Not Gene Therapy, but Genetic Surgery—the Right Strategy to Attack Cancer[1]

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> The whole is simpler than the sum of its parts.
>
> Willard Gibbs

Abstract—In this review, I will suggest to divide all the approaches united now under common term "gene therapy" into two broad strategies of which the first one uses the methodology of targeted therapy with all its characteristics, but with genes in the role of agents targeted at a certain molecular component(s) presumably crucial for cancer maintenance. In contrast, the techniques of the other strategy are aimed at the destruction of tumors as a whole using the features shared by all cancers, for example relatively fast mitotic cell division or active angiogenesis. While the first strategy is "true" gene therapy, the second one is more like genetic surgery when a surgeon just cuts off a tumor with his scalpel and has no interest in knowing delicate mechanisms of cancer emergence and progression. I will try to substantiate the idea that the last strategy is the only right one, and its simplicity is paradoxically adequate to the super-complexity of tumors that originates from general complexity of cell regulation, strongly disturbed in tumor cells, and especially from the *complexity of tumors as evolving cell populations, affecting also their ecological niche formed by neighboring normal cells and tissues.* An analysis of the most widely used for such a "surgery" suicide gene/prodrug combinations will be presented in some more details.

INTRODUCTION

"Cancer is generally unbeatable"—this is the beginning of an interesting review by Felsher [1]. And then the author proceeds with: "However, in a few cancers, cycles of conventional therapies, such as chemotherapy, radiation therapy and hormonal therapy, are sufficient to result in successful treatment. Why some cancers, but not most cancers, respond to conventional therapies is uncertain; the mechanism by which therapies treat cancer is generally not known. The most common human cancers are epithelial cancers and they are the least treatable with conventional therapies. The biology of the type of cancer influences the response to treatment. Even when epithelial tumors do initially respond to treatment, eventually the tumors recur. These observations suggest that cancers may either become resistant or become dormant and later regain their neoplastic properties. Over the last decade, cancer treatment has entered a new era with the development of targeted therapeutics. The presumption was that by targeting the molecular underpinning of cancer with specific drugs, the tumors would respond better to treatment. Targeting tyrosine kinases, in particular, BCR-ABL and c-KIT, Imatinib has proven to be useful in the treatment of CML (chronic myelogenous leukemia) and GIST (Gastrointestinal stromal tumor). Hence,

Imatinib is universally considered an example of a successful targeted therapeutic".

This excerpt expresses the problems of cancer treatment and an emerging strategy of targeted therapy which seems to be a very efficient set of means, if not a panacea, to fight cancer because it is based on growing knowledge of delicate molecular mechanisms and their disfunction in cancer cells.

This set was extended with gene therapy approaches for the treatment of cancer whose development showed a dramatic acceleration in recent years. The two events that have permitted the formulation of the cancer gene therapy concept are the new understanding of the molecular mechanisms underlying oncogenesis, and the development of DNA-delivery vehicles. Many approaches to cancer gene therapy have been proposed, and several viral and non-viral vectors have been utilized. All these approaches are currently united under the term "gene therapy", they include transfer of tumor suppressor genes in tumors, a suicide gene-enzyme prodrug approach, inhibition of dominant oncogenes, immunomodulation approaches, expression of molecules that affect angiogenesis, tumor invasion and metastasis, chemosensitization and radiosensitization approaches etc.

In this review, I will suggest to divide all the approaches into two broad strategies of which the first

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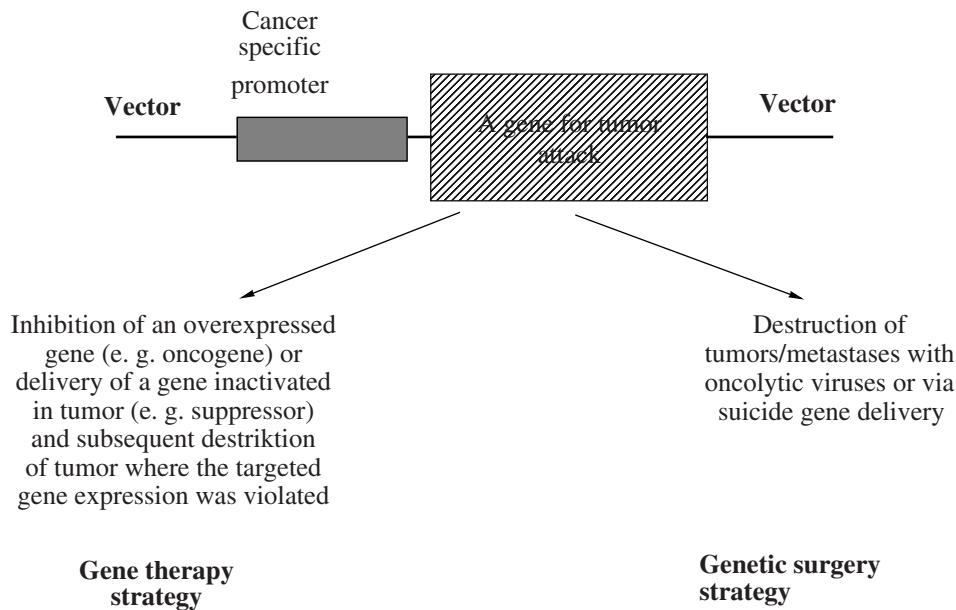
Fig. 1. Basic elements of gene delivery system and outline of the gene therapy and genetic surgery strategies.

one uses the methodology of targeted therapy with all its characteristics, but with genes in the role of agents targeted at a certain molecular component(s) presumably crucial for cancer maintenance (Fig. 1 left). In contrast, the techniques of the other strategy are aimed at the destruction of tumors as a whole using the features shared by all cancers, for example relatively fast mitotic cell division or active angiogenesis (Fig. 1 right) While the first strategy is "true" gene therapy, the second one is more like genetic surgery when a surgeon just cuts off a tumor with his scalpel and has no interest in knowing delicate mechanisms of cancer emergence and progression. I will try to substantiate the idea that the last strategy is the only right one, and its simplicity is paradoxically adequate to the super-complexity of tumors that originates from general complexity of cell regulation, strongly disturbed in tumor cells, and especially from the *complexity of tumors as evolving cell populations, affecting also their ecological niche formed by neighboring normal cells and tissues.* Analysis of the most widely used for such a "surgery" suicide gene/prodrug combinations will be presented in some more details. In view of limited space, I will not consider approaches based on potentiating antitumor activity of the immune system, that could be assigned to the genetic surgery strategy. There are a lot of excellent recent reviews devoted to this approach, see e.g. [2–4].

MALIGNANT TUMORS ARE SUPER-COMPLEX EVOLVING SYSTEMS

A cancerous tumor combines the complexity of cells with their signal cascades, replication, transcription etc., that undergo multiple changes to become cancerous, with the complexity of a growing evolving sys-

tem [5] with all traits and features allowing the tumors to resist antitumor agents and to induce microheterogeneity that makes them unique for every patient [6]. In this respect, cancer is different from all other diseases [5]. To top it all, tumors actively interact with neighboring normal cells, modify them and evolve together.

Super-Complexity of Tumors at the Cellular Level

There are more than 100 distinct types of cancer, not to mention subtypes of tumors in specific organs [7]. Moreover, tumors of any type are heterogeneous both intra- and intertumorally [6].

Normal cellular behavior depends on functional integration of extracellular stimuli with intracellular signal transduction systems. Coupling cell surface message reception to nuclear gene expression is not a simple linear pathway constructed with molecular components acting sequentially to transmit signals to the nucleus. Instead, it is a highly complex network with numerous nodes that integrate signals from different pathways and transmit the outputs to the gene expression control machinery. Although increasing understanding of genetic aberrations and signaling pathway transgressions lead to many novel strategies for targeting cancer cells, often disappointing results from clinical trials suggest that the intimate processes responsible for neoplastic transformation remain largely unexplained [8].

As judged from the frequency of malignant transformation per cell, cancer is a rare disease, approximately one event per 10^{19} cells [9]. It suggests that more than one event within a cell is needed to transform it into a cancer cell [10]. These multiple mutations in the same

cell are necessary to get over a variety of cellular defense barriers that make the cell extremely robust against continuously emerging lesions in various otherwise quite well orchestrated cellular systems maintaining the normal cellular life. Despite tremendous complexity and diversity of regulatory network perturbations operating within a cell autonomously or coupled to the signals that cells receive from their surrounding microenvironment, and despite a tremendous number of known cancer cell genotypes, all tumors seem to have six essential alterations in cell physiology that are jointly necessary (but not sufficient, see below) to dictate malignant growth. These six alterations were called "cancer hallmarks" [7]:

(1) Self-sufficiency in growth signals

(2) Insensitivity to antigrowth signals

(3) Evading apoptosis

(4) Limitless replicative potential

(5) Sustained angiogenesis

(6) Tissue invasion and metastasis

Each of these hallmarks can be acquired in different ways in different tumors, involving different signaling pathways and networks [7]. The accumulating data reveal a more and more complex cellular systems of genes, proteins and complexes involved in tumor initiation and progression.

Tumor Super-Complexity Characteristic of Evolving Systems

In tumors, there are a huge number of different mutant cells that compete with each other for space and food resources, avoid destruction by the immune system, adapt themselves to external effects, and cooperate in the ability of migration and colonization of new organs. The attributes of every evolution, variability and selection, can explain both the emergence of cancer and why it is so hard to cure [5, 11]. Tumors are highly robust and maintain their proliferative potential against a wide range of anticancer therapies. Two aspects of robustness are exploited by tumors—functional redundancy due to cellular heterogeneity, and feedback-control systems that are used to facilitate survival in hazardous environments (for example, due to anticancer drugs or hypoxia). The robustness of a tumor as a system is not equal to the robustness of individual tumor cells. A tumor cell can be more fragile than a non-tumor cell in response to a particular treatment, but heterogeneous redundancy can give rise to robustness at the system level through intercellular genetic variability in the pattern of drug resistance [11].

Tumor Complexity Due to Interaction with Surrounding Tissues (Ecological Niches)

The concept above reduces the problem of cancer origin and development just to evolving tumor cells that accumulate mutations and proliferate. However, it is now accepted to consider a tumor as an integrated ensemble of tumor and surrounding cells, in particular stroma cells. There is convincing evidence for possible mutually beneficial cooperation of partially transformed cells with each other, as well as cooperation with adjacent stroma cells beneficial for progressing tumor cells [5, 9, 12, 13]. The role of mutations in stroma cells in the development of tumors was discussed as late as 1998 [14]. A recently proposed hypothesis of morphostatic field and maintaining it molecules called morphostats [9] suggests that cancer is initiated also due to a disturbance of this field. Within this hypothesis, there are facts that carcinogenic effect results not only directly from accumulation of mutations in an evolving epithelial cancer cell but also from mutually complementary mutations in the cancer cell and surrounding stroma cells. It was also suggested [12] that partially transformed cells in the neoplasm can cooperate through a two-way paracrine exchange of diffusing factors thus providing each other with growth and proliferation resources which enhance the viability of the cells within the tumor, increase the rate of accumulation of mutations and accelerate tumor progression. Thus, cancer is not just a progeny of a particular cell that develops into a tumor due to accumulation of mutations, but a co-evolving cell population containing cancer stem cells, differentiated cancer cells with a limited proliferation potential, normal epithelial cells and surrounding stroma cells that get "activated" due to paracrine signals and must play key roles in driving tumor cell proliferation [5, 7, 15]. Many of the growth signals driving the proliferation of carcinoma cells may originate from the stromal cell components of the tumor mass. These cooperating cells may eventually depart from normalcy, coevolving with their malignant neighbors in order to sustain the growth of the latter [14].

An important feature of this co-evolution is transdifferentiation of progressing tumor cells. The stroma of a tumor includes fibroblasts, myofibroblasts (MF), endothelial cells, and inflammatory response cells associated with the immune system. The origin of myofibroblasts is not definitely clarified, but available data suggest that the main source of tumor MF may be epithelial-mesenchymal transition (EMT)/transdifferentiation of non-cancerous epithelial cells or epithelium-derived carcinoma cells [16, 17]. The myofibroblasts emergent in tumor stroma may produce and modify extracellular matrix, secrete angiogenic and proinflammatory factors, and stimulate proliferation and invasion of epithelial cancer cells [18–20]. A reverse mesenchymal-epithelial transition (MET) is also possible [17]. This is one more level of evolving tumor complexity [9].

A complex system requires adequate complex approaches. However tricky, fastidious and elegant they were, numerous gene therapeutic methods, developed using constantly growing knowledge about the mechanisms of tumor formation and targeted at particular parts of the signal systems affected in a given tumor type (targeted methods), will never provide radical cure of any particular patient. Indeed, it is highly probable

that at least some of a multitude of heterogeneous tumor cells will be resistant to a given treatment. Even if scanty, these cells will survive and give rise to a new tumor, this time fully resistant to the given treatment—a typical bottleneck effect well known in evolution. The situation is aggravated by that tumors of the same type differ in the sets of mutant cells in different patients. Such a difference may a priori lead to different efficacy of therapeutic agents in different individuals. Moreover, different tumor types require different therapies due to disturbance of different signal pathways and networks. As a result, currently about 960 (!) protocols of gene therapy against various cancer types are under clinical trials [21].

Below I will try to substantiate the view that gene therapy (in the sense outlined in the introduction) as well as other targeted approaches to cancer treatment will hardly prove to be a "magic bullet" against cancer. All of them are inadequate to the multilayer complexity of cancer.

Gene Therapy Strategy Outline

The majority of cancer gene therapy approaches use strategies aimed to neutralize molecular causes leading to the emergence of one or a few of the cancer hallmarks: e.g. suppression of activated oncogenes, restoration of the functional tumor suppressor genes expression, and induction of apoptosis in cancer cells by the introduction of pro-apoptotic genes [22, 23]. Many other parts of various signal systems can be targeted with such an elegant approach as noncoding siRNA [24].

As mentioned above, each of the six cancer hallmarks can be achieved by different means in different tumors involving different signaling pathways and different components of these pathways [7]. It is not then surprising that gene therapy allows an incredible diversity of treatment possibilities, relying on the current knowledge about the genetics of cancer formation [25]. Because gene transfer technology encompasses such a diverse set of therapeutic options, it is impossible to describe examples for all treatments. Instead, I will try to provide a couple of typical examples of gene therapeutic treatments.

Delivery of Tumor Suppressors to Tumors Lacking Them: p53 Example

Tumor-suppressor genes play pivotal roles in maintaining genome integrity and in regulating cell proliferation, differentiation, and apoptosis. Their loss-of-function mutations are directly related to tumorigenesis. Thus, use of tumor-suppressor genes as anticancer therapeutics has been rigorously investigated in both experimental and clinical researches. Transfer of various tumor-suppressor genes directly to cancer cells has been demonstrated to suppress tumor growth via induction of apoptosis and cell-cycle arrest. Various studies have also shown that combination of tumor-suppressor

gene therapy with conventional anticancer therapy can yield synergistic therapeutic benefits. Clinical trials with tumor-suppressor genes, especially the *p53* gene, have demonstrated that the treatment is well tolerated, and favorable clinical responses have been observed in a subset of patients with advanced disease or with cancers resistant to conventional therapy [26, 27].

The p53 protein is the most famous tumor suppressor, whose alteration occurs in approximately 50% of human cancers [28, 29] (note that this figure is much higher than for all other known suppressors) and contributes to tumor resistance to a variety of chemotherapeutics [30]. In normal cells, p53 maintains genetic integrity after DNA damage and functions as a gatekeeper of cellular growth, apoptosis and senescence. In cancer gene therapy, several p53-approaches have been developed based on introduction of the *p53* gene into cancer cells with damaged p53.

These studies have indicated that the restoration of p53 function in several different cancers induced growth arrest or obliterated the tumors. In hepatocellular carcinoma, transient p53 expression was sufficient to cause complete tumor regression by a combination of cell cycle arrest and induction of innate immune system activity in athymic nude mice [31]. Promising results were obtained in non-small cell lung cancers [26, 32]. Martins et al. observed that p53 is spontaneously activated when restored in some established lymphomas in vivo, triggering rapid apoptosis and conferring a significant increase in survival. The list of examples can be considerably extended. Nonetheless, reimposition of p53 function potently selects for emergence of p53-resistant tumors through inactivation of p19(ARF) or p53 [33].

Altogether, these studies suggest that although *p53* gene therapy could be useful in the treatment of various tumors one can not expect its very high efficiency in general. First of all, it is applicable only to those cancers (50%) that are deficient in wild type p53, and, in addition, the tumors can be anticipated to develop resistance to p53 restoration (polymorphism and selection in action, see above).

The first gene therapy virus approved for the treatment of head- and neck squamous cell cancer in combination with radiotherapy in China (2003), Gendicine™ (from Chinese Shenzhen SiBiono Genetechnologies), is a replication-incompetent adenovirus containing a *p53* transgene in place of the viral E1 region [34, 35]. A similar product Advexin™ (INGN 201; Introgen; Austin, TX, USA) [36] is pending approval from EMEA [37]. The approval of Gendicine™ by Chinese SFDA, has led to a discussion about the efficacy of the treatment [38, 39]. In a phase I clinical trial with 12 laryngeal cancer patients, only one patient experienced self-limited fever and none of the patients had tumor relapse during the 5 year follow-up after the treatment. Similarly, in phase II/III trials with 132 head and neck squamous cell carcinoma patients, 32% showed fever as the only side-

effect of the treatment. When Gendicine™ was used in combination with radiotherapy, 64% of the patients responded with a complete regression and 29% with a partial regression while with radiotherapy alone, 19% showed a complete regression and 60% a partial regression, suggesting a synergistic effect of the combination treatment [34]. However, the published details of these clinical trials are limited, making comparisons to other cancer trials difficult [39].

Thus, even in the most successful applications the tumor was completely destroyed in only 64% of patients with the same type of cancer. Against the background of the general situation in the field of cancer, it is an excellent result, however, very far from what physicians might dream of.

Nodal Proteins as Targets for Inducing Apoptosis: Survivin as a Raradigm

p53 is actually a nodal protein involved in multiple pathways of the cellular networks [29].

Another attractive nodal protein linking multiple pathways of cellular homeostasis is survivin. Survivin orchestrates integrated cellular networks that are essential for tumor cell proliferation and viability. Apart from being a regulator of apoptosis, it is a regulator of cell division and nonapoptotic cell death, a stress response factor, and a promoter of tumor-associated angiogenesis and chemoresistance [40, 41]. Survivin gene expression is transcriptionally repressed by wild-type p53 and can be deregulated in cancer by several mechanisms, including gene amplification, hypomethylation, increased promoter activity, and loss of p53 function [41].

The overexpression of survivin was reported in many forms of cancer, in contrast to normal terminally differentiated tissues where survivin is either not expressed at all or expressed at a very low level. It seems important that many of the survivin-binding partners themselves behave as oncoproteins. There is a consensus that survivin is an essential cancer gene and an appropriate target for drug discovery. Although survivin is probably essential during development and might also have crucial functions in certain adult tissues, survivin antagonists tested so far were rather well tolerated in clinical and preclinical studies, with modest side effects [40].

Several gene therapy approaches targeting survivin have been developed and passed proof-of-principle in preclinical studies [42]. One approach included delivery of survivin dominant-negative mutants to tumors. Dimerization of these mutants with endogenous survivin may result in accelerated degradation of the complex and sudden loss of survivin levels. In turn, this causes inhibition of cell proliferation, induction of apoptosis, suppression of tumor growth, and enhancement of cytotoxics or immunotherapy in preclinical models [43]. Supported by a favorable safety profile, an original survivin antisense oligonucleotide has now completed a phase I trial in patients with advanced cancers, and a phase II trial has been announced. A parallel strategy to suppress survivin levels in tumor cells involved RNA interference. Another similar approach used short hairpin RNA (shRNA) targeting survivin [44].

Considering these optimistic reports, one should keep in mind that the expression of survivin is not a common feature of the all cancer cells. For example, in a study of its expression in 75 non-small-cell lung tumor samples by immunohistochemical staining, it was found that survivin is expressed in only 81% of the samples tested [45]. This figure is close to that found in our study of this cancer using RT-PCR technique (M. Zinovieva et al., unpublished). Even lower (60%) is the survivin expression in patients with bladder transitional cell cancer (BTCC) [46]. These data clearly demonstrate that it is impossible to expect that 100% patients suffering of cancer can be cured with survivin targeting.

Moreover, survivin is not just a cancer-specific molecule but is also involved in regulating cell function, which suggests that survivin disruption could affect normal cell functioning, particularly the hematopoietic and immune systems. Antisurvivin therapies developed to date have not revealed major systemic toxicities in animal models [47]. However, some caution and additional research are necessary to evaluate the safety of the therapy.

One Target to Hit All 6 Hallmarks: the 90 kDa Heat Shock Proteins (Hsp90)

Stress or heat shock proteins (HSPs) are the most conserved proteins present in both prokaryotes and eukaryotes. Their expression is induced in response to a wide variety of physiological and environmental insults. These proteins play an essential role as molecular chaperones by assisting the correct folding of nascent and stress-accumulated misfolded proteins, and preventing their aggregation. Several HSPs have also been demonstrated to directly interact with various components of the tightly regulated programmed cell death machinery, upstream and downstream of the mitochondrial events. On the other hand, extracellular located or membrane-bound HSPs mediate immunological functions. They can elicit an immune response modulated either by the adaptive or innate immune system [48].

These proteins are proving to be a promising target for the development of novel anti-cancer agents designed to selectively block the growth and proliferation of tumor cells. Since Hsp90 is a molecular chaperone and is responsible for folding numerous oncogenic proteins, its inhibition represents a novel approach toward the simultaneous disruption of multiple signaling cascades. Hsp90 is a key facilitator for the maturation of proteins represented in all six hallmarks of cancer. Currently, a number of novel Hsp90 inhibitors have

been developed and subjected to trials [49–51]. Knockdown of Hsp90 by short interfering RNA resulted in the induction of apoptosis in cancer cells [52]. However, the data on clinical trials of the corresponding gene therapy protocols are still unavailable.

Multiple Targeting

When looking for a universal strategy to attack tumors, one should keep in mind that the genes used by a cancer cell are part of a complete network of inter-locking mechanisms and the cancer cell may use a number of different pathways to develop. This redundancy causes immense pharmaceutical problems, as most or all pathways have to be blocked to achieve effective treatment. Many of the favored targets of modern anti-cancer drug development, including apoptosis, metastasis, and angiogenesis, suffer from problems of redundancy in that they may fail to block other potential pathways of cancer. The redundancy problem could be got over by simultaneous inhibiting several crucial upregulated genes in cancer cells. It can be carried out using coexpression of multiple shRNAs that can simultaneously inhibit multiple genes. Recently, such an approach with multiple shRNAs expression vectors containing different combinations of six shRNA expression cassettes targeted at genes involved in cell proliferation and survival pathways (Bcl-2, Survivin, Akt1, Erk2, CyclinE and NfkappaB) was reported. In HeLa and HEK293 cells, the multiple shRNAs expression constructs efficiently and simultaneously induced inhibition of all six genes. An introduction of multiple shRNAs expression vectors in the human prostate cancer cell line PC3, which contains different cell variants with distinct oncogenic signaling alterations, revealed that the multiple inhibition was much more efficient in inducing apoptosis in PC3 cells. Possibly, such a multitarget shRNAs expression system could be an effective strategy in cancer therapy [53].

Of course, trials described give certain positive results in particular (usually small) groups of patients leading to reduction of tumor size and increase in life span of patients. However, they are far from producing radical cure of cancer in all patients. The problem of cancer is widely acknowledged to be far from being solved [1, 23, 26, 54], even though politically correct authors of numerous reviews usually conclude their works optimistically: "These studies have provided very encouraging signs that current research is on the right path. New delivery methods and more sophisticated gene expression cassettes will create better therapeutic alternatives to make the goal of cancer treatment and eradication achievable…".

However, there are also different statements, for example: "For a disease to be a good candidate for gene therapy, the role of the therapeutic gene in disease pathophysiology must be clearly understood. In this context, single gene disorders have a clear advantage, although assumptions about the desired cell type to be targeted and the appropriate timing of gene transfer have not always proven to be accurate. … but it may not always be reasonable to expect a multifactorial disease to respond to overexpression of a single gene. Likewise, overexpression of a tumor-suppressor gene may have some effect in cancer, but could still be short of curative. It seems likely that in the future more gene therapy targets will be identified, primarily as a result of the rapid ability to identify specific gene associations with human diseases, particularly after the completion of the Human Genome Project. …The future remains uncertain regarding the ultimate clinical impact that gene therapy will have in each of those very distinct situations" [55].

I think that such an opinion reflects the real status of gene therapy better than unjustified overoptimistic enthusiasm, especially in the case of cancer gene therapy. The deeper we get into molecular details and the more we concentrate on specific targets, the more inadequate to the complexity of the system become the methods. Generally, a tumor can be compared to the constantly growing and getting more complex Gordian knot with the increasing in time number of tangles. Even if one succeeds in untangling some tangles, the others continue to grow and entangle. As a result, new tangles replace untangled ones, and these new tangles already can not be untangled as before.

TO CUT THE GORDIAN KNOT IS EASIER THAN TO UNTANGLE IT: GENE SURGERY STRATEGY

When a surgeon operates on a tumor, he is not concerned with its molecular and cellular mechanisms. Using a scalpel as a universal tool, he cuts off the tumor, be it lung, kidney or prostate cancer. A similar, simple, universal and irrespective of molecular mechanisms approach could solve the problem of the radical cure of tumors.

Accordingly, gene surgery is aimed at annihilation of tumors as a whole irrespective of the finest molecular mechanisms of tumor emergence and evolution.

General Features of Genetic Surgery Strategies

I will try to formulate the general features of systems supposed to be considered genetic surgery means.

(1) Universality with regard to tumor type. A truly universal approach should exploit the properties common for all tumors and get round problems due to heterogeneity of tumors and their specific characteristics as an integrated evolving cell population.

(2) Direct targeting at tumorous and tumor-surrounding cells.

(3) Potential possibility of being used for destruction of: (i) tumor, (ii) metastases, and (iii) coevolving stroma cells that constitute a danger of emerging secondary tumors after the annihilation of primary ones.

This is an advantage over the traditional scalpel surgery approach.

(4) To be maximally universal, genetic surgery has to be targeted at the most evolutionary ancient system with the lowest complexity. This might be a system of DNA replication. An alternative, but a more complex system is viruses as naturally selected cell-killing agents.

(5) Finally, considering that 100% transfection of tumor cells by a tumor-targeted gene surgery construction is impossible, it is important that this construction produces product(s) able to migrate into surrounding, non-transfected cells and to kill them.

(6) Evident possibility of perfection based on available approaches.

The most known examples of such an approach are destruction of tumors with oncolytic viruses and so-called suicide gene therapy that initiates tumor self-destruction [23]. These two approaches maximally fit the requirements above. Targeting angiogenesis [56, 57], which is a very universal feature of solid tumors suffers from a number of shortcomings caused by the high complexity of the angiogenesis machinery. These shortcomings are discussed in a recent review [57] and are as follows:

In some cases anti-angiogenic therapies produce initial responses followed almost inevitably by progression. In other cases there is no objective benefit. Increasing evidence supports the proposition that progression and mortality following a period of benefit reflects an adaptive response by tumors, manifesting "evasive resistance" to angiogenesis inhibitors. By contrast, patients for whom there is no tangible benefit indicate that an intrinsic resistance to angiogenesis inhibitors does exist.

Resistance to vascular endothelial growth factor (VEGF) pathway inhibitors (and possibly other angiogenesis inhibitors) involves a number of distinct and interrelated mechanisms that may include revascularization consequent to upregulation of alternative pro-angiogenic signals, protection of the tumor vasculature using various mechanisms, accentuated invasiveness of tumor cells into local tissue to co-opt normal vasculature, and increased metastatic seeding and tumor cell growth in lymph nodes and distant organs.

In addition, being targeted not at the tumor cells per se, the approach does not provide for destruction of tumorigenic cells, like cancer stem cells that can remain intact after destruction of the tumor and then give rise to a secondary tumor. Also, this approach does not provide for the migration of agents used for treatment across tumors and neighboring cells.

Oncolytic Viruses as Emerging Tools for Genetic Surgery

Engineered oncolytic viruses (OV) with various mechanisms of action were developed in the past two decades [58]. There are many excellent reviews devoted to oncolytic viruses, and the most recent of them are [59–62]. Therefore, I will only briefly outline the general principles of virotherapy, its advantages and disadvantages. Oncolytic viruses are replication competent, tumor selective and lyse cancer cells. Their potential for anti-cancer therapy is based upon the concept that selective intratumoral replication will produce a potent anti-tumor effect and possibly bystander or remote cell killing, while minimizing normal tissue toxicity. The viral infection and amplification eventually induce cancer cells into cell death pathways and elicit host antitumor immune responses to further help eliminate cancer cells. The use of OV for killing cancer cells is known as (oncolytic) virotherapy. By their ability to amplify and penetrate into surrounding cells, OV differ from conventional drugs that do not amplify themselves and are needed at very high concentrations to affect all tumor cells. Different RNA and DNA viruses are used in virotherapy. Oncolytic virotherapy is a promising form of genetic surgery for cancer, employing nature's own agents to find and destroy malignant cells. Some of the newest additions to the panel of oncolytic viruses include the avian adenovirus, foamy virus, myxoma virus, yaba-like disease virus, echovirus type 1, bovine herpesvirus 4, Saimiri virus, feline panleukopenia virus, Sendai virus and the non-human coronaviruses [60].

Mechanisms of the Selective Permissiveness of Cancer Dells for Viruses

Virotherapy works against cancer by a combination of different mechanisms. Viruses may be naturally oncolytic or be engineered for oncolytic activity, and different mechanisms can be used to provide tumor selectivity [59–61].

Neoplastic cells are selected for growth advantage, but the mutations that determine the advantage can lead to defects in anti-viral defense, altered expression of receptors, expression of novel receptors, and disruption of intracellular signaling pathways (Vähä-Koskela et al., 2007) [61]. Mechanisms of tumor selectivity can be broadly categorized into five groups [61]:

(i) defective anti-viral defenses which are particularly exploited by naturally oncolytic viruses,

(ii) targeting at the receptors unique to or overexpressed on tumors,

(iii) use of tumor or tissue specific promoters,

(iv) viral gene deletions or mutations restricting viral replication in normal tissue, and

(v) proteolytic processing of viruses in the tumor microenvironment.

Below is a very brief description of some examples demonstrating the above approaches.

Defective Cancer Cells' Anti-Viral Defense Systems Exploited by Engineered and Naturally Oncolytic Viruses

Anti-viral defense mechanisms are inactive in many tumors. In normal cells, double stranded RNA (dsRNA) dependent protein kinase (PKR) and type I interferon (IFN) pathways are important anti-viral mechanisms [63]. The type I interferons, IFNα and IFNβ, have anti-proliferative properties, and many tumor cells manifest a faulty antiviral response due to disturbances of the interferon (IFN) response system critical components [60]. Defects in these pathways were found in tumors, where they promote cancer growth. Several oncolytic viruses, in particular RNA viruses such as RV, Newcastle disease virus (NDV) and vesicular stomatitis virus (VSV) exploit these defects in the IFN pathways. For example, NDV is able to inhibit IFN signaling in avian but not human cells, and is therefore not a human pathogen. NDV can, however, infect and lyse human tumor cells lacking an intact type I IFN response [61]. A deletion of viral genes that counteract IFN has been used also to design oncolytic herpes virus (DNA virus) and influenza virus (RNA virus).

Reovirus (RV) is an example of a naturally occurring oncolytic virus, whose tumor selectivity involves a defective double stranded RNA (dsRNA) dependent protein kinase (PKR) anti-viral response. RV has been found to replicate more rapidly in Ras-activated cells. In normal cells, viral dsRNA induces activation and phosphorylation of PKR, which in turn inhibits translation of viral transcripts via phosphorylation of translation initiation factor 2α (eIF2α) [64]. In susceptible Ras-transformed cells, PKR is not phosphorylated in response to RV infection [65]. However, there are also other mechanisms of permissivity for RV replication in Ras-transformed cells [66].

Targeting to Receptors Unique to or Overexpressed on Tumors

Viruses can become tumor-specific if tumors expose receptors that can serve as viral receptors unique to tumor cells. The Edmonton strain of measles virus enter to cells via the CD46 receptor, which is overexpressed in some tumors [61]. The A21, ICAM-1 and DAF receptors for coxsackievirus are overexpressed in many cancer cells. Many human cancer cell types overexpress the CD155 receptor which is a poliovirus receptor involved in the cellular poliovirus infection in primates. This receptor allows the virus to selectively infect the corresponding tumor cells. Other examples can be found in the above cited reviews. Some viruses use receptors abundant also on normal cells, such as the above mentioned ICAM-1, but still preferentially amplified in cancer cells. In this case, some intracellular mechanisms are responsible for the selectivity, in particular impaired antiviral response (see above) [60].

Viruses can also be retargeted to tumors by modifying virus attachment proteins [61]. For example, the infection efficiency of adenoviral vectors in tumor cells can be improved by insertion of an arginine-glycine-aspartic acid (RGD-4C) peptide sequence into the HI loop of the adenovirus fiber domain, thus allowing the virus to use the $\alpha_v\beta_3$ integrin as an alternative receptor. Such a modification increased the transduction efficiency of an adenoviral vector in CAR-deficient tumor cells [59, 67, 68].

Use of Tumor or Tissue Specific Promoters

Another way to increase tumor selectivity of DNA viruses is genetic engineering replacement of essential virus promoters with cellular promoters that are over-activated in tumor cells. Viruses mutated to be replication dependent on certain molecular features characteristic of cancer cells were called conditionally replicating viruses.

Promoters for tumor antigens such as prostate specific antigen and alpha fetoprotein have been incorporated into genetically modified adenoviruses [61]. A recent example [69] used an oncolytic adenoviral vector, in which the adenoviral critical gene E1 A promoter was replaced by a human tyrosinase enhancer (HTE)/promoter specific for melanoma cells.

Two promising cancer specific promoters activated in most tumor cells are those of the *BIRC-5* gene encoding survivin and the *TERT* gene encoding the telomerase catalytic subunit. It was reported [45] that the survivin promoter in vivo was more than 200 times more cancer specific than the cytomegalovirus promoter. Recently, conditionally replicative adenoviruses (CRAds) with the tumor specificity regulated by the survivin promoter were constructed, and their viral infectivity was enhanced by an RGD capsid modification in the adenovirus fiber region. These CRAd agents effectively target cholangiocarcinoma cells (a highly malignant neoplasm with no effective treatment), induce strong cytotoxicity in these cells in vitro and inhibit tumor growth in a murine xenograft model in vivo [70]. Another CRAd with a modified hTERT promoter (also expressing granulocyte macrophage colony-stimulating factor, GM-CSF) showed strong anti-tumor efficacy in nude mice with human head/neck and hepatocellular carcinoma xenografts, as well as strong tumor-cell selectivity [71].

Viral Gene Deletions or Mutations Restricting Viral Replication in Normal Tissue

Cancer cell specific viruses have also been engineered using viral gene deletions or mutations restricting viral replication in normal tissue. The most known example are adenoviral vectors deleted in the E1B gene in order to restrict their replication to cells in which the p53 protein function is impaired (see above). Whereas the E1B protein of wild-type adenovirus binds p53 thus

preventing apoptosis induction in response to infection, E1B-deleted vectors can replicate productively only in cells lacking functional p53, though this requirement is not absolute [60]. (The mechanism of tumor selectivity now appears more complex, with evidence that E1B has a function in the export of viral RNA. Tumor cells but not normal cells appear to be able to provide the missing export function of E1B). The adenovirus mutant ONYX-015 engineered to lack the E1B protein was one of the first modified viruses to enter clinical trials.

Vaccinia virus is another example of a deletion-modified virus: with the viral thymidine kinase (*tk*) gene deleted it lacks the ability to synthesize nucleotides. The modified vaccinia virus can only replicate in cycling cells, such as transformed cells, with an abundant supply of nucleotides [72]. Similarly, Herpes viruses lose the ability to infect non-dividing cells due to deletion of the *tk* gene that forces them to prefer tumor cells over normal cells.

Permissiveness of tumor cells with activated Ras and inhibited PKR for oncolytic herpes simplex viruses (HSV) can be achieved by deleting the main neurovirulence c34.5 gene whose product normally allows to overcome the restriction on replication posed by functional PKR. One more strategy to enhance the tumor cell specificity of oncolytic viruses is to mutate them in order to induce a more potent IFN response. While this reduces the replication of such viruses in normal cells, cancer cells remain permissive to the viruses because they are unable to respond to IFN signaling [73].

The list of examples can be extended further, but the principle of the approach is hopefully clear.

TARGETING THE TUMOR MICROENVIRONMENT

An alternative approach is to use the in vivo tumor environment to enhance selectivity. For example, it is possible to engineer viral vectors with therapeutic transgenes that target the key components of the tumor microenvironment (e.g. the tumor vasculature or matrix). An oncolytic adenovirus encoding the matrix-degrading protein relaxin was able to enhance viral spread without causing significant toxicity [58].

Another approach is based on the proteolytic environment of the tumor. One can engineer oncolytic viruses whose replication is activated by tumor matrix metalloproteinases (MMP). For example, the fusion (F) protein of measles virus (an enveloped virus) facilitates viral entry into cells by mediating fusion of the viral and cellular membranes, and is normally produced as an inactive precursor that is naturally cleaved by furin (an enzyme which belongs to the subtilisin-like proprotein convertase family) to expose a fusion peptide. A virus was constructed containing F-protein with the furin cleavage site replaced by a matrix metalloproteinase 2 (MMP2) cleavage site. As many cancer cells secrete high levels of proteases like MMP2, such a

modified virus will be preferentially activated in the vicinity of tumor cells [59, 61]. Further studies are required to determine whether minor alterations, such as insertion of protease cleavage sites, have any effects on virus replication and the infection process.

RV normally infects cells of the gastrointestinal tract, where proteases can convert the noninfectious RV into an infectious form called the intermediate sub-viral particle (ISVP). When given intravenously, RV is not efficiently processed to the infectious form. However, it is possible to select for variants that have been converted into ISVP by the action of proteases overexpressed in the tumor microenvironment [59].

Additionally, oncolytic viruses can be further engineered to delete immunosuppressive viral components and to insert transgenes that enhance antitumor immunity [74].

COMBINATION OF ONCOLYTIC VIRUSES WITH GENE THERAPEUTIC GENES: ARMED ONCOLYTIC VIRUSES

A new trend in virotherapy is the construction of oncolytic viruses armed with transgenes encoding various therapeutic products capable of forming "oncolytic pharmacophores". A pharmacophore was first defined by Paul Ehrlich in 1909 as "a molecular framework that carries (phoros) the essential features responsible for a drug's (=pharmacon's) biological activity" (Ehrlich. Dtsch. Chem. Ges. 1909, 42: p. 17). Oncolytic vectors can be used to spread anti-tumor genes, or transgenes can be used to help virus replication or spread. For example, transgenes that promote apoptosis can increase virus spread.

Many types of viruses are used for such purposes [59]. Genetically engineered, conditionally replicating herpes simplex viruses type 1 (HSV-1) can transfer and express foreign genes in host cells. Recently, a new-generation oncolytic HSV-1 was described suited for functioning as a vector for expressing foreign molecules that allow relatively easy insertion of transgene(s) into the viral genome [75, 76].

A new therapeutic class of oncolytic poxviruses has recently been developed that combines targeted and armed approaches for treating cancer. The viruses were armed with therapeutic transgenes, such as a gene encoding granulocyte-macrophage colony-stimulating factor (GM-CSF), to stimulate antitumoral immunity. The resulting agents have been shown to be highly selective for cancers and to have a high degree of systemic efficacy by multiple mechanisms of action in preclinical testing. Initial preclinical and clinical results show that products from this therapeutic class can systemically target cancers in a highly selective and potent fashion using a multi-pronged mechanism of action [62].

RNA interference (RNAi) is a powerful tool for gene knockdown purpose and seems to be promising for the treatment of cancer. Combining shRNA gene

therapy and oncolytic virotherapy can enhance antitumor efficacy as a result of synergism between oncolysis and shRNA antitumor responses. A novel oncolytic adenovirus-based shRNA expression system for Ki-67 gene silencing that allows efficient tumor-specific viral replication was shown to induce the apoptosis of tumor cells effectively in vitro and in nude mice [77].

A similar approach was applied for reducing hTERT expression in hope to inhibit proliferation of cancer cells [78]. This system also did not prevent tumor-specific viral replication and effectively induced the apoptosis of tumor cells in vitro and in nude mice.

Clinical Trials

Clinical studies require a high-titer production of viruses according to Good Manufacturing Practices. It is now about 13 years since the beginning of the first clinical trial in which cancer patients were treated with an oncolytic virus. By 2008, oncolytic viruses from several different species have been taken to phase I and II clinical trials in over 800 cancer patients [58]. These included genetically engineered vaccinia, herpes simplex virus (HSV), adenovirus (all based on human adenovirus serotype 5), and measles, as well as non-engineered viruses such as NDV and RV [79]. Two agents have been most extensively tested in clinical trials (>400 patients by 2008): a first-generation RV (Reolysin, Oncolytics), and a second-generation adenovirus (ONYX-015, an E1B-55 kDa deletion mutant) [62]. Mechanisms which provide viruses with tumor selectivity do not completely preclude infection of normal tissue and consequent toxicity. That is why in the majority of clinical trials the virus was administered by intratumoral (i.t.) injections [79]. Intratumoral delivery of oncolytic viruses provides a further direct physical restriction to enhance tumor selectivity. Although the safety results were encouraging, efficacy of OV as single agents was rather low [58, 80] after both direct intratumoral and intravenous (i.v.) injection, and no systemic spread to distant tumors was detected [61, 62] (see also discussion below). Studies with i.t. administration have generally not demonstrated activity against distant non-injected lesions [79], greatly limiting the potential of local viral therapy in the treatment of metastatic disease. In an exception to this observation, in patients with metastatic melanoma receiving i.t. injections of GM-CSF armed vaccinia virus (JX-594, JENNEREX), regressions in distant non-injected dermal metastases were noted in four of seven patients treated. These regressing lesions were found to be heavily infiltrated by T lymphocytes. Similarly, in a phase I study of intratumoral administration of a modified HSV expressing GM-CSF, non-injected distant tumor sites became inflamed in 4 of 30 patients [61]. Most of these vectors have proven safe, and phase II studies are ongoing [60]. In total, the observed level of anti-tumor activity, although low, provides encouragement for future oncolytic viral therapy if the route of administration,

potency, tumor selectivity and immune interactions can be optimized. In contrast to conventional phase I drug trials, the maximum tolerated dose of virus is commonly not reached, and dose is limited by technical restrictions in the quantity of virus which can be produced [59].

A limited number of studies have investigated i.v. delivery of virus. Studies using i.v. administration of modified adenoviruses have not shown systemic activity [79], however, systemic efficacy following i.v. administration has been demonstrated using NDV and RV. In a phase I study of i.v. administered RV in patients with advanced cancer, viral replication was demonstrated in post-treatment tumor biopsies and stable disease was reported in 6 of 32 evaluable patients, demonstrating the feasibility of systemic viral delivery to tumors [61]. Systemic delivery is associated with more severe flu-like symptoms, although the toxicity profile remains favorable compared with conventional cancer therapy. One treatment-related death following treatment with a naturally modified NDV has been reported in a patient with reduced lung capacity. Post-mortem examination suggested that rapid tumor lysis had led to respiratory failure [61]. Similar to conventional cancer therapy, this case demonstrates the need for adequate performance status and functional reserve [61].

Oncorine™ (H101; similar to ONYX Pharmaceutical's discontinued Onyx-015) from Shanghai Sunway Biotech (China) is a conditionally replicative adenovirus, which gained marketing approval in China in 2006 for treating head and neck cancer and is in clinical trials for non-small-cell lung cancer. Oncorine™ contains a deletion in the E1B region and together with ONYX-015 has provided a plethora of data in various types of cancer e.g. glioma, head and neck, pancreatic and ovarian cancers. In phase I trials, Oncorine™ showed safe and effective tumor shrinking in 3 patients out of 15. In phase II trials in 53 patients, the treatment group (two cycles of i.t. injection of Oncorine™ showed a 28% response rate against the 12% rate seen in controls. The agent was well tolerated in the responsive patients (3 with complete regression and 11 with partial regression) [27].

It should be noted that in the case of the most investigated Onix-015 no objective responses to single-agent therapy occurred in patients with pancreatic, colorectal or ovarian cancers. In view of the rarity of clinical responses to Onyx-015, combination therapy with chemotherapy was explored for Oncorine™. A synergistic interaction with chemotherapy in multiple tumors types and by multiple routes of administration was observed. Thus, in phase III trials with 123 patients in the treatment group (combination of chemotherapy and Oncorine™) or control (chemotherapy alone), the response rate in the treatment group was 72.7%, as compared to 40.4% in the control group. The complications included fever, injection site pain, nausea, alopecia, leucopenia and flu-like symptoms [27].

Due to clinical trials, virus species-specific characteristics are coming to light. Intravenous efficacy has been shown with vaccinia, measles, NDV, and RV. In contrast, i.v. adenovirus dosing of 150 patients on trials was negative for responses. Also, after i.t. treatment of >150 patients with group C adenoviruses, systemic efficacy against distant tumors has never been reported. Toxicities varied between virus species [79].

Obstacles

Although promising, virotherapy still faces many obstacles that need to be addressed:

(1) Highly effective non-specific host mechanisms to clear virus following systemic delivery [61].

(2) The interaction with the host immune system is complex: though virally-induced cell lysis releases tumor associated antigens in a "dangerous" context, and limited evidence suggests that this can lead to the generation of a specific anti-tumor immune response, an anti-viral immune response may limit efficacy by rapidly clearing the virus.

(3) Many tumor types are characterized by small groups of tumor cells surrounded by large areas of tumor-associated fibroblasts and connective tissue. In this environment intratumoral spread is blocked. Locally administered virus does not generally spread to other tumor sites [81].

(4) The lack of universality. Different viruses have evolved very different life cycles in their hosts. Virus species, therefore, vary dramatically as "pharmacophores" [79].

(5) A common problem of treatment-emergent resistance due to the selection of virus-resistant tumor cells by evolving tumors. Although oncolytic viruses were claimed to kill cancer stem cells [58] possibly responsible for sustaining tumor growth and metastasis, and also importantly contributing to resistance to chemo- and radiation therapy [82], there is so far no direct evidence of anticancer stem cells efficacy of this approach in vivo in tumors. At the same time, OV can leave some cells intact due to the lack of necessary receptors.

(6) The effect of virotherapy on stromal cells is unclear, although these cells are a very important participant of tumor progression.

(7) Safety issues of clinical use of live replicating viruses is a problem of great concern. The potential toxicity of these agents to patients, staff, patients' immediate relatives and shedding into the environment have resulted in rather stringent regulatory guidelines and risk assessments. This process combined with the considerable costs when compared to standard cancer chemotherapeutics has led to questioning of the viability of virus based approaches [61].

(8) Clinical experience has so far provided evidence of limited efficacy though a favorable toxicity profile.

A number of novel strategies are now under investigation to overcome the barriers. The immune response barrier may be alleviated using a transient immune suppression with immune suppressors or chemotherapy.

Intratumoral barriers could probably be overcome through expression of hyaluronidases and proteases from oncolytic viruses. Bystander effects mediated by prodrug-activation genes could also enhance the spread of oncolytic viruses. Combination therapy with chemotherapy or radiotherapy represents a promising avenue for ongoing translation of oncolytic viruses into clinical practice [60, 61]. No doubt that oncolytic viruses are moving closer to fulfilling their clinical potential and may augment the means of care for certain cancer scenarios in the future [61].

But it is also evident that OV use too complex and too variable particular and generally uncommon features of cancer cells and therefore will hardly become a universal and reliable cancer killer.
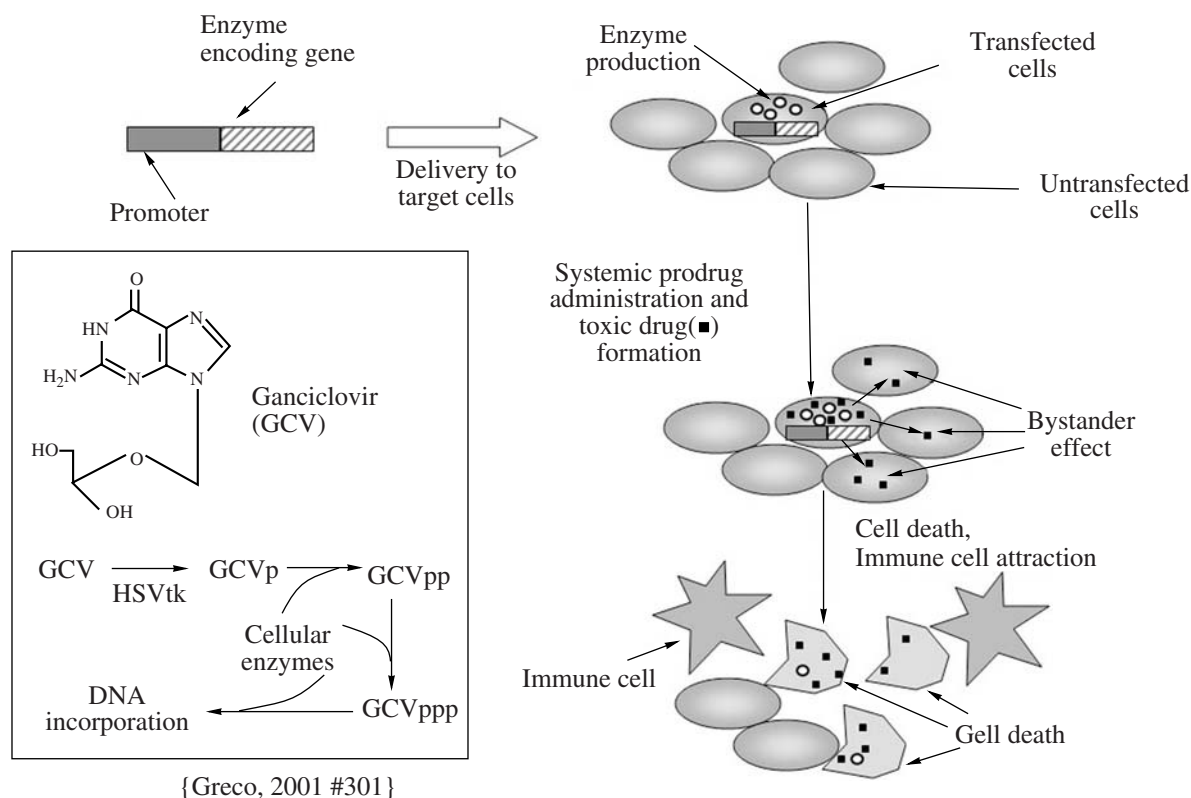
GENE-DIRECTED ENZYME PRODRUG THERAPY AND THE BYSTANDER EFFECT

Another strategy of genetic surgery is so called gene-directed enzyme prodrug therapy (GDEPT). This is a two step approach. In the first step, a transgene encoding an enzyme is delivered into the tumor and expressed. In the second step, a prodrug is administered and selectively activated by the expressed enzyme (Fig. 2). The first GDEPT system described was the thymidine kinase gene of the Herpes Simplex virus (*HSVtk*) in combination with the prodrug Ganciclovir (GCV). A large number of experiments have been performed with this system in different types of tumors, and initial studies in animal models were very promising [83]. I will use this system as a paradigm to describe the advantages and limitations of the approach. As to other systems, the information can be found in numerous reviews [23, 84, 85].

In this kind of genetic surgery, it is very important that even if only a small portion of cancer cells contain therapeutic genes, the deadly products of the prodrug conversion by the enzyme are spread to neighboring cancer cells. This bystander cell killing may greatly improve the efficiency of cancer killing.

When compared to classical chemotherapy, GDEPT offers a number of advantages in addition to the bystander effect. In particular, using tumor-specific regulatory elements, the gene of the prodrug converting enzyme can be made to be expressed predominantly in cancer cells and not in unmodified human cells [86]. The toxic agent can thus be concentrated in tumor cells further reducing off-target toxicity [84, 85].

One can formulate the requirements for an "ideal" suicide gene/prodrug combination [85]. For the gene product:

Ganciclovir (GCV)

$$GCV \xrightarrow[\text{HSVtk}]{} GCVp \rightarrow GCVpp$$

Cellular enzymes

DNA incorporation ← GCVppp

{Greco, 2001 #301}

Fig. 2. Outline of the gene-directed enzyme prodrug therapy (GDEPT) as a paradigm of the gene surgery strategy and succession of steps in HSVtk/GCV transitions (framed).

(i) to be either absent from the human genome or expressed only at low levels in healthy organs, and to be intrinsically non-toxic,

(ii) to be both necessary and sufficient for full activation of the prodrug without the necessity for further catalysis by endogenous enzymes which could be mutated in some tumors,

(iii) to be monomeric and small, allowing the use of expression vectors in which the transgene choice is size-restricted,

and, for the prodrug:

(i) to have high affinity for the enzyme encoded by the suicide gene and low affinity for endogenous enzymes outside the tumor mass,

(ii) to be able to penetrate into a solid tumor and be efficiently taken up by tumor cells,

(iii) to exhibit no toxicity prior to activation,

(iv) the toxic form(s) of the drug should be capable of intercellular diffusion to allow killing of tumor cells also via the bystander effect,

(v) to have a half life long enough to maximize the bystander effect within the tumor but short enough not to cause off-target toxicity following diffusion to surrounding regions.

I think that the toxicity of the activated prodrug is desirable to be DNA replication dependent, though some authors express a contrary point of view [85]. Their opinion is based on the fact that only a fraction of tumor cells are actually proliferating at any given time. However, exactly this proliferating fraction includes stem cells [82, 87–92] and causes tumor to evolve and produce metastases. Therefore, to destroy the whole tumor it may be sufficient to kill this fraction.

Bystander Effect

The bystander effect is the major driving force behind any GDEPT strategy. Numerous studies performed with a variety of suicide gene/prodrug combinations have demonstrated that complete tumor eradication is possible even when the suicide gene product is expressed by less than 10% of the tumor cells [93]. Bystander effects can either be exerted by free diffusion of toxic metabolites or can rely on intercellular communication via gap junctions [22, 87, 94–96]. It is worth mentioning that interactions between cells are primarily mediated by four types of structures in the plasma membrane: gap junctions, tight junctions, desmosomes, and adherence junctions [97]. Gap junctions are intercellular plasma membrane domains enriched in channels that allow direct exchange of ions and small molecules between adjacent cells. Gap junction channels are composed of a family of transmembrane proteins called connexins [98]. Many tumor cells do not have or have

impaired functional gap junctions [98–100], therefore free diffusion may have advantages for cancer gene therapy, however, it may also cause off-cancer toxicity due to systemic diffusion.

It is important, that the bystander effect can be further potentiated by the immune system, when released foreign and/or tumor antigens from dying cells stimulate the immune system to eliminate tumor cells which do not express the suicide gene, even when these are separated either spatially (the so-called "distant bystander effect") or temporally (the so-called "vaccination effect") from the suicide gene expressing cells [85], phenomena which could also lead to the destruction of metastases originating from a primary tumor.

HERPES SIMPLEX VIRUS THYMIDINE KINASE/GANCICLOVIR AS A CLASSICAL ENZYME/PRODRUG PAIR FOR GENETIC SURGERY

The 376 amino acid protein thymidine kinase (tk) encoded by the herpes simplex virus 1 (HSV1) genome is responsible for initiating the phosphorylation of deoxythymidine to deoxythymidine triphosphate for incorporation into nascent DNA. HSVtk has a different catalytic specificity compared to mammalian thymidine kinases, exhibiting around 1000-fold greater efficiency in monophosphorylating ganciclovir (GCV) (Fig. 2) [85]. Expression of HSVtk to activate ganciclovir was used for the first proof-of-principle of suicide gene therapy [101]. Since then, the HSVtk/GCV combination and variations thereof remain the most widely used systems in both clinical and experimental GDEPT applications. Following the monophosphorylation of GCV to GCV-MP by HSVtk, GCV-MP is subsequently further phosphorylated to its diphosphate (GCV-DP) and triphosphate (GCV-TP) forms by endogenous guanylate kinase and several other enzymes such as phosphoglycerate kinase. The cytotoxic effect of GCV results from incorporation of GCV-MP into DNA using GCV-TP as a substrate. The GCV-terminated strands of DNA are not substrates for DNA chain elongation, therefore the elongation of DNA strands is prevented, leading to cell death. The precise mechanism of HSVtk/GCV mediated cell death is still not completely understood. Apart from a few possible exceptions with certain cell types, the death is apoptotic and is probably independent of the p53 pathway. Indeed, tumor cell lines exhibiting mutated p53 are also sensitive to HSV-tk vectors, hence this therapeutic strategy can be applied irrespective of the p53 status of the tumors [93].

Other mechanisms of HSV-tk/GCV cytotoxicity also appear to operate. Independent of cell replication, some toxicity of GCV to quiescent cells expressing HSV-tk, such as thyrocytes and hepatocytes, has been reported. The mechanism underlying this phenomenon is not yet clearly understood but could be the consequence of mitochondrial DNA polymerase inhibition. GCV-TP, the most active toxic metabolite of GCV, apart from incorporating into growing DNA chains also exerts its cytotoxic effects intracellularly by inhibiting cellular DNA polymerases. However, replicating DNA is considered to be the major target in cancer cells for GCV activated by HSVtk [83, 85, 93].

Although apoptosis seems to play a major role in this process, non-apoptotic mechanisms may sometimes also be involved, often seemingly dependent on the specific cell type to which the system is applied [83].

Bystander and Distant/Vaccination Bystander Effect in the HSVth/GCV System

Compared to some other suicide gene/prodrug combinations, one of the main disadvantages of using the HSVtk/GCV system for GDEPT is that, although the prodrug can passively diffuse into target cells, the cytotoxic GCV-TP is highly charged and therefore insoluble in lipid membranes, which means that it cannot diffuse out of the cell and into neighboring cells to exert its toxic effects. In spite of this, however, a bystander effect is often still observed both in vitro and in vivo [96]. This is generally due to the transfer of GCV-TP across gap junctions [93], though some reports indicate that the presence of gap junctions is not obligatory for the bystander effects [22]. The intracellular TK level might influence the bystander effects. The efficiency of GCV killing and the magnitude of the bystander effect were compared for cell lines containing single and double-copy TK. The two copy expressing cells metabolized GCV more efficiently, were more sensitive to GCV, and demonstrated improved bystander killing [22].

In addition to gap junction mediating bystander effect, it has been reported that in certain cases the bystander effect might occur not only via intercellular communications, but also by phagocytosis of apoptotic bodies, through the activation of the immune system, or by releasing cytotoxic metabolites [22, 85]. Such distant bystander effect may affect even cells at distant metastases [102].

CLINICAL TRIALS

A number of reviews summarize the clinical trials results for the HSVtk/GCV system delivered by different vectors for different tumor treatments [25, 83, 85, 103, 104]. In the review of 2003 [83], the author wrote the following about this system: "A large number of experiments have been performed with this system, in different types of tumors and initial studies in animal models were very promising. This encouraged investigators to move into clinical trials although poor results have been obtained so far". This review summarizes the results of clinical trials published up to 2001. The results of clinical trials during last several years can be found in a recent review [85]. An initiation of several new clinical trials using the HSVtk/GCV system with a number of tumors, including melanoma, pancreatic cancer, glioma, retinoblastoma and prostate cancer, was

reported in the Journal of Gene Medicine clinical trials database (www.wiley.co.uk/genetherapy/clinical/). However, the results of these trials are still mostly unavailable.

Briefly, the existing data reliably demonstrated the safety of this approach and a large number of clinical phase I/II trials carried out using several different vector types for gene delivery to a variety of malignancies have all demonstrated that HSVtk/GCV gene therapy is well tolerated. There is also evidence that this combination does indeed prolong patient survival time in some settings. In the last five years, updates and results from several further clinical trials have been published documenting the application of retroviral producer cells for glioma treatment, adenoviral vectors for glioma, prostate cancer, malignant pleural mesothelioma, retinoblastoma and ovarian cancer treatment. Several of these trials which used high-titre or replication-competent adenoviral vectors for HSVtk delivery demonstrated encouraging results, including long-term tumor regression and patient survival in retinoblastoma, malignant pleural mesothelioma and glioma therapy. An important result was reported concerning treatment of patients with operable primary or recurrent malignant glioma resulting in statistically significant increase in patient survival times [105]. In this study, 36 patients were enrolled, 17 of which were randomly selected to receive an HSVtk-expressing adenoviral vector injected into a resected tumor site. Subsequent twice-daily intravenous delivery of GCV for 2 weeks led to an increase in mean survival time from 39 weeks in the control group to 71 weeks in the treated group.

However, despite the undoubted value of the information which has been gathered from a wide variety of preclinical and clinical studies in which GDEPT systems have been evaluated to date, including those in which real clinical benefit has been demonstrated, success rates even approaching those which are routinely achieved in preclinical studies have yet to be attained in clinical applications. The question is—why? Clearly, a number of factors are responsible for this discrepancy, starting with a very simple one—toxic side effects associated with GCV, especially against bone marrow cells. The GCV doses which can be administered to humans (about 10 mg/kg/day) are much lower than those used in animal experiments (up to 300 mg/kg/day) [106]. Other reasons might be inefficient gene transfer, a larger size of human malignancies as compared with those in murine models, and a slower growth rate of spontaneous tumors compared to experimental xenografts [85, 106].

But the main role probably belongs to the fundamental biological differences between mouse and man. In their comprehensive review [107], the authors remind us that mice are not small people and then point out at parallels and discrepancies between mouse and human carcinogenesis at the cellular and molecular levels and indicate limitations that are inherent in the mouse models used for elucidating the human disease process. In particular, they note a marked decrease in age-specific cancer rates that has accompanied the substantial increase in lifespan that has occurred during the past 80 million years of the mammalian evolution that led via the primate lineage to humans. This decrease in cancer susceptibility has been accomplished through the development of several distinct antineoplastic mechanisms, many of which are intrinsic to human cells. The authors indicate also that if transformation of murine cells requires far fewer genetic and/or epigenetic alterations to the cell genome, then multistep tumor progression occurring in mice must involve far fewer changes and be far simpler than the comparable processes in humans. Stated differently, these discrepancies in the organization of cell-autonomous regulatory pathways must dictate markedly different courses of tumor progression in the two species. Moreover, the substantially greater number of pathways required to transform human cells means that most of the cell-autonomous, anticancer protective mechanisms that are present in human cells must have been developed or at least perfected during the time since our evolutionary lineage diverged from that of rodents.

In addition, I would think that both naturally and artificially induced or grafted tumors due to their fast progression in mice have not enough time for harmonizing tumor evolution with the environment and therefore may be less robust and less evolutionary adapted to their ecological niches. This in turn can lead to higher sensitivity of mice tumors to antitumor treatments. Thus, mouse models are rather a proof-of-principle than a real step forward to the practical use of GDEPT.

Therefore, much needs to be done to translate the proved principle into human clinical practice. Nevertheless, GDEPT remains a highly promising system for the genetic surgery of solid tumors. Development of new approaches to optimize enzyme/prodrug interactions, potentiation of the bystander and especially distant bystander effects and immune response, as well as combination of different suicide systems to overcome resistance of some cells to single drugs, allow one to hope that real clinical success using GDEPT genetic surgery will be reached in the near future and provide the most universal means to win the terrible war with cancer [85].

PROSPECTS FOR IMPROVEMENTS

Increasing Bystander Cell Killing Potential

Enhancement of bystander effect might increase the anti-tumor effect of genetic surgery. Several possibilities were tested to this end.

Enhancement of Gap-Junctions

One of the central players in the gap junctions formation are their structural proteins (connexins) [108].

Therefore, in order to improve GJIC (gap junctional intercellular communications) in target cells for more efficacious HSVtk/GCV GDEPT, strategies have been developed to enhance the expression of connexin (Cx) proteins in cancer cells [22, 109–111]. It can be achieved by introduction of connexin genes together with HSVtk. In most of the studies, connexin-43 was used for this purpose [110], although successful transfections of other connexin genes were also reported [85].

There are several other possibilities to improve GJIC. For instance, all-transretinoic acid can increase connexin-43 expression in various tumor cell lines and facilitate GCV-induced bystander cell killing both in vitro and in vivo [112]. Other low molecular mass reagents were also described as effective means of gap junction improvement (for recent review see [22, 85]).

Augmenting the Immune-Related Anticancer Response

Investigation of the distant bystander effect mechanisms revealed that the immune response following application of the HSVtk/GCV included an infiltration of CD4+ and CD8+ T-cells and macrophages into the treated tumor, combined with increased levels of IL-2, IL-12, IFN-c, TNF-α and GM-CSF [102, 113]. Several groups have quite successfully attempted to potentiate the distant bystander effect and the vaccination effect associated with the HSVtk/GCV system by co-expressing immune stimulatory molecules, such as IL-2 [114], IL-12 [115], GM-CSF [116], TNF-α [117] and TNF-related apoptosis-inducing ligand (TRAIL) [118].

Linking Thymidine Kinase to Other Proteins to Promote the TK Protein Intercellular Traffic

In an attempt to overcome the limitations imposed upon the efficacy of the bystander effect by its dependence on functional GJIC, several groups have constructed fusions consisting of HSVtk and proteins known to promote intercellular import/export, such that the bystander effect is mediated by intercellular transport of the activating enzyme rather than of the activated prodrug. In one of such attempts, the *tk* gene was linked to the gene of another herpes virus protein, VP22. The VP22 protein has been shown to pass freely between cells by an unknown mechanism [22, 119]. It can spread to almost every cell in a monolayer from only a few producer cells. VP22 fusion proteins might function as potent protein delivery systems. Therefore, a VP22-TK construct was tested on different tumor cell lines in vitro and in vivo to improve bystander killing. It has been reported that VP22-TK chimeric proteins spread between cells in quantities sufficient to induce cell death in response to GCV treatment, not only in the primary TK+ cells but also in surrounding TK-cells. This effect was observed in vitro after GCV treatment of transfected tissue culture cells, and in vivo after GCV treatment of mice injected with tumor cells transduced with VP22-TK fusion genes. However, more recent findings indicate that any differences between the action of VP22-TK and wild-type HSV TK are not actually mediated by intercellular trafficking of the fusion protein [120]. However, the idea seems to be fruitful, and recently Luo et al. [121] constructed recombinant *HSVtk* chimeras fused with the arginine-rich (RXP) repeat of herpes simplex virus type 2 (HSV-2) US11 protein and examined their activity of intercellular trafficking and cytotoxicity. The US11 gene product of herpes simplex virus is an abundant virion structural protein with RNA-binding regulatory activity. Its carboxyl-terminal half consists of tandem tripeptide repeats of the RXP sequence. It was demonstrated that the US11 protein had intercellular trafficking activity and that the RXP repeats were responsible for this activity. These same properties were also observed in cells expressing a fusion protein linking US11 to the green fluorescent protein [122]. When examining the immunofluorescence staining patterns of RXP-TK fusion proteins in transfected COS7 cells, the RXP chimeras revealed a conservation of the trafficking activity of RXP [121].

Fusion proteins consisting of HSV TK and 8–11 amino acids from the human immunodeficiency virus (HIV)-1 TAT protein were reported to have full catalytic activity while also being capable of gap-junction independent intercellular trafficking [123]. Moreover, the TAT-TK fusion proteins seem to be more stable than wild-type HSV TK, probably due to being protected from cytoplasmic degradation enzymes as a result of their nuclear localization following intercellular translocation [85, 124]. However, most recent results did not confirm these data [125]. The authors concluded that although some degree of enhancement by TAT was shown in certain tumor cells in vitro, it is unlikely that TAT peptide linked to a suicide protein could be a useful booster of in vivo gene therapy trials. Future studies will hopefully reveal the reason for such a discrepancy.

Enhancement of the HSV TK Catalytic Activity

As mentioned above, the GCV associated toxic side effects dictate the use of rather low doses of agents, which is probably one of the reasons for rather poor effects observed with *HSVtk*/GCV in clinical trials. Another antiherpes drug, aciclovir (ACV), is less immunosuppressive and generally much better tolerated than GCV, but HSV TK displays a much higher K_m value for ACV than for GCV. Therefore, HSV TK mutants with increased specificity for GCV or ACV have been found and characterized, and some of them are already in clinical use. Of these, the most extensively evaluated one is 43-fold more sensitive to GCV and 20-fold more sensitive to ACV than the parental HSV TK [126]. New mutant enzymes are being revealed [127, 128], one of them displaying 124-fold decrease in K_m with aciclovir as the substrate [127]. Such new "prodrug kinases" could provide benefit to genetic surgery making it feasible to use relatively non-

toxic aciclovir at nanomolar concentrations or ganciclovir at lower, less immunosuppressive doses.

Using a fusion protein of HSV TK and guanylate kinase (GMK, a cellular enzyme converting GCV-DP to GCV-TP following phosphorylation of GCV by HSV TK) to improve the therapeutic effect of the *HSVtk*/GCV system, Willmon et al. managed to decrease the IC50 of GCV by 175-fold as compared to HSV TK alone [129].

Enhancement of HSV TK Substrate Efficacy

One more evident way to improve the *HSVtk*/GCV system efficiency is to find better substrates with higher bystander effect, lower toxicity etc. The search was performed among several antiherpes purine and pyrimidine nucleoside analogues. All pyrimidine nucleoside analogues, including (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU), showed low, if any, bystander killing effect. In contrast, purine nucleoside analogues, such as ganciclovir, were endowed with a pronounced bystander killer effect. Lobucavir (Cyclobut-G), a ganciclovir analogue, displayed a two- to three-fold more pronounced bystander killer effect than ganciclovir, eliminating at a concentration of 10 µM 75 and 90% of a cell population that contained 5 and 10% *tk* gene-transfected cells, respectively. On the other hand, BVDU metabolites were less prone to pass the gap junctions than GCV metabolites [95].

To enhance the efficacy of HSV TK/GCV genetic surgery for nasopharyngeal cancer, long-circulating liposome-encapsulated GCV known to gradually release GCV was tested. Pharmacokinetics studies in mice showed that intravenous and intraperitoneal injections of such GCV led to long circulation in plasma and were significantly more effective than GCV solution in inhibiting tumor growth [130].

HSV TK Potentiators

The efficacy of HSV TK can be enhanced by the action of certain low-molecular weight compounds. For example, it was recently found that a diterpenoid, scopadulciol (SDC), may play a role in the HSV TK/prodrug administration system because it produced a significant increase in the active metabolite of ACV. The bystander effect was also considerably augmented by the combined treatment with ACV/GCV and SDC. SDC significantly enhanced the cell-killing activity of prodrugs. These findings are especially valuable with respect to the use of GCV in lower doses and less toxic ACV. The novel strategy of drug combination might provide benefit to HSV TK/prodrug genetic surgery [131].

Overriding Resistance to GCV

The susceptibility of cancer cells to HSV TK/GCV treatment varies dramatically, and the mechanism of this variability is unknown. The endogenous p53 status does not correlate with chemosensitivity to HSV TK/GCV treatment [132]. Sometimes GCV-resistant tumors develop after functional loss of the TK gene [133–135], but in other cases this process does not appear to reflect the level of HSV TK expression [136]. It was suggested that repair of GCV incorporated in DNA may be a factor involved in GCV resistance and that the repair enzyme β-pol exerts protection of cells against the cytotoxic and genotoxic effects of GCV [137]. Therefore, altered expression of β-pol during carcinogenesis [138] could be responsible for the resistance variability.

It was reported that the multidrug resistance protein MRP4, a member of the ATP-binding cassette superfamily [139], conferring resistance to purine-based antiretroviral agents may be responsible for GCV resistance. Cells overexpressing MRP4 had markedly increased resistance to the cytotoxicity of GCV [140].

These data lead to several recommendations on rational use of the HSV TK/GCV system:

• To avoid *tk* gene inactivation, one should preferentially use vectors that do not integrate into the cell genome like adenoviral or non-viral type instead of retroviral or lentiviral vectors.

• To diminish repair dependent variability in the GCV sensitivity, it is desirable to enhance the *tk* gene expression as much as possible in order to saturate the reparation system and enforce cells to choose apoptotic death.

• Finally, to avoid the MDR mediated GCV resistance, it is possible to inhibit MDR genes via RNA interference by inserting into the vector the corresponding shRNA encoding DNA together with the *HSVtk* gene. Such an attempt with promising effect was reported recently [141].

UTILIZATION OF TWO ENZYME/PRODRUG COMBINATIONS

An evident way to overcome the GCV resistance is the use of HSV TK/GCV genetic surgery with another enzyme/prodrug combination. One of the promising complements is the bacterial or yeast cytosine deaminase (CD) gene, associated with the prodrug 5-fluorocytosine (5FC), which also is a most widely used suicide system in gene therapy [69, 84, 85, 106]. Cytosine deaminase (CD) is found in bacteria and fungi, but not in mammalian cells. It catalyzes the deamination of cytosine to uracil and ammonia. Both bacterial and yeast CD deaminate 5-FC, a low-toxic drug used in human medicine to control fungal infections, to 5-fluorouracil (5-FU), a chemotherapeutic agent with radiosensitizing properties used to treat carcinomas in humans. 5-FU is then either converted via a multistep process to 5-fluoro-2'-deoxyuridine-5'-monophosphate (5-FdUMP), an irreversible inhibitor of thymidylate synthase, or phosphorylated to 5-fluorouridine-5'-triphosphate (5-FUTP). Thymidylate synthase inhibition by

5-FdUMP results in thymidine starvation, which subsequently prevents DNA synthesis. 5-FUTP can be incorporated into RNA in place of UTP, resulting in the inhibition of nuclear processing of rRNA and mRNA. In addition, the precursor of 5-FUTP, 5-FUDP, can be further metabolized to 5-FdUTP, which is subsequently incorporated into nascent DNA, resulting in DNA damage. There are also other ways of 5-FU catabolism but the reader can find them in the above cited reviews. Recent studies on the problems of efficiency and resistance to the treatment with either HSV TK or CD systems suggest that TK/GCV and CD/5-FC-induced apoptosis does neither require p53 nor death receptors, but converges at a mitochondrial pathway triggered by different mechanisms of modulation of Bcl-2 proteins [142]. GDEPT using CD also involves a strong 5-FU mediated bystander effect, and, since 5-FU can readily move out of and into cells by non-facilitated diffusion, direct cell to cell contact and functional GJIC are not prerequisites for this process. There are data indicating that besides the 5-FU-mediated bystander effect, a distant bystander effect involving natural killer cells as the major immune component contributes to the success of this therapy [85].

Clinical trials aimed at evaluation of the CD/FC system therapeutic potential are underway.

ONCOLYTIC VIRUSES CARRYING SUICIDE GENES

One of the ways to potentiate genetic surgery is to combine both main lines of its application, oncolytic viruses and enzyme pro-drug suicide gene therapy. Some examples of such an integration were reported. For example, a prostate-restricted replicative adenovirus (PRRA) AdIU1 armed with the herpes simplex virus thymidine kinase has been developed. In vitro, the viability of a prostate cancer cell line, CWR22rv, was significantly inhibited by treatment with the virus plus GCV. In vivo assessment of AdIU1 plus GCV treatment revealed a stronger therapeutic effect against CWR22rv tumors in nude mice than treatment with AdIU1 alone. These results demonstrate the therapeutic potential of specific oncolysis and suicide genetic surgery [143].

VECTOR TARGETING AND PATHOTROPISM PRINCIPLE IN TREATMENT OF TUMORS AND METASTASES

A major obstacle that limits the potential of human gene therapy and genetic surgery is the inefficiency of gene delivery to appropriate sites in vivo. The specificity can be markedly enhanced when tumor targeting approaches are used. Tumor cells and tumor environment, e.g. vasculature, both offer specific molecular targets that can be utilized for site directed delivery of therapeutic genes. Targeting has been intensively reviewed in [144–150]. Viral vectors, which usually do not have a natural tropism for tumor tissue, were gener-

ated to carry tumor targeting molecules on their surface. Targeting to receptors unique to or overexpressed on tumors was discussed above in the part devoted to oncolytic viruses. Synthetic gene delivery vectors based on cationic lipids or cationic polymers were biochemically modified to incorporate ligands specific for tumor cells or tumor vasculature.

As solid tumors exceeding a certain size rely on blood supply, the administration of particulate gene delivery vectors via the bloodstream is a promising concept [149]. An approach to the therapy of solid tumors which looks attractive is to attack the endothelial cells of the tumor vascular system rather than the tumor cells themselves, which circumvents the problem of poor penetration into tumor masses because the endothelial cells are freely accessible through the blood whereas the tumor cells are, for the most part, inaccessible. Also, endothelial cells are similar in different tumors, making it feasible to develop a single reagent for treating numerous types of cancer [151, 152].

Principle of Pathotropism

One of the widely accepted characteristics of tumors and their metastases is wound like features of their host environmental tissues, e.g. incessant angiogenesis. This observation has led Dvorak [153] to characterize tumors as "wounds that do not heal".

Angiogenesis is associated with remodeling of subendothelial extracellular matrix components, resulting, in particular, in the appearance of newly exposed collagens that normally do not contact blood. Exposure of subendothelium initiates several events including platelet deposition mediated by the interaction of the glycoprotein (GP) Ib-V-IX complex with von Willebrand factor (vWF) immobilized on exposed collagens [154–157]. The structural peculiarities of the "wounds" can be used to target vectors, be they viral or nonviral by their nature. Such targeted to wound-like lesions vectors can be considered "pathotropic" [158–165].

The capacity of vWF to bind exposed collagen (http://en.wikipedia.org/wiki/Von_Willebrand_factor#Function) have been used for construction of targeted vectors delivering genes to vascular lesions in sites of tumors and metastases. The Moloney murine leukemia virus (MoMLV) envelope (env) protein was engineered to incorporate a high-affinity collagen-binding domain derived from vWF. The chimeric virions were capable of binding collagen matrices, and retained their infectivity [158]. When administered by portal vein infusion, vector particles accumulated in the angiogenic tumor vasculature within 1 hour of infusion [159, 160, 164, 166]. The "pathotropic targeting" introduces a new paradigm in cancer gene therapy. An exposed collagen targeted vector carrying a dominant mutant cyclin G1 gene as a therapeutic gene, aimed at the suppression of the cyclin G1 cellular function, was referred to as Rexin-G. The ability of Rexin-

G to reach and safely impact metastatic disease was demonstrated in preclinical and clinical trials [161–165].

CONCLUSIONS

Animal experiments provided an enormous amount of data that cancer *gene therapy* targeted at certain genes or proteins incorporated in a cellular functional modules which seem to be crucial for cancer development, might be an efficient way for cancer treatment. However, the ongoing clinical trials proved only the safety of these treatment modalities, but no significant increase in the survival of cancer patients. The development of new vector systems and other improvements of techniques within the strategy may certainly give new, additional opportunities for more successful clinical applications. But being in itself a version of the targeted therapy, the gene therapy strategy will never reach high levels of versatility and will be always restricted to the cancer type, the patient's genetic variability, and the intratumoral heterogeneity, and thus will never exclude selection for cells resistant to the treatment with consequent growth of secondary resistant tumors.

On the other hand, the strategy referred to here as *genetic surgery* is directed at killing the tumor as a whole, irrespective of delicate mechanisms of its initiation and evolution. Two implementations of such a total killing strategy were outlined here: (i) oncolytic viruses and (ii) gene-directed enzyme prodrug therapy (GDEPT).

Despite the ingenuity of the OV usage for cancer cell killing and their capacity of spreading across the whole tumor, this approach suffers from the same limitations as gene therapy: it is based on certain molecular changes characteristic of cancer but not of normal cells. Inasmuch as different cancer types have different characteristic changes and each of them represents an evolving heterogeneous cell population, this approach is neither universal (that is not applicable to all types of cancers) nor having a chance to completely annihilate even a certain tumor, because it also leaves intact some portion of tumor cells resistant to OV. In addition, the technologies based on this approach are expected to be rather cumbersome and expensive. However, the approach will hopefully bring considerable improvement in modern lines of battle with cancer.

GDEPT seems the most suitable for the role of a universal and merciless killer of cancerous tumors and metastases, provided that it is targeted at continually replicating DNA—the only general cancer cell feature shared by all types of cancers and by all cells within a tumor that actually serves as the driving force for tumor development. The HSV TK/GCV pair is a paradigm of such an approach, another perspective pair is CD/5-FC. It will certainly take much time and effort to translate the idea to practical clinical technologies. But "Paris is worth a mass". The trajectories of improvements are beginning to emerge, and the final clinical tool is supposed to possess the following features:

- Ability to direct interruption of DNA replication
- Strong bystander and distant bystander effects
- Non-viral vectors
- Systemic delivery
- Pathotropic behavior.

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