Energetic/Mitochondrial	**- Nutritional**
- Plasma membrane	**- Hormonal**
- Signal transduction	**- Neuronal**

Table 48 Examples of documented reversions of dyregulations

i) Immune system mediated
cachexia reversion
primary tumor rejection
tumor tissue pH correction
wound healing after primary tumor rejection
liver metastasis eradication
liver regeneration
ii) other means
reversion of liver fibrosis via oncolytic NDV treatment
reversion of liver fibrosis via miRNA treatment

CHAPTER VIII. POTENTIAL NEW TARGETS FOR THERAPY: HOST CELLS AND THE STROMAL SUPPORT NETWORK

This Chapter tries to elucidate potential new targets for therapy. The targets are dysregulatory mechanisms described in the previous Chapter. The aim is to derepress epigenetic dysregulation in cancer cells and to interrupt the support of cancer provided by host cells and stroma network.

A. TARGETING EPIGENETIC DYSREGULATION

The most characterized mediators of epigenetic inheritance are gDNA methylation and histone posttranslational modifications. These processes cooperate to alter chromatin state and genome transcription. Different "epigenetic drugs" are able to revert such "epimutations" (1). Drugs that reverse DNA methylation, such as 5-azacytidine and 5-aza-2`-deoxicytidine and histone deacetylase inhibitors that target the histone deacetylation component of gene silencing are already in the clinic (2,3) and approved by the FDA for certain diseases.

A randomized controlled trial with azacytidine was performed in patients with myelodysplastic syndrome (4). The encouraging results indicate that at least some of the clinical effects were due to true reversal of epigenetic targets: i) clinical efficacy was accomplished at far lower doses than the ones initially used, ii) emerging data suggest that the efficacy of the aza-cytidines correlates with the acute reversal of gene silencing (5). Feasibility for prolonged drug treatment regimens appears possible for the aza-cytidines (6).

Epigenetic mechanisms play an important role in the regulation of tumorigenesis. Hypoxia-induced epigenetic changes may be critical for the adaptation of cancer cells to the hypoxic microenvironment of solid tumors. L Poellinger demonstrated that inhibition of the H3K9 methyltransferase G9A (by the small molecule inhibitor BIX-01294) attenuates oncogenicity and activates the hypoxia signaling pathway (7). In similar ways tumor cells may adapt to all kinds of oxidative and metabolic stress conditions (8).

The group of TW Mak is studying new ways to combat cancer: targeting metabolic adaptations, manipulating a cancer cell`s response to excessive oxidative stress, and exploiting aneuploidy (9).

The epigenetic machinery affects not only protein-coding genes but also expression of miRNAs (10). miRNA expression is regulated by multiple transcriptional networks as well as by the epigenetic machinery. Also, miRNAs can themselves repress key enzymes that drive epigenetic remodeling. miRNAs can directly modulate gene transcription in the nucleus through the recognition of specific target sites in promoter regions.

Regulatory circuits linking epigenetics and miRNAs have a major impact in genome transcription and cell physiology. Tumor-associated aberrations in the miRNA or epigenetic machineries are widely distributed in human cancer. We are only beginning to understand their relevance in diagnosis, prognosis or therapy (10).

The era of epigenetics thus is an exiting one and will have a major impact on cancer control.

B. TARGETING HOST CELLS HELPING EXTRAVASATION AND METASTATIC NICHE FORMATION

i) HOST-TUMOR CELL INTERACTIONS

Many cancer cells, including cancer stem cells, that are carried through the circulation form small aggregates (microthrombi) that lodge by passive mechanical or active mechanisms in arterioles and capillaries of various tissues. Platelets and the coagulation system have been shown to promote survival of circulating tumor cells (CTCs) in the bloodstream by conferring resistance to the shear stress and to attack from natural killer cells (11).

Platelet activation has been associated with EMT, while Tissue Factor (TF) protein expression by cancer cells correlated with hypercoagulable state and metastasis (12). Platelets were also found to promote or maintain the state of EMT on CTCs through secretion of TGFß in response to CTC activation (13). Platelets also secrete CXCL5 and CXCL7 to recruit granulocytes (14) and govern pre-metastatic tumor communication to bone (15). Two drugs, aspirin

and a P2Y12 inhibitor, were found capable of attenuating platelet-induced ovarian cancer cell invasion (16).

Studies from B Qian et al (17) suggest that a distinct population of CD11b+ macrophages may recognize emigrating tumor cells and assist them with the extravasation process. After gaining access to the underlying tissue parenchyma, extravasated tumor cells establish reciprocal signaling networks with stromal cells to promote their own growth. Recruitment of monocytes/macrophages by tissue-factor (TF)-mediated coagulation was found to be essential for metastatic cell survival and premetastatic niche establishment in mice (18). M2/repair-type macrophages predominate in human cancers and actively stimulate tumor growth. Targeting the modulation of M2/repair-type macrophages into M1/kill-type macrophages would be a breakthrough (19).

Oxygen sensing prolyl-hydrolyse (PHD) proteins by T cells have been described to be involved in establishing an immunologically tolerant metastatic niche in the lung (20). Pharmacologic inhibition of PHD proteins limits tumor colonization of the lung (21). Anti-tumor effects can also be achieved by supplemental oxygenation. This would weaken the hypoxia-A2-adenosinergic immunosuppression in the tumor microenvironment (22).

Hepatic stellate cells (HSC) in the space of Disse play an important role for induction of a pre-metastatic niche in the liver (23). They become activated by acidic tumor microenvironment and promote the metastasis of hepatocellular carcinoma (HCC) via osteopontin (23). PDGF receptor-α (PDGFRα) and TGFß are required for HSC activation during liver metastasis. PDGFRα promotes TGFß signaling by regulation of TGFß receptors (24).

Other findings suggest that HSCs play an important role in liver mestastasis of colon cancer cells by the action of the SDF-1/CXCR4 axis. Blockade of this axis would be a target for antimetastatic therapy (25). Interestingly, the Chinese herbal compound "Songyou Yin" was found to attenuate hepatoma cell invasiveness and metastasis through downregulation of cytokines (IL-6) and growth factors (TGF-ß, VEGF, hepatocyte growth factor) secreted by activated HSCs (26). Inhibition of the SDF-1/CXCR4 axis was also reported to be possible by kisspeptin-10 (KP-10). KP-10 also inhibited in MCF-7 breast cancer cells EMT (27).

KP-10 was also demonstrated to be capable of inhibiting the Warburg effect in breast cancer. By activating the Smad signaling pathway, KP-10 induced mitochondrial injury (28).

Another potentially interesting target may be Tissue-Inhibitor-of-Metalloproteases (TIMP). Pancreatic premalignant lesions were reported to secrete TIMP-1. This then activates HSCs via CD63 signaling to create a pre-metastatic niche in the liver (29).

Activated HSCs also play an important role in liver fibrosis. As reported in Chapter VII, reversion of liver fibrosis could be achieved, in model systems, by treatment with oncolytic virus (NDV) and by treatment with a distinct miRNA.

ii) THE USE OF MABs OR SIMs

a) Capillary endothelial cells may be targeted by anti-VEGF and anti-VEGF-R antibodies, small molecule VEGF-R inhibitors, VEGF-Trap, Ang2/Tie2 blocking antibodies as well as by endogenous angiogenesis inhibitors and inhibitors of epithelial precursor cell (EPC) recruitment.

b) Pericytes may become inhibited by anti-PDGF antibodies, PDGF-R inhibitors and inhibitors of Ang-1/Tie2 signaling.

c) Fibroblasts may become inhibited by inhibitors of HGF or its receptor c-Met. This may also be true for inhibitors of CXCL12/SDF-1, PDGF/PDGF-R or of fibroblast activation protein (sibrotuzumab). Innate immune cytokines may also be interesting agents to regulate fibroblast behavior (30).

d) Neutrophils, macrophages and mast cells may be affected by anti-inflammatory inhibitors, cytokine and chemokine inhibitors and by inhibitors of NF-kB, IKK and TNF-α.

e) Lymphatic cells may be targeted by inhibitors of VEGF-C, VEGF-D, VEGF-R3, or PDGF/PDGF-R.

Several strategies aim at blocking immunosuppression by Tregs:

i) elimination by targeting CD25 (IL-2Rα) via mab or via an IL-2-toxin fusion protein (Ontak),

ii) elimination by lymphocyte depletion with cytotoxic drugs, such as low dose cyclophosphamide,

iii) blocking the mediators of suppression, e.g. IL-10, TGF-ß and CTLA-4,

iv) activation of DCs to express IL-6 that will block the function of Treg,

v) targeting TLR8 expressed on Treg to block their inhibitory function.

Cancer treatments known as immune checkpoint inhibitors unleash the immune system to attack cancer. New immune checkpoint inhibitor therapies prevent the PD-L1 checkpoint protein from attaching to the PD-1 checkpoint receptor. This is a perfect example of a clinically successful interference with a cancer-derived dysregulation of the adaptive specific anti-tumor T-cell response.

Since the first remarkable reports of immune checkpoint inhibitors shrinking advanced melanoma in 2011, research in this area has taken off at an incredible pace. In 2016, the FDA approved five new uses for immune checkpoint inhibitors: lung cancer, head and neck cancer, bladder cancer, kidney cancer, and Hodgkin lymphoma (HL). In 2017, ASCO has named Immunotherapy 2.0 as the advance of the year!

Further details about progress obtained with checkpoint inhibitors have been mentioned in Chapter IV (Immunotherapy) and Chapter VI (Combination Therapies).

C. TARGETING INVADOPODIA, INVASION ENZYMES AND THE ECM AT THE INVASION FRONT

i) TARGETING INVADOPODIA

The vascular basement membrane (BM) is a thin and dense cross-linked ECM layer that covers and protects blood vessels. New evidence has mechanistically linked the breaching of vascular BM with the formation of specific cellular micro-domains known as podosomes and invadopodia (31).

Invadopodia are actin-rich organelles that protrude from the plasma membrane and contact and locally degrade the ECM. They represent key cellular structures that are used to coordinate and regulate the various components of the process of cancer invasion (32). Invadopodia formation accompanies the mesenchymal mode of migration on firm matrices and is facilitated by Rac1 activation (33). Invadopodia represent a new therapeutic target to block cancer metastasis (34).

It is satisfying to remember that oncolytic NDV targets exactly Rac1 (35) as we described in Chapter V.

ii) TARGETING INVASION ENZYMES

The plasminogen system has been implicated in clot lysis, wound healing, tissue regeneration, cancer and many other processes that affect health and disease. The urokinase receptor uPAR was originally thought to assist the directional invasion of migrating cells. Now it becomes increasingly evident that this proteinase receptor elicits a plethora of other cellular responses (36).

Urokinase plasminogen activator (uPA): The inactive, pro-enzyme pro uPA is released by stromal cells and binds to its cognate receptor (uPAR) displayed at the surface of a cancer cell at the invasion front. This binding converts the pro-enzyme into active uPA which then converts the serum protein plasminogen from local blood vessels to the active plasmin form. The latter functions as a protease to cleave pro-enzyme forms of matrix metalloproteases (pro-MMPs) into active MMPs and latent TGF-ß1 into its active form.

Recently, novel selective inhibitors of uPA have been discovered (37).

iii) INHIBITION OF ECM DEGRADATION

Disruption of the basement membrane is a hallmark of malignancy. Proteolytic enzymes of many classes, implicated in tumor cell invasion (uPA, plasmin, cathepsins, MMPs), contribute to matrix degradation. Other matrix-degrading enzymes such as heparanase, which cleaves heparin sulfate proteoglycans, and hyaluronidase, which cleaves hyaluronic acid, have also been associated with tumor progression and invasion.

L Liotta observed that metastatic potential correlates with the degradation of type IV basement membrane collagen by metal-dependent enzymes (38). These metalloproteinases (MMPs) are now recognized as MMP-2 and MMP-9.

MMPs are overexpressed in most types of cancer and correlate with advanced tumor pathology. An increase in their expression and activity often correlates with tumor angiogenesis, metastasis, and poor prognosis. Most MMPs are not expressed by the cancer cells themselves but instead are expressed and activated in the stroma. Recent data indicate that the linkage between matrix remodeling, adhesion, and growth signaling may drive soft tiisue sarcoma bone metastases. This can be the basis for prognostic and therapeutic strategies (39).

An example of an MMP that promotes carcinogenesis is MMP-3/stromelysin-1. Overexpression of MMP-3 alters epithelial cell adhesion by cleaving E-cadherin, inducing EMT, and promoting premalignant and malignant lesions. MMP-3 induces Rac1b, an alternatively spliced variant of Rac1, which then stimulates increased levels of mitochondrial reactive oxygen species (ROS) and thereby DNA oxidative damage (40).

Development of inhibitors of MMPs has been fraught with challenges. Current research employs innovative approaches for drug delivery methods and allosteric inhibitors (41).

iv) ENZYME INHIBITORS

Inhibitors of matrix turnover include suramin and dalteparin. Inhibitors of proteases, such as uPA and MMPs have already been delt with.

Another enzyme of great significance for ECM degradation is heparanase (Hpa). This endo-ß-D-glucuronidase which cleaves heparan sulfate side chains from HSPGs on cell surfaces and from the ECM, has pro-metastatic, pro-

angiogenic and pro-coagulant functions (42). The search for selective Hpa inhibitors has been long and sometimes frustrating.

Phosphomannopentaose sulfate (PI-88) is a drug that suppresses angiogenesis by downregulating Hpa and VEGF (43). The administration of PI-88 at 160 mg/d was reported to be safe, well tolerated and to confer significant clinical benefits for patients with HCC (44).

v) MABS AS ECM CONTACT INHIBITORS

Tumor cell contact to ECM is often mediated via integrins. Among these, integrins αvß3, αvß5, α5ß1, or α6ß4 seem particularly suited as targets for contact inhibition by respective mabs.

The interaction of cells with the ECM can be facilitated (e.g. CD44, integrin ligands osteopontin and periostin) or inhibited (e.g. the ECM glycoprotein tenascin). Thus, mabs against osteopontin or periostin might be usefull to inhibit facilitation of contacts of tumor cells to the ECM.

D. TARGETING COMMUNICATION BETWEEN SEED AND SOIL

i) CANCER CELL-DERIVED EXOSOMES

Exosomes (endosome derived vesicles) are small (30- to 100-nm) vesicles of cells that carry a variety of bioactive molecules. Such molecules include proteins, lipids, RNA, as well as DNA molecules. They serve important roles in cellular communication, both locally and distally.

The exosomal process is abnormal in cancer (45). Glypican-1 was reported to identify cancer exosomes and to detect early pancreatic cancer (46). In 2001, tumor-derived exosomes were proposed as a souce of shared tumor rejection antigens for CTL cross-priming (47).

Exosomes appear to play a significant role in different stem cell niches such as the mesenchymal stem cell niche, cancer stem cell niche and pre-metastatic niche. Cancer cell-derived exosomes participate in crucial steps of metastatic spread of a primary tumor, ranging from oncogenic reprogramming of malignant cells to formation of pre-metastatic niches. Such

effects are achieved through the mediation of intercellular cross-talk and subsequent modification of both local and distant microenvironments in an autocrine and paracrine fashion.

Exosomes also orchestrate multiple systemic pathophysiological processes. Examples are coagulation, vascular leakiness, and reprogramming of stromal recipient cells to support pre-metastatic niche formation and subsequent metastasis. Pancreatic cancer-derived exosomes were taken up by liver Kuppfer cells and caused hepatic stellate cell activation to generate a fibrotic environment with immune cell infiltrates that favours metastases (48).

There is a potential for clinical application of cancer cell-derived exosomes, both for diagnostic as well as for therapeutic purposes (49 -54). Exosomes play also a role in immune regulation (51). Dendritic cell-derived exosomes have been proposed as immunotherapies in the fight against cancer (52). Efficacy of vaccination with tumor-exosome-loaded dendritic cells combined with cytotoxic drug treatment has been reported in pancreatic cancer (55).

In 2013 JE Rothman, RW Schekman and TC Südhof received the Nobel Prize for Physiology or Medicine for their "discovery of a machinery regulating vesicle traffic, a major transport system in our cells".

iii) miRNA

microRNA (miRNA) are small highly conserved noncoding RNAs. They play an important role in the complex network of gene regulation, in particular with regard to gene silencing. miRNAs regulate gene expression in a highly specific way at the post-transcriptional level. miRNAs normally consist of 21 to 23 nucleotides (nt), but sometimes they can also consist of hundreds of nts. miRNA binds to the 3`untranslated region (3`-UTR) of mRNA of a distinct target gene. This leads either to inhibition of translation of such mRNAs or to its degradation. miRNAs were first described 1993 but the name was introduced only in 2001. In human, more than 1.800 different miRNAs have been identified. They can be checked in a respective library (miRBase.org).

Recent research indicates that some miRNAs are important for maintaining pluripotency and self renewing capacity of embryonal stem cells (56-58). The following examples demonstrate the relevance of miRNAs for cancer.

microRNA-335 was reported as metastasis suppressor targeting the formin family of actin nucleators (59). It was also reported as a potential suppressor of metastasis and invasion in gastric cancer (60). Furthermore, it promoted cell proliferation by directly targeting Rb1 in meningiomas (61) .

microRNA-145 was reported to regulate cancer stem-like properties and EMT in lung carcinoma-initiating cells (62). In these cells, miR-335 suppressed proliferation by targeting OCT4 (63). It also was found to inhibit lung cancer cell metastasis (64). Epigenetic silencing of miR-145-5p contributed to brain metastasis (65).

microRNA-302 increased reprogramming efficiency via repression of two transcription factors, NR2F2 and OCT4 (66). Anti-miR-302 inhibitor abrogated the production of hyaluran-CD44v3-mediated cancer stem cell functions (67).

Every tumor has specific miRNA alterations, i.e. some are overexpressed and others downregulated. These altered miRNAs can be used as tumor-specific signature. Specific miRNAs can be targeted using oligonucleotide sequences corresponding to the altered miRNAs. These are referred to as "antagomirs". In this way, one could design targeted therapies for personalized medicine in patients (68).

iv) TARGETING HIJACKED PATHWAYS OF ORGAN METASTASIS

Lack of environmental oxygen at the tumor site leads to activation of the transcription factor HIF-1. This factor induces genes like erythropoietin or VEGF with a role in neo-angiogenesis. The integrin ligand osteopontin is another mediator of angiogenesis which is secreted into the blood circulation. HIF-1 has been described as a master regulator of breast cancer niche formation. Hypoxia also induces lysyl oxidase which is critical for bone marrow cell recruitment and pre-metastatic niche formation.

Tumor signaling via PD1 on T cells and expansion of MDSCs are major mechanisms of tumor immune escape. CXCR2 was identified as a novel target for modulating tumor immune escape (69).

MDSCs and TAMs form an important component of the hypoxic tumor microenvironment. Hypoxia caused a rapid, dramatic, and selective up-

regulation of PD-L1 on MDSCs, macrophages, dendritic cells and tumor cells (70). Blockade of PD-L1 under hypoxia enhanced MDSC-mediated T cell activation and was accompanied by the down-regulation of MDSC-secreted IL-6 and IL-10. Neutralizing mabs against IL-10 under hypoxia abrogated suppressive activity of MDSCs. Simultaneous blockade of PD-L1 along with inhibition of HIF-1α may represent a novel approach for cancer immunotherapy.

Proteins of relevance for the metastatic niche formation appear as promising targets. These can be transcription factors (e.g. HIF-1), growth factors (e.g. VEGF, TGFß, HGF), chemokines (e.g. CCL2) and their receptors (e.g. CXCR4), enzymes (e.g. lysyl oxidase) or enzyme inhibitors (e.g. TIMP-1) and cytokines (e.g. TGFß, IL-6). We must be aware, however, that these targets derive from normal physiological regulatory systems. Therefore, there targeting should be done only transiently and in critical steps of tumor development.

Building of the metastatic niche is facilitated via S1PR1-STAT3 signaling to recruit MDSCs (71). An immunosuppressive environment is promoted by carbonic anhydrase IX (72) and complement C5a receptor (73). TIMP-1 creates a pre-metastatic niche through SDF-1/CXCR4-dependent neutrophil recruitment (74). Neutrophils compete with monocytes for access to the chemokine CCL2 for control of the metastatic niche (75). The liver environment appears tolerogenic with regard to antigen-presenting cell function (76). This might also facilitate liver metastasis.

A balance between TIMP-1 and MMP-9 plays an important role for the viability of alveolar macrophages (AMs) in chronic obstructive pulmonary disease (COPD). A chinese drug, in form of Liuweibuqui capsules, was reported to be capable to inhibit the release of inflammatory cytokines, promote viability in AMs, and regulate the expression of MMP-9 and TIMP-1 (77).

TGFß is an important signaling molecule. It is a local multifunctional cytokine and can exert direct anti-inflammatory effects by inhibiting Th1 helper cells. It also plays an important role in wound healing. In addition to its effects on epithelial proliferation, survival, and differentiation, it is also an important regulator of the cell-matrix interaction (78). It may also play a role

in the myofibroblastic stromal reaction and lymph node metastasis in invasive breast carcinoma. Future research on fibrocytes, myofibroblasts, TGFß and mechanisms of stromal changes are essential in future and may lead to new treatment approaches (79).

Chapter VIII

Key points:

1. Based on the previous chapters, numerous potential new targets for cancer therapy are being pointed at.

2. One direction concerns the interference with epigenetic mechanisms of cancer cell dysregulation.

3. The others aims at interruption of cancer support from host cells and from the stromal network.

4. The interference with host support of tumor growth and metastasis represents a change of paradigm: away from the focus on the tumor with all its variability.

5. Target directed interference can be exerted trough the use of monoclonal antibodies or various inhibitory molecules, including TKIs, enzyme inhibitors or miRNAs.

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CHAPTER IX FROM THE PAST TO FUTURE DIRECTIONS OF CANCER THERAPY

A. FROM THE BEGINNINGS TO STANDARD THERAPY

This book can be considered like a journey. A journey through history of cancer treatment and cancer research. Also a journey through a variety of different research areas. From surgery to physical treatment (radiotherapy), further to chemical treatment (chemotherapy), then to physiological treatment (hormone therapy).

These standard therapies of cancer were developed about 100 years ago. At that time nothing was known about cancer metastasis and very little about tumor-host interactions as exemplified by tumor immunology. So it is not surprising that in absence of a scientific basis, various concepts of treatment developed into dogmas which later turned out to be wrong.

B. FASCINATING DISCOVERIES OF THE LAST 60 YEARS FROM CANCER RESEARCH

Meanwhile cancer research has led to discoveries which provide a much deeper understanding of molecular and cellular processes and their physiological regulation or dysregulation in case of cancer development. Our journey takes us from the beginnings of molecular biology to the discovery of oncogenes and tumor suppressor genes. This leads to new insights into the cell cycle, its clock-wise function and control. The decision concerning cell growth or quiescence, cellular senescence or programmed cell death is taken at the restriction (R) point during the G1 phase.

Our journey includes auto-biographical notes because this was the time period that I can witness from my own scientific career. Cancer metastasis, tumor immunology and cancer immunotherapy were three research fields which in the 1970s were still very much in the dark. Since I felt that these

areas were of great relevance for the future development of cancer treatment, I tried to get engaged with these questions.

A milestone discovery in metastasis research was what is called the EMT: the transition of an epithelial cancer cell into a mesenchymal phenotype. This environmental signal-induced change of phenotype is based on epigenetic re-programming. It facilitates cancer cell invasion and dissemination via the blood circulation or through the lymphatic system. Once arrived in an organ with a suitable "soil", the establishment of secondary growths (metastases) is facilitated by a reversion of EMT called mesenchymal-to-epithelial transition (MET).

Tumor virologists, molecular biologists and cell biologists eventually found out that products from oncogenes and tumor suppressor genes often function via affecting signal transduction by cellular growth factor receptors. This then became the area of so-called targeted therapies. While cytostatic drugs interfere with tumor cell proliferation by inhibiting enzymes within the tumor cells, targeted therapies interfere with transduction of signals from outside the cell via growth factor receptors. Tumor characteristic protein tyrosine kinases associated with such receptors are inhibited by small molecule inhibitors developed by pharmaceutical companies. Gleevec was the first approved drug of this kind.

C. IMMUNOTHERAPIES ARISING

i) MONOCLONAL ANTIBODIES

Like the research of molecular biology and virology, the research of immunology in the last 60 years had a fascinating and successful development. This is witnessed by many Nobel Prizes for discoveries concerned with the genes and proteins that characterize the antigen-specific receptors of B- and T-lymphocytes. In both cases the high diversity and antigen specificity is generated from a restricted germline pool of variable and constant domain genes. Somatic rearrangement mechanisms during B-cell development in bone marrow and T-cell development in the thymus enabled in vertebrates the development of the adaptive arm of the immune system with its billiards of different receptor specificities.

The first clinically relevant success story is that of the development of monoclonal antibodies, which are products of B lymphocytes. The first FDA-approved mab was trastuzumab (Herceptin). This antibody targets the cell surface receptor HER2 expressed for instance by breast cancer cells. Meanwhile dozens of therapeutic mabs are available for application in patients with a large variety of cancer types.

The second clinically relevant success story turned up only in recent years: the development of mabs targeting immune regulatory receptors on T cells, such as CTLA-4 or PD-1. These receptors deliver negative signals to activated T cells to stop their activity at the end of their response. Tumors are able to hijack this physiological regulatory mechanism to their own advantage. They thereby shut-off anti-tumor reactivity coming from tumor-infiltrating T cells.

The clinical application of checkpoint inhibitory mabs, that interfere with this tumor immune escape mechanism, has resulted in a proportion of melanoma and carcinoma cancer patients with an improvement of long-term survival. Such results were received with great surprise because this had never been achieved before, neither with cytostatic drugs nor with small molecule inhibitors.

ii) IMMUNE T-CELLS

Further milestones from immunology came from research on T-cells and Dendritic cells. The MHC restriction of cytotoxic T lymphocytes was discovered in 1973, the genes coding for the TCR in 1984 and the first human tumor-associated antigen (TAA) recognized by CTLs as a peptide-MHC complex was identified in 1991.

The identification of HIV as the causative agent of the Acquired Immunodeficiency Syndrome (AIDS) opened the way for therapeutic targeting and also elucidated the importance of the immune system for maintenance of general health. The discovery of Toll-like receptors (TLRs) triggering innate immune reactivity as well as that of Dendritic cells functioning as professional antigen-presenting cells (APCs) for T-cells were further milestones in the new millenium.

Adoptive T-cell transfer therapies involve allogeneic donor cell transfers to achieve Graft-versus-Leukemia effects. They can also transfer autologous immune T-cells, especially memory T cells from bone marrow.

A new recent development consists of modern gene transfer technologies which allow to produce T-cells with transfected TAA-specific TCRs or with chimeric TAA-specific receptors (CARs) consisting of antibody binding sites fused to T cell receptor signaling chains.

iii) ANTI-CANCER VACCINES AND ONCOLYTIC VIRUSES

Immunotherapies based on T-cell immunity include active immunization with cancer vaccines and adoptive cellular therapies. Oncolytic viruses (OVs), also developed in the past 60 years, are very promising new biological agents. They have the capacity of tumor selective replication and of tumor selective toxicity.

They also support the development of post-oncolytic anti-tumor immunity. Therefore, the combination of OVs with cancer vaccines has its own logic. Two types of such vaccines have been developed by our teams, ATV-NDV in Heidelberg (1985-2005) and VOL-DC in Cologne (from 2010 up to now).

D. COMPARING IMMUNOTHERAPIES WITH OTHER THERAPIES

i) IMMUNOLOGICAL TOLERANCE

During evolution, the immune system evolved starting with innate immunity mechanisms. These were able to identify, via innate immunity recognition receptors (e.g. TLRs, RLRs) foreign from self-molecules and to react against these.

Later, during vertebrate development, the adaptive immunity system evolved. This is characterized by lymphoid organs specialized for development of new cell types with a broad variety of cell surface receptors for antigen recognition. Special recombinase enzymes were developed to generate a great variety of new antigen-specific receptors from a set of germline genes during somatic B- and T-lymphocyte maturation. Before

these cells are allowed to leave their respective lymphoid organs, they are selected against reactivity towards self antigens (proteins from the body`s own cells).

The term *Horror autotoxicus* was introduced by Paul Ehrlich at the beginnings of the 20th century to describe that the humoral immune system has developed mechanisms to avoid self destruction. In the 1950s, Sir Macfarlane Burnet described a cellular mechanism for the avoidance of auto-immune reactions and introduced for this the term self-tolerance.

In 2017, M Feuerer and colleagues described in Nat Immunol (1) that Treg cells not only maintain self-tolerance. Their other function is to support organ homeostasis by differentiating into specialized tissue Treg cells. A genome-wide DNA-methylation landscape analysis revealed more than 11,000 regions that were methylated differently in pairwise comparisons of tissue Treg cell populations and lymphoid T-cells. Tissue Treg cells integrate multiple waves of epigenetic reprogramming that define their tissue-restricted specialization (1).

For cancer immunotherapy, this discovery means that Treg cells are by no means an enemy to cancer-reactive T-cells. They help to maintain self-tolerance and organ homeostasis.

The immunotherapies I dealt with for over 40 years did not interfere with Treg cells and were always characterized by low side effects.

ii) IMMUNOLOGICAL MEMORY

We mentioned above already that immune system reactivity is based on excluding anti-self reactivity. Sophisticated mechanisms of central and peripheral tolerance serve this purpose. This is one advantage of immunotherapy in comparison to other therapies with their side effects.

When comparing immunotherapy with other therapies there is another characteristic difference, namely that of a memory function. Immunological memory serves the purpose of obtaining long-term protection and long-term health effects. The details of the immunological memory function are not yet entirely understood but it is likely that they are connected to special niches in

the bone marrow and to a state of regulation that is reminiscent to that of stem cells.

Metabolic activity of T cells regulates and is regulated by cellular signaling pathways and epigenetics (2). Thus, T cell longevity and function in immunotherapy is profoundly influenced by metabolic regulation (2). Another recent discovery is that of mitochondrial priming by CD28 (3). Early CD28-dependent mitochondrial engagement is needed for T cells to remodel cristae, develop enhanced spare respiratory capacity (SRP), and rapidly produce cytokines upon restimulation. These are all cardinal features of protective memory T cells (3).

Neither radiotherapy nor chemotherapy have a memory function. If the cancer is not entirely destroyed, the remaining cancer cells, in particular the cancer stem cells, can develop new growths.

iii) INDIVIDUALITY OF THE CANCER-REACTIVE MEMORY REPERTOIRE

When we analyzed the repertoire in breast cancer patients of bone marrow derived memory T cells against TAAs, each patient showed a different pattern of reactivities. There was a multitude of target molecules that were recognized in individual patients. This did not cause any problem with side effects.

This finding is of relevance not only with regard to the question of one or multiple target antigens. The individuality of the immune memory repertoire seems to mimic the individuality of the cancers that arise in patients.

iv) POLYSPECIFICITY

Another relevant question concerning future strategies of cancer treatment relates to that of targeting one or multiple molecules, molecule domains or molecule epitopes. This question is connected with that of development of cancer resistance mechanisms.

Knowing that cancer is characterized by high heterogeneity and variability and can adapt to challenges like targeted therapies by developing escape

strategies, the question is how to cope with this situation. One idea was to target a molecule such as a protein tyrosine kinase (TK) not just by one but by two TKIs.

Unfortunately, a TKI has side effects not much different from a cytostatic drug. Let us assume that there are six types of side effects per TKI. The addition of two TKIs may reduce the likelihood of resistance development from the side of the cancer. But the cancer patient is exposed to 2x6 side effects. Nobody can predict, in which way the side effects will develop in an individual patient and possibly potentiate each other. At the end, the situation may not be much better than with cytostatic drugs.

The situation is quite different with immunotherapy. Thanks to the invention of immunological tolerance to self-tissues, immune T-cells reacting towards TAAs produce only low level side effects. Only when interfering with immune regulatory mechanisms there is the risk of development of autoimmune mechanisms.

E. TRENDS TOWARDS PERSONALIZED AND INDIVIDUALIZED THERAPIES

i) PERSONALIZED TREATMENTS BASED ON GENOMICS

There exists at present a development towards so-called personalized medicine. In medical oncology, this means individual tumor typing by procedures such as genomics, proteomics or pharmacogenomics and then adjusting the currently available drugs to the derived patterns of signaling pathways. In this way the drugs could be better targeted to subsets of patients. These should be responding according to respective algorithms. This would be a step forward, no doubt.

But the effort is enormous and expensive. There exist already chemosensitivity tests *ex vivo* which allow to estimate the likelihood of drug response for a patient`s tumor cells. Although such tests are less expensive than those mentioned above, they have not been introduced into standard care.

ii) INDIVIDUALIZED TREATMENTS BASED ON IMMUNOLOGY

The concept of this kind of treatment is fundamentally different from that of personalized treatments. Each patient receives his own drug in form of a patient-derived autologous anti-cancer vaccine.

The rationale is multifold: Apart from the individuality of mutation-derived TAAs and the individuality of the cancer-reactive T cell memory repertoire, there exists the individuality of MHC molecules and the phenomenon of MHC restriction of TAA recognition by T-cells.

iii) OVs HELPING TO BREAK CANCER RESISTANCE MECHANISMS

Oncolytic viruses (OVs), in particular NDV, were shown to be able to break tolerance of a tumor-specific T helper cell line. NDV was also able to exert oncolytic activity against hypoxic cancer cells and to induce immunogenic cell death (ICD). In addition, NDV targets Rac1 protein, which is important for glioblastoma migration and invasion.

The combination of OVs with immunotherapies thus has great potential for the future of cancer therapy.

F. FUTURE IMMUNOTHERAPY

i) ADAPTATION OF STUDY PROTOCOLS AND EVALUATIONS

Immunotherapies are different from all other kinds of cancer therapies. They obey different rules, have other kinetics and should be evaluated in a different way.

To see the difference, let us compare the sophisticated system of foreign antigen recognition and avoidance of self-reactivity (via central and peripheral tolerance mechanisms) developed by evolution with that developed by chemists and pharmacists to fight cancer: cytostatic drugs and small molecule inhibitors.

The latter have tried to develop drugs which should be as cancer-specific as possible. The tremendous list of side effects generated in cancer patients and documented in this book unfortunately speaks another language. One

wonders why certain drugs from the past became approved at all. Perhaps because there was no other treatment available. All these drugs were developed without regarding their effect on the immune system. The dominating paradigm in those times was a focus on cancer and to cause as much damage to it as possible without considering the host.

Nowadays, we are in an entirely different situation with all the new discoveries and inventions and yet these old drugs are still being used.

We have very strict regulations before a new drug or treatment can be approved. These new regulations did not exist when the old cytostatic drugs were approved.

So there exists a discrepancy between the judgement towards those old drugs, with their lack of rational design and lack of proof of efficacy according to modern standards, and the hurdles which new drugs or treatments have to take. This dilemma neads to be solved in the future in order to replace drugs with side effects of grade 3 and 4 by immunotherapies with side effects ranging between grade 1 and 2.

Monitoring the immune status and the immune reactivity of a cancer patient should become standard procedure, before and during cancer therapy. Immune parameters which already are of predictive relevance, for instance in colorectal carcinoma, should be introduced. A computational approach has been developed to comprehensively analyse tumor immunity (4). Radiologists may learn to distinguish immune reactivity from other phenomena.

The imaging manifestations in patients on immune therapies have just been reviewed (5). They appear to be distinct from those typically seen with conventional cytotoxic therapies. Patients on immune therapies may demonstrate delayed response, transient tumor enlargement followed by shrinkage, stable size. There may also be initial appearance of new lesions followed by stability or response. These newer patterns of response to treatment have rendered conventional criteria such as WHO and RECIST suboptimal in monitoring changes in tumor burden. Newer imaging response criteria such as immune-related Response Evaluation Criteria in Solid Tumors (irRECIST) and immune-related Response Criteria (irRC) are being

implemented in many trials to effectively monitor patients on immunotherapies (5). Stable disease should be considered as a criterium for response evaluation by immunotherapies.

Immunotherapies should get a chance to be evaluated in a situation in which the patient`s immune system is still competent. This is more likely the case in the adjuvant than in the advanced disease situation. In Germany, we were able to convince ethical committees to agree to anti-cancer vaccination in a variety of cancers in the post-operative adjuvant situation. This was possible mainly because of a proven lack of severe side effects.

In most countries new therapies can only be tested in patients that have already received standard treatment. These are usually late-stage patients with reduced immunocompetence. Adoptive immunotherapies with activated T-cells are more likely to function in this clinical situation than active specific immunotherapies.

ii) IMMUNOTHERAPY AS NEW STANDARD TREATMENT

At present, cancer patients receive standard therapies like before. Sometimes immunological agents like tumor-targeting mabs or checkpoint inhibiting mabs are being added. This, however, does not mean that immunotherapy has meanwhile become a standard therapy.

Evidence-based medicine has developed rules how to perform clinical studies. These rules have their value in drug development.

Whether the same rules can be used for development of sophisticated immunotherapies, such as multimodal immunotherapies, is, however, questionable. This is not only a question of money and logistics. It is also a question of principle.

Above we stated that each cancer has its own individual history of development. Results from cancer genomics support this conclusion. We also mentioned the individuality of the repertoire of cancer-reactive memory T cells and the individuality of MHC molecules and their recognition by T cells.

Considering these facts, one may wonder what sense it would make to randomize patients into groups that either receive immunotherapy or not and then to compare them. When biometricians developed those rules of phase I to phase IV clinical studies, they were obviously not aware of the facts of individuality.

During our journey through the history of development of different forms of cancer therapy we have seen several developments that have led to dead ends. Often these were due to a lack of knowledge and dogmatic thinking.

There is no guarantee that the future development of cancer therapy will be devoid of dogmas and dead ends.

G. HOW TO REDUCE SIDE EFFECTS OF TREATMENT

This is the second of the three main objectives of this book. In Chapter VI we provided examples of how to reduce side effects.

Sometimes a lower dose and other ways of application (e.g. metronomic chemotherapy) may be sufficient. In other situations, a combination of different types of therapy can cause synergistic effects, which then allow for dose reduction.

In any case, physicians should become aware of not using drugs which can cause grade 3 to 4 severe side effects. Such side effects destroy not only the immune system and the proper functioning of organs. They destroy the mere goal of any cancer therapy, namely to improve OS.

H. EFFICACY OF TREATMENT

Efficacy of treatment can be estimated in different ways. For instance by calculating the percentage of patients who are alive at a defined time period after treatment (e.g. 5-year survival rate). It could also be the rate of progression-free or metastasis-free survival.

Overall survival (OS) is perhaps the most important parameter for the individual patient. Benefits in OS have astonished oncologists when the first

results came in with checkpoint inhibitory antibodies. Such a result is not so astonishing for a tumor immunologist. Once the tumor-induced break has been relieved by such mabs, the TILS can go on doing their job in killing tumor cells via CTL activity and by surveilling the tumor and its metastases via effector memory T cells. In contrast to cytostatic drugs, the effect of the application of such mabs is rather long-lasting, which has been a further surprise to oncologists.

For the cancer patient, quality of life (QoL) is another important aspect. Since QoL is very much affected by the side effects of therapies, we have discussed this issue intensively in this book.

I. A PSYCHO-NEURO-IMMUNOLOGICAL PERSPECTIVE

Immunology is also of relevance for neuroscientists. They know that the first defined cytokine, interleukin 1 (IL-1), activates a discrete population of hypothalamic neurons. This interaction leads to the release of glucocorticoids from the adrenal gland.

Lymphocytes also synthesize acetylcholine, the first formally recognized neurotransmitter. There are now in the area between immunology and neurobiology 37 cytokines and their receptors, at least 60 neurotransmitters and over 50 neuroactive peptides (6).

Sleep has a critical role in promoting health. Neuroendocrine and autonomic neural underpinnings link sleep disturbance with immunity and inflammatory biology. This area may also have an impact in future to reduce the risk of infectious disease, cardiovascular disease and cancer (7).

Psychotherapy is already part of care of cancer patients. Its scientific basis rests on the psycho-neuro-immunological network.

J. PROMISING FUTURE DIRECTIONS

The approval by the FDA in recent years of targeted therapies with small molecule inhibitors and with monoclonal antibodies indicates that we are in a

process in which things relating to cancer therapy are moving. Nevertheless, biological cancer therapies are not yet part of standard therapies.

Chapter VIII described potential new targets of cancer therapy. Targeting of the tumor`s stromal network was suggested as a good concept. In the meantime, we know that cancers depend on a support system. Interruption of this support pathway may be as efficient as targeting the cancer cells themselves. This view represents a change of paradigm in comparison to standard therapy.

Of course, the tumor itself needs to be targeted as well, in particular the cancer stem cells. Standard therapies target the cancer itself and try to reduce the number of dividing cancer cells. Eventually, the cancer will not only re-grow but it may have developed resistance mechanisms.

To overcome such resistancies, several strategies can be applied. Above we mentioned already oncolytic viruses. Another strategy is to target epigenetic mechanisms of cancer: DNA methylation, chromatin and nucleosome positioning (8). Promoter DNA hypermethylation, a frequent phenomenon, can cause disruption of gene function and facilitate development of cancer resistancies. Inhibitors of DNA methyltransferases or of histone deacetylase had effects at low doses in hematopoietic-related neoplasms (9). Many agents targeting epigenetic regulation are under development and entering clinical trials (8).

We predict that targeting epigenetic mechanisms in the cancer and activating the immune system towards TAAs at the same time should be a good concept. In our own experience from 1986 (more than 30 years ago !), treatment of tumor cell immune escape variants (TAA loss variants) by 5-aza-cytidine caused re-expression of the TAAs. So there is a rational basis for combining epigenetics with immune activation (10).

The role of epigenetics in immune evasion has exposed a key role for epigenetic modulators in augmenting the tumor microenvironment. Epigenetic modulators allow to restore immune recognition and immunogenicity (11). Recent studies suggest that epigenetic drugs prime the immune response by increasing expression of TAAs and immune-related genes (12).

Our ability to predict what will be happening in the future with regard to cancer treatment is, of course, limited. Science is a very innovative discipline and many new developments may occur.

From my present point of view, I predict the following areas of research as particularly promising : targeting Epigenetics, the Tumor microenvironment, and the Immune system. These are three areas of research from which I expect many new exciting results in the future.

This view is apparently shared by others (13,14).

Chapter IX

Key points

1. With regard to future directions of cancer therapy, it is important to compare therapies to evaluate their advantages or disadvantages.

2. In this chapter we compare immunotherapies with other therapies.

3. Immunological tolerance is a sophisticated control system to avoid auto-immune reactivity and to keep tissues and organs in a healthy state. This might explain that immunotherapies have lower side effects than other therapies.

4. Immunological memory is another characteristic of the adaptive immune systems. It serves the purpose of obtaining long-term protection. This is of great relevance for long-term overall survival. None of the other therapies has a memory function.

5. In many cancer patients exists a cancer-reactive memory repertoire. This is the result of spontaneous immune T-cell reactivity against the autologous tumor. This memory repertoire is highly individual and polyspecific. If it can be re-activated it should contribute considerably to the efficacy of treatment.

6. New immune-related Response Evaluation Criteria in Solid Tumors (irRECIST) and immune-related Response Criteria (irCR) are being implemented in trials of immunotherapy.

7. Promising future directions of cancer research and treatment are Epigenetics, Tumor microenvironment and Immune system activation. From a combination of these targets we expect in future new exciting results.

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CHAPTER X. AUTOBIOGRAPHY NOTES AND SCIENTIFIC OEVRE

A. AUTOBIOGRAPHY BOXES

BOX 1

Auto-Biography 1 1962 Biochemistry

Diploma study of Biochemistry in Tübingen

In 1962, the University of Tübingen was the first in Germany to inaugurate in the Faculty of Natural Sciences a Diploma study of Biochemistry. Until this time, this discipline had been designated as "Physiological Chemistry" and belonged to the Medical Faculty.

In this year, I had started to study Chemistry and Biology at the University of Hamburg, closest to my home town Wentorf, south-east of Hamburg. As soon as I became aware of the existence of a Biochemistry Study in Tübingen, I asked several Professors of chemistry from Hamburg about their opinion. Their answers were rather negative and biased. So I decided not to follow their advice. Being convinced that this was the right thing for me, I took the train to Tübingen and tried to register at the University for this discipline.

It was a big surprise for the Professor of anorganic chemistry when he met me. He told me that there existed a "numerus clausus" and all slots had been given away already to the best students. But I did not give up. Eventually, I could convince him to take an oral examination next morning. After a two-hours test he was convinced of my qualification, agreed that I could study biochemistry and even offered me a place in his practical training course. So this was a good start of my career.

BOX 2

Auto-Biography 2 1969 Immunology

PhD thesis at Cologne, Germany, supervised by Prof *Klaus Rajewsky*

I had finished my study of biochemistry with a diploma thesis work on competitive inhibition of the enzyme phosphoribosyl-transferase by imidazole derivatives. Next I had to decide about an interesting Institution where to perform the PhD thesis work. One option was to work at the Max-Planck Institute in Freiburg (Germany) on a topic of immunochemistry, namely to analyze the complement factor C`9, which catalyzes the production of membrane pores in the process of antibody and complement-mediated cell lysis.

An alternative was in Cologne the University Institute of Genetics, where Prof *K Rajewsky* was just building up a new Division of Immunology. The topic was to study the interaction of carrier protein-specific T cells with hapten-specific B cells in the secondary anti-hapten antibody response. I decided for Cologne because this topic was at the for-front of modern immunology. In retrospective, I do not regret this decision in favor of cellular immunology. The seminars in this Division were lively and arguments had to be put forward or against a certain hypothesis. This was excellent basic education in science.

BOX 3

Auto-Biography 3 1972 Cellular Cytotoxicity

Post-doc at the Karolinska Institute, Stockholm, Sweden

With the help of a stipend from the Deutsche Forschungsgemeinschaft (DFG), I spent my Post-Doctoral research period at the Department of Tumor Biology under the supervision of the young Prof *Hans Wigzell*. He had impressed me at my first International Congress in Finland where I presented my results from the PhD thesis in Cologne.

He had developed an anti-immunoglobulin (Ig) column to separate T lymphocytes (T cells) from B lymphocytes (B cells). It was the time of the

"elusive" nature of the T cell receptor (TCR) for antigen and many laboratories worldwide competed for solving this important question. In contrast to the B cell receptor (BCR) for antigen which was made up of Ig, T cells expressed a receptor which was not Ig. While it was known that B cells recognize three-dimensional epitopes of a protein, we and others had found that T cells were able to recognize denatured protein epitopes.

Together with my post-doc colleagues, the french *P Golstein* (who later discovered the famous CTLA-4 regulatory receptor) and the danish *B Rubin*, we designed experiments to study antigen-specific cytotoxic T lymphocytes (CTL) in order to find out more about the nature of their TCR. Mice were immunized with hapten-coupled albumin and their immune spleen cells re-stimulated with the same antigen in culture for several days. Thereafter the re-activated immune cells were tested for CTL activity by exposing them to ^{51}Cr-labeled hapten-coupled erythrocytes for 4 hours and testing their release of radioactive ^{51}Cr. In this way we indeed discovered hapten-specific cell lysis. It soon turned out, however, that it were not T cells which killed because they could not be inhibited by anti-T cell antibody.

So the chase began after the type of killer cell: The title of the publication was "Chasing the killer cell ". It revealed an antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism. In this, immune B-cells produce anti-hapten IgG antibody which binds to the target cells and natural killer (NK) cells recognize the Fc portion of IgG thereby becoming activated towards cytotoxic activity. Neither NK cells, nor the ADCC mechanism had been known at this time. It were exiting times even though we had missed our goal of dealing with the TCR of CTLs.

Later it became clear why we failed. The choice of erythrocytes as target cells was wrong. They do not express MHC molecules which T cells need to recognize antigen, but this was not known yet.

PS: In 1972, I had the chance to attend the Nobel Prize Ceremony for *G Edelman* and *RR Porter* at the "Stadhuset" in Stockholm. Together they had resolved the structure of an IgG immunoglobulin antibody molecule. While the american scientist *G Edelman* was very self-confident and presented

himself as super-hero of modern immunology, the british *RR Porter* did the opposite with typical british humor and understatement.

BOX 4

Auto-Biography 4 1973 Immunogenetic Research in London,

Senior Research Fellow at the London Hospital Medical College, London (UK)

The time in Sweden had been fruitful not only with regard to my scientific experience but also with regard to my family life. In 1969 I had married *Barbara (birth name Ziemssen)*. Now in Sweden, two Schirrmacher girls were born: *Tanja* in 1971 and *Elise* in 1973. They were both healthy and sweet.

When the time of the Stipend from the DFG was over, *H Wigzell* recommended me to join Prof *H Festenstein*`s group in London in order to get more experience in immunogenetics. *Hilliard Festenstein* was one of the few researchers who were capable of in vitro culture immune cells from mouse and man. He had discovered an important minor histocompatibility locus in mice coding for the minor lymphocyte stimulating antigen (Mls). A mixed lymphocyte-culture (MLC) stimulation reaction was observed when co-culturing spleen cells of two strains of mice with the same MHC, for instance cells from DBA/2 mice (H-2^d, Mls^a) with those of strain B10.D2 (H-2^d, Mls^b). Much later it was discovered that Mls antigens were identical with viral superantigens (vSAG), in this case relicts in the DNA of mouse mammary tumor viruses (MMTV). DBA/2 mice exert a central tolerance reaction in their thymus against endogenous self antigen Mls^a by deleting reactive T cells expressing a vß6 TCR chain. This is not the case with B10.D2 mice because these express endogenous Mls^b. About 1:10 of B10.D2 T cells express a vß6 TCR chain. This frequency of SAG-reactive T cells is much higher than the frequency of T cells against a conventional pMHC antigen, like a TAA or a viral antigen (1: 10 000 to 1: 100 000).

In my later tumor immunological research at DKFZ, Heidelberg, this immunogenetic knowledge played an important role. It enabled breaking T cell tolerance and it helped to establish an adoptive cellular immunotherapy effective even in late-stage metastatic disease.

BOX 5

Auto-Biography 5 1973 MHC restriction of CTL

British Society of Immunology Meeting in Brighton

A milestone for me was in 1973 the discovery by *Rolf Zinkernagel* and *Peter Doherty* of the MHC restriction of CTLs. They had studied the specificity of CTLs from mice infected by a *murine cytomegalovirus virus* (MCMV). What was surprising was that the CTLs, in order to be able to kill an infected target cell line, had to recognize not only a distinct viral antigen but also the MHC type of the mouse strain, in which the CTLs were generated. Today we know that CTLs recognize small peptides from target antigens (from viruses or tumor cells) as complexes associated with the CTL host cells` MHC molecules.

I was chairing at the annual British Society for Immunology (BSI) Congress in Brighton a Session on "Cellular Immunology". From the Abstracts that had been sent in, I had selected the best ones for oral presentation within the 2 hours Session. At the last minute before the start, a then unknown Rolf Zinkernagel approached me and showed me his abstract of his latest findings. In recognizing the importance of these new findings, I agreed immediately to an oral presentation. In 1996 *R Zinkernagel and P Doherty* received the Nobel Prize for their important discovery.

BOX 6

Auto-Biography 6 1976 Start in Heidelberg, Germany

Head of Division Cellular Immunology

In 1976, I was appointed as Head of the Division "Cellular Immunology" at the German Cancer Research Center (DKFZ) in Heidelberg, Germany. This National Institution had been founded by the surgeon Prof *KH Bauer*. With 33 years, I was rather young to become a "Wissenschaftlicher Rat und Professor" in Germany.

The Institute of Immunology was the last of 9 Institutes which, together, represented the Science at DKFZ. The Institution DKFZ is funded to 90% by the State of Germany and to 10% by the Land Baden-Württemberg. Together with my colleagues *K Eichmann* and *W Dröge*, who were appointed as Heads of two other Immunology Divisions, our task was to build up a new Institute at the 7th floor of the DKFZ building. Later, *PH Krammer* joined the Division of *K Eichmann* and *G Hämmerling* was appointed as Head of the fourth Division. When *K Eichmann* became Director of a Max Planck Institute in Freiburg, *PH Krammer* succeeded as Head of the respective Division.

It was the time of the Social-Liberal Government of *Willy Brandt* and *Walter Scheel*, who encouraged for daring more democracy. So, our new Institute (07) became the first at DKFZ not being directed by one chairman. We, the Heads of the Divisions, together with DKFZ administration at the time, decided to introduce a rotating Directorship. Since all Heads of the Divisions had studied abroad, we agreed that the management stile of the New Institute should be different from previous ones, more informal and colleagual.

We are still proud of the following established traditions which supported a corporate identity for our Institute. It was based on competition between the four Divisions with regard to the following: to organize

i) Unusual Christmas parties with unique creativity in winter,

ii) Immuno-Moonshine Sessions on major topics of immunology ,

iii) Immuno-Retreats for all scientists, and

iv) Immuno-Soccer-Cups in summer.

BOX 7

Auto-Biography 7 1980 Chamber music in Paris

Immunologists performing chamber music at the Opening Ceremony of the 4th International Congress of Immunology (ICI) in Paris.

1980: Over 6000 immunologists attended the opening ceremony at the Congress Center in Paris. The Congress was organized by the Nobel Laureat Jean Dausset and the French Society of Immunology (SFI). My french colleague Herve (Wolf) Fridman had known that I had the hobby of playing the travers flute and that the Belgian immunologist Kees Melief similarly enjoyed playing chamber music with the cello. So he asked us, about three months before the event, whether we might like to play some chamber music at this occasion. We had agreed under the assumption that chamber music would be performed in a small chamber as a side program. To our surprise, however, we were informed just one week before the Congress the Organizing Committee had decided that we should perform in the middle of the official Opening ceremony in the main Hall in front of 6000 people.

We only had time for rehearsal one day before the event. The other musicians were professionals: Spedding Micklem (piano), Antonio Nunez (first violin), Marian Nienhuis (second violin) and Dieter Leicht (first violoncello). We played Wolfgang Amadeus Mozarts Flute Quartett in D major, Robert Schumanns Piano Quintett and Franz Schuberts String Quintett. I did not have much contact before the event with the organizers, nor with the other musicians. I drove my car from Heidelberg to Paris, where I stayed with friends.

Kees Melief sent me in august 2015 a digitalized soundtrack of our performance from 35 years before. In his accompanying letter he memorized the following: " It was a memorable congress, if only because servants in uniform with white gloves served all participants real champagne with very tasty French appetizers in the "Palais du Luxembourg". To have such long stretches of classical music as evident from the soundtrack was another unique aspect of the congress. This may have happened at the behest of Jean Dausset, but was in practice made possible by the herculean efforts of Herve Fridman and his colleagues. I remember taking the train with my fellow musician Marian Nienhuis. At that time in the first class train compartment it was possible to have a five course luxurious dinner in a restaurant on the second floor of the train with panoramic views left and right and plenty of time before arriving at the "Gare du Nord". A nostalgia is elicited by this recording, which is not optimal, but one can hear that the musicianship is in many ways excellent and brings back the joy of these moments."

BOX 8

Auto-Biography 8 1982 Heparanase

German-Israel Cooperation Project

International collaboration is an important aspect in science. In 1982, I received a grant from the German-Israel Cooperative Initiative and invited Dr Israel Vlodavsky from Jerusalem to study in Heidelberg the interaction of our Eb/ESb tumor cell variants with his culture system of blood vessel endothelial cell monolayers and their subendothelial extra-cellular matrix (ECM). Thus, technologies from cell biology were combined with those from tumor biology. It became a very fruitful cooperation for both sides and the reciprocal sympathy turned into a long-lasting friendship.

Only the metastatic variant ESb and not its parental line Eb was capable to penetrate the blood vessel endothelial monolayer. Only ESb cells were also capable to degrade the subendothelial ECM. This degradation involved a lymphoma derived protease as well as a new enzymatic activity, which caused the removal and degradation of heparan-sulfate side-chains from ECM-derived heparan-sulfate-proteoglycans (HSPGs). This enzyme, heparanase (Hpa), could eventually be identified. In contrast to other ECM-degrading enzymes, it was later discovered that there was only a single gene in the human genome coding for this enzyme. Inhibitors of Hpa had the potential to inhibit angiogenesis. Much of Vlodavskys later work was devoted to identifying such inhibitors.

As immunologists, we in Heidelberg became interested to find out whether Hpa might become a new target for T-cell mediated immunotherapy. From respective algorithms for searching peptides fitting into HLA-A2 molecules, we derived hundreds of peptides from Hpa. The ones with the best HLA-A2 affinity were synthesized and tested in a memory-type ELISPOT assay with bone-marrow derived T cells from breast cancer patients. Many such patients had in their BM a repertoire of specificities highly enriched for Hpa peptides. Their frequency turned out to be higher than specificities for other TAAs, possibly due to the fact that the product of Hpa activity was activating DC activity (PMID: 16885374).

We wrote and handed in a patent application for the use of Hpa-derived peptides for the use in future cancer vaccines. The patent became granted, but the DKFZ patent specialists could not find any pharmaceutical company to be interested in it. So the idea could not mature and the patent was not followed any further.

BOX 9

Auto-Biography 9 1982 – 1988 Honorable Prizes

Aronson Prize (82), Meyenburg Prize (82), German Cancer Award (88)

1982 was a year in which I received two awards: The Aronson Prize, given by the town of Berlin, and the Meyenburg Prize, given by the Meyenburg Foundation Hamburg via the DKFZ in Heidelberg. The latter Prize was handed over to me by the then new Director, Prof H zur Hausen. The prizes acknowledged our systematic research in animal models on cancer metastasis and in basics of cellular immunology.

In 1988 I received the "German Cancer Award" for our experimental work on post-operative active-specific immunotherapy (ASI) of cancer metastasis. It had been successful because we had used a bird virus, Newcastle Disease Virus (NDV) to infect autologous tumor cells to create a live cell anti-tumor vaccine.

The concept of ASI had been studied in the USA by W Cassel from Atlanta in the 1960s and 1970s and by M Hanna Jr in the 1980s and 1990s. Nevertheless, it had never been heard of in Germany in the 1980s in circles of medical oncologists.

I was convinced that pioneers were needed to develop entirely new concepts of cancer treatment which were highly specific and had only low side effects. However, some clinicians were afraid of "invadors" of their territory. Unfortunately, in my conviction for ASI and in spite of the German Cancer Award, I was not backed-up by Prof zur Hausen, the Head of the Cancer Research Center and the later Nobel laureate.

BOX 10

Auto-Biography 10 1988 Surgeons and immunotherapy

1988 compared to 1978

1978: I was invited by the surgeon Prof Grundman to Münster (Germany) to give a talk about cancer metastasis. The audience were all surgeons. At the end, during the discussion, I was asked by one of them about my opininon about a new type of therapy, called immunotherapy. Obviously he had not known that this was my own field of research. I asked back, whether anybody in the auditorium had heard about the american surgeon *Steve Rosenberg* from the National Cancer Institute (NCI) in Washington. Nobody had heard about him. So I explained how this surgeon was pioneering the field of immunotherapy by treating patients with lymphokine-activated killer (LAK) cells. I further mentioned that the aim was a kind of systemic immune cell therapy.

The surgeons looked at me as if I had been ET coming from another planet talking in a foreign language. In the break, thereafter, none of the surgeons had any further question about this Steve Rosenberg. While turning their backs to me, they rather preferred to talk among themselves about their careers.

1988: The attitude of surgeons towards immunotherapy seemed to have changed somewhat. I was able to cooperate with the surgeon Prof Peter Schlag, who had just received a Professorship for Surgical Oncology at the University Hospital in Heidelberg. He aggreed to perform a joint clinical feasibility study in colorectal cancer patients of post-operative immunotherapy with a virus (NDV)-modified autologous tumor cell vaccine.

It was also the year, in which I was invited to give a talk about immunotherapy at the annual conference of the German Surgical Society, chaired by Prof C Herfarth from Heidelberg.

BOX 11

Auto-Biography 11 1990 – 2008 Clinical studies

From 1990 onwards we entered into clinical trials of post-operative immunotherapy via anti-cancer vaccination with the autologous vaccine ATV-NDV. Studies in primary operated breast cancer patients were performed, without funding, under the Directorship of Prof G Bastert of the Heidelberg University Clinic of Gynaecology in cooperation with my former student Dr T Ahlert. Studies in primary operated colorectal cancer (CRC) patients were performed, without funding, in cooperation with Dr D Ockert from the University Clinic of Surgery in Mannheim. Studies in CRC patients of stage IV with operable liver metastases were performed, with funding, in collaboration with Prof P Schlag from the University Clinic of Surgery in Heidelberg.

I am very grateful to the many engaged clinicians who were interested and capable to perform such innovative studies. These required an enormous amount of conviction, good will and new logistics. Nowadays, such innovations need much more control, money and personel.

Most immunotherapy studies worldwide up to this time had been done in melanoma patients, because melanoma was considered an immunogenic and immune-responsive type of tumor. In 1990, we had applied for financial support for studies of active-specific immunotherapy (ASI) in 5 different types of human cancer. 4 of the 5 grant applications were turned down by the reviewers, all clinicians practicing chemotherapy. After heavy disputes, only one of the tumor entities was recommended for funding, namely colorectal carcinoma (CRC). If we were to succeed in this important tumor, so was the official argument of the clinical reviewers, then the concept would be considered likely to work also in other carcinomas. Most of the clinicians were convinced that we would fail because this tumor was considered inert to immunotherapy.

For nearly 15 years, I was the driving force for testing new immunotherapy strategies in cancer patients, while Prof zur Hausen, the Scientific Director of DKFZ (for 4x5 = 20 years), decided from the beginning to function mainly as retarding element. As virologist he was aiming at prophylactic viral vaccines against virus-associated cancers and could not imagine that the immune system was capable of recognizing and reacting against TAAs which had nothing to do with viruses. So there was a lot of struggeling and arguing.

One can imagine therefore why only very few applications for financial support of ASI studies were granted. This explains among others why it all took so long.

When the institutional pressure against my engagement for clinical studies continued to rise in the years 1990 to 1995, I contacted worldwide many experts in the field to ask for their advice. Was the present time right for performing translational work on immunotherapy or not ? Was it ethical and justified ?

I received many letters of support. Some colleagues became worried and wondered whether, with regard to ASI, I was being "*harassed by the Inquisition*" (letter from Prof JJ Oppenheim, National Cancer Institute, USA, 22.12.1994). Prof JV Nossal (Sir GUSTAV NOSSAL), Director of the WALTER AND ELIZA HALL Institute of Medical Research, Melbourne, Australia , wrote to me 22.05.1995, "the chief result of our review of your work in 1991 resulted in the suggestion to get a small advisory group which could help you in the design of clinical trials and in their eventual evalution".

Some experts suggested to me to look for another place for my studies.

In spite of the long-lasting pressure, I did not give up. For the following reasons:

i) I was convinced that I was on the right path and doing research at the right Institution,

ii) I thought of the many cancer patients that were suffering and deserved better treatments as soon as possible,

iii) with regard to ethics, my position was: when you have a new treatment with a scientific rationale, with positive pre-clinical results and with low side effects, it would be *un-ethical* not to offer it to cancer patients suffering now,

iv) not knowing about the outcome from ASI studies, I tried to keep the risk of failure to myself. I did not force any of my research assistants to engage in this type of applied research.

An External Evalution of the ASI-Project, Division "Cellular Immunology", Deutsches Krebsforschungszentrum, took place March 27, 1995. Peer

reviewers were Prof JO Armitage (Omaha, USA), Prof HF Oettgen (New York, USA), Prof RA Reisfeld (La Jolla, USA), Prof HK Selbmann (Tübingen, Germany) and Prof RM Zinkernagel (Zürich, Switzerland). The Expert Report , submitted April 5, 1995, answered 4 of the 5 questions as good as they could and commented the fifth one: "Do you have specific recommendations ?" as follows: "The committee feels that DKFZ should make an effort to enlist the collaboration of capable senior principal investigators who have undisputed control of eligible patient populations in their institutions. This should not be left to Prof Schirrmacher. The perceived lack of institutional support in this regard has in fact been a major source of frustration for him."

The results of most of our clinical ASI studies were positive, even though they were of only Phase I/II type and not randomized. Prof zur Hausen maintained that he could be convinced only by positive results from randomized-controlled prospective studies.

In 2008, we published the results from such a randomized-controlled prospective study. It had been performed in stage IV colon cancer patients. In the ASI arm there was a significantly improved 10-year survival. When I presented these new results in the context of the last Peer-Reviewing of my Division, no time was allowed for discussion. Also, afterwards, there was no feedback from within the DKFZ. Nobody wanted to take any notice nor discuss the truth, so "political" had the topic "ASI" already been made during the years 1990 - 2008. It was an atmosphere, as if people were afraid of hearing the truth. The dogma prevailed over the truth.

For me, however, the results were re-assuring. We had seen similar improvements in OS (as large as 30% !) in most of our previous Phase II clinical studies. So, apparently, there had not been a bias in the previous study protocols.

Many years after his retirement, Prof zur Hausen admitted, in public, that now he was convinced of the value of immunotherapy. This was easy to say after having received to Nobel Prize. Based on suggestions by others, he eventually awarded prizes for checkpoint inhibitory antibodies and for CAR-T cells.

I do not want to judge.

My achievements were meant to serve the cancer patient. I am aware that the achievements were not possible without the discoveries of my scientific ancestors in this particular research area. They also would not have been possible without the engaged work of my co-investors and clinical cooperation partners.

BOX 12

Auto-Biography 12 2006 GBM immunotherapy

An innovative case of multimodal treatment of Glioblastoma multiforme.

In 2006, I was approached by a Physician who came from New York to Heidelberg to ask for my help with regard to post-operative immunological treatment of a rich business man who had been diagnosed at the age of 50 with GBM. After successful operation in his home country Belgium, the only additional option of treatment that was offered was radio- and chemotherapy (temodal). Since our GBM vaccination study with ATV-NDV had already been closed, there was no possibility to offer participation in a clinical study. Also, since the patient had already been operated, no autologous viable tumor material was available for preparation of an autologous virus-modified vaccine. The physician had already organized a consortium of specialists concerning surgery (Brussels, Belgium), chemotherapy (MD Anderson, Houston, Texas, USA) and systemic oncolytic NDV virus therapy (Hebrew University, Jerusalem, Israel).

I was asked to design an individual protocol for immunotherapy which I did. The responsibility for this was by the Head of Neurosurgery of the University Clinic Heidelberg. His doctors cooperated in the vaccine preparation and application. Dr Herold-Mende from the Neurosurgery Department had a collection of about 30 self-established GBM cell lines. Out of these we selected 4 lines which closely matched with the tumor of the patient. Next we negotiated with the other specialists to obtain a two-week window per months for the immunotherapy treatment. We insisted that there should not be any interference by the other treatment modalities.

The immunotherapy consisted of

i) priming with a DC vaccine pulsed with NDV oncolysate of two selected GBM cell lines and

ii) boosting one week later with a live NDV-modified tumor cell vaccine consisting of the two other selected cell lines.

The immune response was followed by immune monitoring via ELISPOT using DCs loaded with either autologous tumor lysate, lysate from the defined cell lines or with defined TAAs. Before the immunotherapy, the patient`s T cells did not react against his own tumor. Six months after vaccination, however, we had established such an autologous anti-tumor immune response, in spite of the ongoing chemotherapy. The consortium met every 3 to 6 months to further discuss the mode of treatment. This way the patient was accompanied for as long as about 6 years. He eventually died in the seventh year.

The patient has gained a number of years of life. We have learned a lot about how to generate an immune response against an autologous tumor with allogeneic tumor material and in combination with oncolytic virustherapy and chemotherapy.

BOX 13

Auto-Biography 13 2008 Retirement Symposium and Farewell Party

2008 has been the year of my official retirement from DKFZ (Heidelberg) at the age of 65. Having served the Cancer Research Center for more than 32 years, this event was celebrated by a Special Symposium. All speakers had been members or guests of my Division of Cellular Immunology. The Symposium and the Farewell Party afterwards took place at the "Palais Prinz Carl" in the old town of Heidelberg.

Here follows the list of talks given at the Symposium at April 5, 2008.

1) Jim Dennis (Toronto, Canada): "Sweet memories of Heidelberg-Glycosylation past and present"

2) Achim Krüger (München, Germany): "A Genetic Tag`s Odyssey and Distant Accomplices of Metastasis"

3) Mina Fogel (Rehovot, Israel): "L1-CAM: From Bench to bedside"

4) Georg Brunner (Münster, Germany): "Gene expression analysis of malignant melanoma"

5) Susanne Sebens (Kiel, Germany): "Interaction in tumorigenesis and cancer research"

6) Hans-Jörg Schild (Mainz, Germany): "Regulation of adaptive immune responses"

7) Kash Khazaie (Chicago, USA): "The dark side of immune response to cancer"

8) Markus Feuerer (Boston, USA): "Thoughts on Peripheral Tolerance"

9) Manfred Lutz (Würzburg, Germany): "Peripheral Tolerance Induction by Dendritic Cells"

10) Brigitte Gückel (Tübingen, Germany): "Cell based vaccination strategies for breast cancer"

11) Andreas Kaufmann (Berlin, Germany): "Prophylactic and therapeutic HPV vaccines"

12) Klaus Bosslet (Berlin, Germany): "Recombinant oncolytic NDV virus: Expression of a Prodrug activating enzyme and a therapeutic antibody"

A phantastic Farewell Party crowned this memorable event: Italian Food served in the Art Deco "Spiegelsaal", a selection of good wines, international communication among old friends, exchange of gifts, music and dance until midnight.

B. SCIENTIFIC OEVRE (V Schirrmacher)

** This sign identifies publications of particular significance

MAJOR RESEARCH TOPICS

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ACKNOWLEDMENT

I like to thank first of all Dr. Wilfried Stuecker who provided me with the possibility to join the IOZK following my retirement at DKFZ Heidelberg. I thank the Editor Anna Gaina from Lambert Academic Publishing for her idea to contact me and for her help and support. Furthermore, I acknowledge the help of Christine Siegfried with computer program work and the help of Ute Wagner with text corrections. The cover picture was taken by photographer Roland Baege.