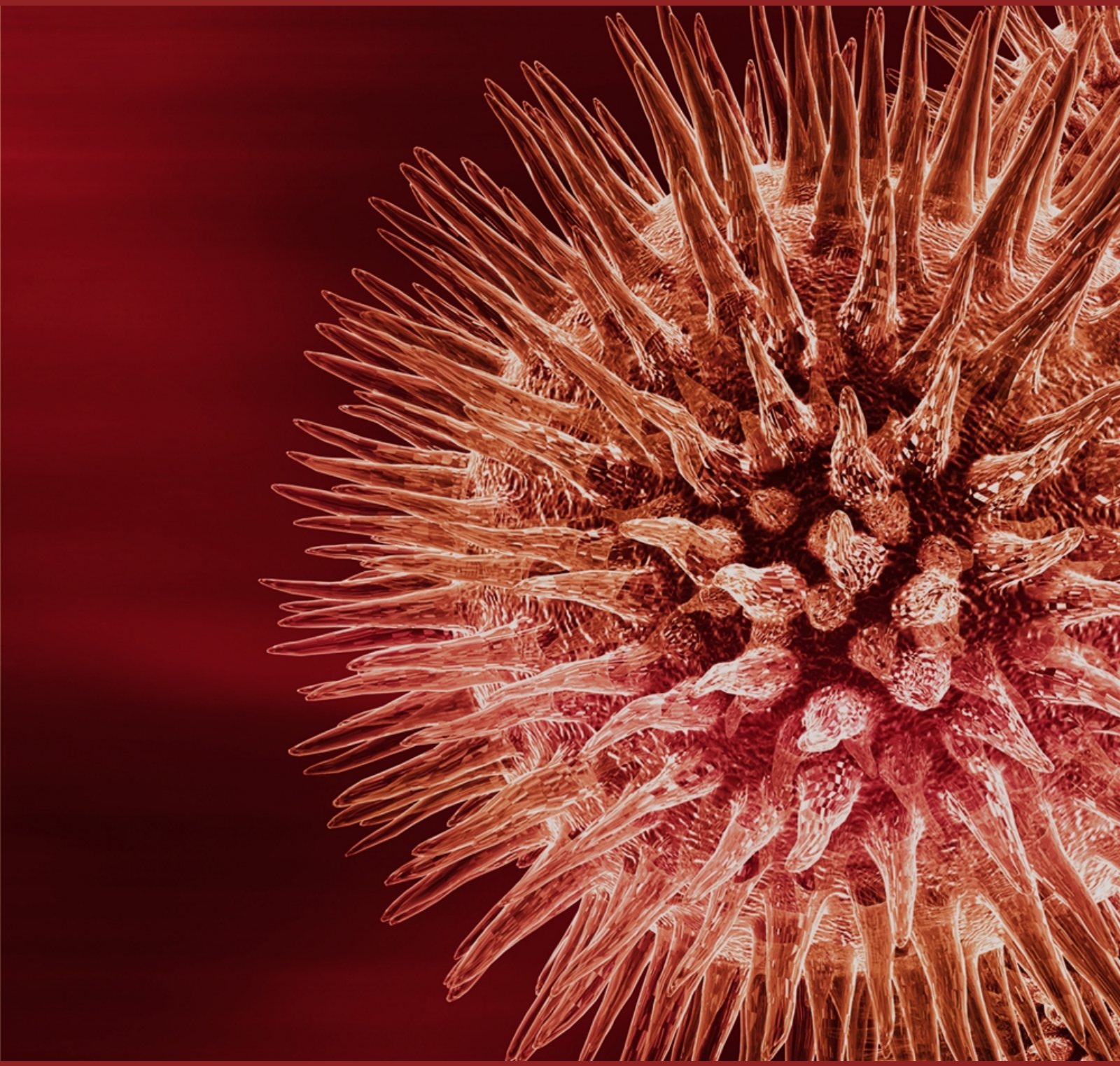
Journal of Biomedicine and Biotechnology

Gene Therapy—Part I

Guest Editors: Nicol Keith and Claude Bagnis



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EDITORIAL

Why a Special Issue on Gene Therapy?

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Going both from the organism to the DNA and from the DNA to the organism, the biology of the 20th century has paved the way for a new type of biologist who has emerged from the laboratory during the last two decades: the gene therapist. At the beginning of the nineties, much of this young field was based on a relatively simple concept: a deficient function can be replaced with an artificial gene. For twenty years now, gene therapists have been learning how wide the gap is between a concept and its practical reality. However, according to the increasing numbers of papers published which relate to gene therapy, and the huge interest generated by meetings of the American or the European Society of Gene Therapy, we are certainly learning a lot.

So, what have we learnt? Firstly, biotechnology is the key to link the bench to the bed of the patient. The biotechnology industry is ideally placed to deal with the concept of biotherapeutics and the range of manufacturing issues including good manufacturing practice (GMP), safety, toxicology, product scale-up to generate large batches of vectors, and testing new gene transfer tools. The close contacts between biotech companies and the development of gene therapy highlight the technological aspects of gene therapy. Indeed, delivery of the gene therapy has emerged as essential area for improvement. Gene delivery is a major growth industry with approaches ranging between almost-naked DNA to miniviruses and viral vectors gutted of most if not all of the viral sequences of their original genome. In addition, there has been increased and imaginative use of unexpected viruses as gene transfer agents, as highlighted by N. Maitland et al in their review of baculovirus to transfer therapeutic genes. Where is the universal vector everybody was running after 10 years ago?

We have also learned that between the needle and the arm of the patient there is a world called "Biosafety." There is no "zero risk" when considering a biotherapeutical strategy. In addition, gene therapy is a complex biological issue which generates specific risks as recently evidenced during the treatment of genetic diseases with adenoviral and retroviral vectors. It can be suggested that gene therapy is not toxic because gene therapy is not efficient. This opinion may hold some truth, but the use of increasing doses, the use of more complex strategies, and the use of more potent genetic activities is now leading us to reach the borderline between safety and toxicity making the biosafety a major determinant in the future of this therapeutical approach. Biosafety has to consider both the design of a gene transfer product and the possibility for this product to bypass the safety keys set up by the investigator to avoid the induction of unexpected deleterious effects. In this respect, in this two-part special issue T. Robson and D. G. Hirst (part II) focus on transcriptional targeting and A. G. Schätzlein (part II) focuses on the targeting of synthetic gene delivery. These reviews discuss one of the most important aspects of biosafety: the possibility to restrict the expression of the gene of interest to the selected tissue. In addition, A. Van den Broeke and A. Burny (part I) remind us that gene transfer vectors have to be considered in appropriate animal models by taking advantage of an elegant sheep model that allow us to revisit the concept of retroviral vector stability and recombination potential.

We learned that there is not always a simple solution. Cardiovascular disease is a complex multiparameter disorder. However, innovative biological therapies as discussed by K. L. Dishart et al (part II) highlight some very exciting advances in the field of cardiovascular disease. It will be of interest over the next few years to analyze whether clinical results will reach the expectations in this domain bearing in mind the lessons of gene therapy for cancer. The treatment of cancer, makes up almost 60 to 70% of the gene therapy trials worldwide, and includes some very elegant approaches (reviewed by D. H. Palmer et al and by S. M. Scholl et al (part I)). Nevertheless, gene therapy for cancer remains a challenging issue to address despite 15 years of effort and experience as exemplified by the gene therapy of malignant glioma (T. Kanzawa et al (part I)) and pediatric cancer (E. Biagi et al (part I)). Improvements however, can be reassured through our understanding of tumour biology and advances in chemistry. Examples of such improvements involve the synergy

between therapeutical strategies (R. J. Mairs and M. Boyd (part II)), the use of cascade mechanisms such as strong bystander effects (W. A. Denny (part I)), or the boosting of the immune system (S. M. Scholl et al (part I)). The possibility to target the expression of a gene at the desired time and location as reviewed in this issue will obviously help us to improve the therapeutic impact of these approaches.

Measuring the success of a new therapy is a challenge in itself. The traditional criteria used to evaluate cancer therapies may not be applicable to molecularly driven therapeutics. As we enter the era of mechanism-based therapeutics, it will be essential to show that the new genetic therapies reach their desired target and interacts with the target in a specific fashion. An essential part of this process is therefore the development of new approaches to visualize the localization of the transgene and the extent of its effects. The green fluorescent protein or the bacterial beta-galactosidase encoded by the *LacZ* gene gave some insight to product localization. But moving into animal models highlights the need for new detection technologies such as high sensitivity detection of light and PET imaging. These advances in noninvasive imaging in vivo are discussed by G. Vassaux and T. Groot-Wassink (part II). This is an emerging field which faces the challenge of detecting and mapping a few if not single cells, in a 3D context in the next two decades. A nice bet.

Finally, it is encouraging that individual groups worldwide are addressing local requirements to ensure the progress of fledgling genetic therapies into the clinic. However, it is becoming clear that a greater cooperation and unification of regulatory procedures would enhance the prospects of successful clinical trials for gene therapy. Amati et al discuss how this might be achieved within Europe; a major but worthwhile task.

In summary, we learned a lot over the past twenty years. It is a particularly exciting time to be involved in gene therapy. There has been immense progress in the field and a realistic expectation that gene therapy will make a difference to the patient. However, we still have much to learn and this is one of the goals of this two-part special issue on gene therapy of the Journal of Biomedicine and Biotechnology.

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Claude Bagnis is the Head Scientist of the Department of Cell and Gene Therapy of the Etablissement Français du Sang Alpes Méditerranée in Marseille, France. His projects mainly focus on therapeutic gene transfer in the field of oncology both considering hematopoietic cells or solid tumor cells as targets and retroviral vectors as gene transfer tools. He started his works in 1986 with avian leukemia virus-derived vectors in the École Normale Supérieure of Lyon before he joined in 1991 the Centre Régional de Lutte Contre le Cancer in Marseille, where he switched to murine leukemia virus-derived retroviral vectors as a tool to genetically modify human hematopoietic stem cells with marker genes in the clinical context. Since 2001, as the Head Scientist of a task group dedicated to the involvement of the Etablissement Français du Sang in the onset of clinical trials using the viral vector technology, Claude Bagnis is mainly developing the use of human immunodeficiency virus-derived vectors to transfer killer genes such as prodrug-dependent suicide genes, proapoptotic genes, or immune response inducers for cancer gene therapy strategies.

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REPORT

EMEA and Gene Therapy Medicinal Products Development in the European Union

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The evaluation of quality, safety, and efficacy of medicinal products by the European Medicines Evaluation Agency (EMEA) via the centralized procedure is the only available regulatory procedure for obtaining marketing authorization for gene therapy (GT) medicinal products in the European Union. The responsibility for the authorization of clinical trials remains with the national competent authorities (NCA) acting in a harmonized framework from the scientific viewpoint. With the entry into force of a new directive on good clinical practice implementation in clinical trials as of 1 May 2004, procedural aspects will also be harmonized at EU level. Scientifically sound development of medicinal products is the key for the successful registration of dossiers and for contributing to the promotion and protection of public health. The objective of this paper is to introduce the EMEA regulatory processes and scientific activities relevant to GT medicinal products.

INTRODUCTION TO THE EMEA AND THE EU MEDICINES NETWORK

The legal basis for the operation of the EMEA (European Medicines Evaluation Agency) was established by the Council Regulation 2309/EC adopted by the European Council of Ministers [1] in July 1993.

The main objective of the EMEA mission is to contribute to the protection and promotion of public and animal health by

(i) mobilizing scientific resources from throughout the European Union to provide high quality evaluation of medicinal products, to advise on research and development programmes and to provide useful and clear information to users and health professionals;

(ii) developing efficient and transparent procedures to allow timely access, by users, to innovative medicines through a single European marketing authorization;

(iii) controlling the safety of medicines for humans and animals, in particular through a pharmacovigilance network and the establishment of safe limits for residues in food-producing animals.

The EMEA is a technical agency acting as a central point of coordination of the existing scientific resources in the European Union (EU) for the regulatory and scientific evaluation of medicinal products both for human and veterinary use [2, 3, 4]. The operations started in January 1995. The main tasks carried out by the EMEA can be grouped in three categories:

(1) regulatory and scientific contribution to the life cycle of medicinal products,

(2) harmonization of technical requirements for marketing authorization at EU and international level,

(3) support to the European Commission on policy matters relevant to medicinal products.

Moreover, the agency is responsible for the efficient running of the operations at EU level including development and maintenance of the necessary IT tools (such as databases) and networks to support the communication and coordination among the stakeholders.

New legislation adopted since 1995 has added a number of responsibilities, in the context of orphan designation, with the creation of the Committee for Orphan Medicinal Products (COMP) [5]. Other tasks relate to the conduct of clinical trials, as well as pharmacovigilance activities for medicines for veterinary and for human use, including the design and the operating of various systems, such as a clinical trial database and the EudraVigilance database for the transmission of adverse drug reactions [7, 8].

The scientific competences of the EMEA on medicines for human use, are based within its scientific committees, the Committee for Proprietary Medicinal Products (CPMP), which is responsible for evaluating quality, safety, and efficacy and the COMP, which is responsible

for the evaluation of requirements for the designation of the orphan medicinal product status.

THE EMEA IN THE LIFE CYCLE OF GENE THERAPY MEDICINAL PRODUCTS

Regulatory framework

Whilst the EMEA has no remit for the authorization of the conduct of clinical trials in the EU, it is agreed that a scientifically sound development is necessary in order to demonstrate the efficacy of the product and to exclude any major safety problem incompatible with the safe use of the product.

GT products must be authorized through the centralized procedure [1]. The application for marketing authorization (MA) must be made to the EMEA as described in the Notice to Applicants [9]. At the conclusion of the scientific evaluation undertaken in 210 days, the opinion of the scientific committee is transmitted to the European Commission to be transformed into a single marketing authorization applying to the whole European Union.

At present, the presentation of the MA application dossier for GT products must fulfil the same administrative and scientific requirements as for any other medicinal product as laid down in the legislation [6]. These include requirements relating to establishments, in which GT medicinal products are manufactured [10], and considerations on the environmental impact of the use of gene transfer products on the deliberate release of genetically modified microorganisms. At the level of marketing authorization applications, the CPMP will ensure that all appropriate measures are taken to avoid adverse effects on human health of the environment, which might arise from the deliberate release of placing on the market genetically modified organisms (eg, genetically modified viruses acting as gene transfer vectors).

The environmental impact of the use of gene transfer products as far as their contained use is concerned, has implications for the authorization and conduct of clinical trials as they may present biosafety issues related to intrinsic safety properties and also the safe handling in relation to environments and the wider human population. These aspects are taken into consideration by the competent authorities in member states when authorizing the conduct of clinical trials.

Guidelines and scientific advice

Technical requirements useful for scientifically sound development of GT products are established at EU level as reference tool not only for sponsors but also for the bodies in charge of the authorization for conducting clinical trials within the member states, and for the final premarketing regulatory evaluations.

One of the tasks of the EMEA is to advise companies on the conduct of the various tests and trials necessary to demonstrate the quality, safety, and efficacy of medicinal products.

The advice is generally given in the form of guidelines or scientific advice on individual products. The guidelines are produced with the support of specific CPMP working groups, such as the Biotechnology Working Party, the Safety Working Party, the Efficacy Working Party, and the ad hoc group on GT. Relevant guidance documents are also produced within the framework of the International Conference for Harmonization (ICH)—see "Future Developments" section.

Since gene transfer products contain genetic and other materials of biological origin, many of the quality considerations for recombinant DNA (rDNA) products and other biologicals manufactured by modern biotechnological methods will apply to the manufacture of gene transfer products.

Biotechnology guidelines are made available on the EMEA web page, http://www.emea.eu.int.

In order to address the specificities of GT, the CPMP has developed a multidisciplinary note for guidance, which was adopted in April 2001 [11]. The objective of the CPMP note for guidance is to provide recommendations and assistance in generating data on quality, nonclinical, and clinical aspects of gene transfer medicinal products, intended to support marketing authorization applications within the European Union. The current version maintains the focus on general aspects whereas guidance on specific therapeutic applications is addressed in other relevant documents. Experts regularly involved at national level in the evaluations concerning the clinical trials authorization, provide the scientific contribution on the issuance of such guidelines. Further input from interested parties is then provided during the consultation period, which is the usual procedure undertaken by the EMEA before finalising the regulatory recommendations.

As for any new technology, a flexible approach for the control of these products is being adopted so that recommendations can be modified in the light of experience gained from production and use, and from further developments.

Whilst the recommendations set out in the GT guideline should be considered to be generally applicable, individual products may present specific quality control and safety concerns, for example, as in the case of DNA vaccines intended for prophylactic use in a large number of healthy individuals.

The production and control of each product will be considered on a case-by-case or product-specific basis reflecting the intended clinical use of the product.

As regulatory experience with this treatment modality is still growing, in order to address product specific issues and more generally scientific issues not covered by or deviating from existing guidance, the CPMP is able to provide Scientific Advice to Applicants on quality, nonclinical and clinical aspects of their development program. The CPMP scientific advice is made available to the sponsors on request. The scientific advice is not binding for the sponsor or for the CPMP, but deviations from the advice

may need to be justified and discussed at the time of the evaluation of the dossier for marketing authorization. Details of the procedures are provided on the EMEA web page.

The request for scientific advice may be put forward to the EMEA at any stage in the development of a medicinal product. Presubmission and briefing meetings with the sponsors are also organised at the EMEA to streamline the preparation of the dossier for the available regulatory procedures. So far, out of more than three hundred scientific advices provided on development, four sponsors developing GT products have used this procedure.

This reflects the early stage of development of this innovative approach more often carried out in academic institutions as research initiatives than by the pharmaceutical industry.

The national authorities experts responsible for the approval and supervision of GT provide their input to the EMEA scientific advice process. The authorization of clinical trials in Europe takes place at national level. In the USA, all clinical trials are submitted for central authorization to the FDA through the IND mechanism.

Only very few candidate products have a development plan oriented towards regulatory procedures ultimately leading to the EU-wide issuance of a marketing authorization. Sixteen percent of GT clinical trials are conducted in EU and 80% in USA; more than 87% of worldwide GT clinical trials are in an early development (phase I–II) [12].

The GT scientific advices issued so far have addressed specific aspects of phase III clinical trial design, and also the design and adequacy of nonclinical development strategy. The medicinal products concerned were in all cases viral vectors (adenoviral or retroviral vectors) for therapeutic applications in oncology, cardiovascular diseases, rare Mendelian conditions, and infectious diseases.

It is expected that in the future, more requests for scientific advice will be lodged by sponsors, taking into account the potential opened up by the reading and annotation of the human genome, the refinement of vector designs and types and the progress of GT research from the early clinical phase into Phase II–III trials.

Orphan medicinal product designation and procedures

The EU legislation on orphan medicinal products provides additional support for the development of those GT product intended for the treatment of rare and serious diseases [5]. The EMEA within the COMP has established a procedure for providing the scientific basis for the orphan medicinal products designation [13].

Orphan designation can be obtained for medicinal products intended for human use in specific medical conditions following submission of a valid application to the EMEA. Opinions on orphan medicinal products designations are based on the following cumulative criteria: (i) the seriousness of the condition, (ii) the existence or not of alternative methods of diagnosis, prevention, or treatment, and (iii) either the rarity of the condition (considered to affect not more than five in ten thousand persons in the community) or the insufficient return of development investments.

The EU supports research, development, and marketing of orphan medicinal products, by providing incentives such as protocol assistance and scientific advice from the EMEA, direct access to the Centralized Procedure, fee reduction for centralized applications, priority access to EU research programs, and ten years of market exclusivity from the date of marketing authorization.

So far, three GT medicinal products have been designated as orphan medicinal products and for one of them a protocol assistance process has been initiated in 2002, encompassing scientific advice on development as well as regulatory advice.

Summaries of the designated orphan medicinal products are now made available to the public on the EMEA website as of 2002. The outcomes of the EMEA activities in the field of orphan medicinal products are regularly updated and made available on the EMEA web page within the COMP press releases.

Marketing authorization

Up to date no marketing authorization has been issued for GT medicinal products.

Under the centralized procedure, applications are made directly to the EMEA, in charge of coordinating the core operations. Independent assessment of the data on quality, safety, and efficacy is carried out by designated evaluation teams located within the national competent authorities. A subsequent scientific peer review process is carried out by the EMEA scientific committees. The outcome of the procedure is a CPMP scientific opinion, which is either favourable or unfavourable. This is communicated to the European Commission, which in turn is responsible for converting CPMP opinions into legally binding decisions (eg, the granting of a marketing authorization in case of a favourable opinion). For every favourable CPMP scientific recommendation to the European Commission on the granting of a marketing authorization, a comprehensive European public assessment report (EPAR) reflecting the scientific review and conclusion of the CPMP is published on the EMEA web page for information to the public. The EPAR excludes commercially confidential information. The outcomes of the EMEA centralized operations since January 1995 till June 2002 are summarized and updated regularly in the CPMP monthly reports and in the annual reports available on the EMEA website.

FUTURE DEVELOPMENTS

The International Conference on Harmonization of technical requirements for registration of pharmaceuticals and the CPMP GT expert group

The International Conference on Harmonization (ICH) was initiated in April 1990 in Brussels and is a joint

initiative involving six parties, in scientific discussions of the technical requirements to be satisfied to ensure quality, safety, and efficacy of medicinal products.

The six members of ICH are the regulatory bodies of the USA (Food and Drug Administration—FDA), EU (European Commission—EMEA), Japan (MHLW), the relevant federations of research based pharmaceutical industry, namely, the Pharmaceutical Research and Manufacturers of America (PhRMA), The European Federation of Pharmaceutical Industry (EFPIA), and the Japanese Pharmaceuticals Manufacturers Association (JPMA). The observers are representatives of the World Health Organisation (WHO), European Free Trade Association (EFTA), and Canada.

The objective of the ICH exercise is to promote international harmonization of the requirements for registration of pharmaceuticals among the three regions EU, USA, and Japan so that medicines are developed and made available in a timely and efficient manner to promote public health, preventing unnecessary duplication of clinical trials in humans, and minimizing the use of animal testing without compromising safety and effectiveness. More information on the ICH activities can be found at the ICH website, http://www.ich.org/ich1bis.html.

In May 2001, in the context of the ICH, the status of the regulatory guidance documents for GT medicinal products already issued and available in the three regions (EU, USA, and Japan) was discussed.

While there are differences in the format of the documents, the underlying scientific principles are not fundamentally different. For this rapidly evolving area, there is a need to continue fostering the exchange of information amongst the regions particularly in relation to emerging scientific findings, promoting harmonized scientific understanding.

Areas of scientific importance that warrant the attention of ICH as a matter of priority, are the need to review the dose definition and standardization of viral vectors, to facilitate the comparison of dose-related findings in nonclinical studies and to facilitate a better understanding of clinical data.

Of importance is the collaborative work, involving industry, academia, and FDA, that is being conducted at laboratory level to establish wild-type virus reference standard materials for adenovirus Type 5 and adeno-associated virus. A reference standard for a retrovirus is currently available. Plans to develop other standards will be considered. The results of the scientific review on GT standardization approaches and on new emerging GT technology, to be conducted at regional level may be shared in the framework of ICH in the form of scientific workshops. The first one took in September 2002 in Washington DC.

In order to optimize the review of GT developments, the CPMP established and ad hoc expert group on GT. The expert group avails itself of multidisciplinary competence from the CPMP working groups (Biotechnology Working Party, Efficacy Working Party, Safety Working Party, Scientific Advice review group) and external expert on a case-by-case basis depending on the topics on agenda.

The group provides the CPMP with updates on current development in the area of GT research, and gives advice on the trends of such developments, and recommendations for producing technical guidelines in the form of supplementary explanatory notes or position papers when required. The creation of the group was considered important for ensuring that the CPMP guidelines are up-to-date and reflect the state-of-the-art of the technology and to prepare EU scientific positions in such a rapid evolving field.

Within the ICH activities, the group will provide substantial contribution to scientific workshops and public debates.

The scientific reports of the group are made available on the EMEA web page thus keeping the interested public up to date with the EU scientific and regulatory position (eg, http://www.emea.eu.int/pdfs/human/genetherapy/584302en.pdf).

EU directive on clinical trials

The recently approved "GCP directive" addresses a number of fundamental issues relating to the implementation of GCP in clinical trials. The directive is applicable to all clinical trials (phase I–IV) conducted in the EU [14].

Sponsors are required to obtain an authorization from the competent authority of the member state(s) in which the clinical trial is going to be conducted. For most drugs, this can be granted implicitly, if the authority concerned has not informed the sponsor of any grounds for nonacceptance, within a maximum of 60 days. However, written authorization is necessary for trials involving medicinal products intended for GT, somatic cell therapy including xenogenic cell therapy, and all medicinal products containing genetically modified organisms (GMOs). Similarly, whilst ethics committees are required to give a reasoned opinion within a maximum of 60 days for the purpose of commencing a trial, for GT, somatic cell therapy, or medicines containing GMO's, an extension of 30 days will be permitted with a further 90 days in the event of consultation of an expert group. In the case of xenogenic cell therapy, there will be no time limit to the authorization period. No GT trials may be carried out which result in modifications to the subject's germ line genetic identity.

The legislation also establishes the basis for exchange of full information on the conduct of any clinical trial between the national health authorities, the EMEA, and the European Commission through the creation of a comprehensive European clinical trials database.

The GCP directive is about to bring important changes to harmonize the key legal requirements and procedures for conducting clinical trials on medicinal products for human use in the EU. Member states are required

to implement the directive into national legislation by May 2003 and the directive is to become effective as of 1 May 2004.

CONCLUSIONS

During the first seven years of EMEA operation (1995–2002), a number of initiatives have been set up in order to facilitate the dialogue on the development of medicines, and to streamline relevant regulatory procedures.

Only a very limited number of GT medicinal products have been submitted through the EU network and this reflects from one side the ongoing efforts in research, and on the other side the status of early development in the clinical applications of GT. Scientific advice has been provided for GT products intended for the treatment of oncology conditions, immunological disorders, and infectious diseases. The newly established procedure on orphan designation has provided incentives and advice to drug development for rare diseases.

The CPMP has mobilized important scientific resources in order to meet the regulatory challenges of this rapidly evolving field. State-of-the-art scientific advice has been provided on a case-by-case basis. Moreover, in order to keep abreast with the current advances and needs in the GT field, the CPMP has established an ad hoc expert group to provide additional recommendations not only at EU but also at international level. CPMP GT guidelines will be updated or supplemented with explanatory notes to reflect the progress in experience.

On the basis of the exchange of information and experience at ICH level, the group might provide suggestions for future recommendations, position papers, or statements of principle for certain specific topics.

The coming in to force of a new EU legislation to regulate clinical trials authorization and the establishment of the European database for clinical trials are foreseen to provide further opportunities for cooperation in the development of GT medicinal products.

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COMMENTARY

Retroviral Vector Biosafety: Lessons from Sheep

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The safety of retroviral-based systems and the possible transmission of replication-competent virus to patients is a major concern associated with using retroviral vectors for gene therapy. While much effort has been put into the design of safe retroviral production methods and effective in vitro monitoring assays, there is little data evaluating the risks resulting from retroviral vector instability at post-transduction stages especially following in vivo gene delivery. Here, we briefly describe and discuss our observations in an in vivo experimental model based on the inoculation of retroviral vector-transduced tumor cells in sheep. Our data indicates that the in vivo generation of mosaic viruses is a dynamic process and that virus variants, generated by retroviral vector-mediated recombination, may be stored and persist in infected individuals prior to selection at the level of replication. Recombination may not only restore essential viral functions or provide selective advantages in a changing environment but also reestablish or enhance the pathogenic potential of the particular virus undergoing recombination. These observations in sheep break new ground in our understanding of how retroviral vectors may have an impact on the course of a preestablished disease or reactivate dormant or endogenous viruses. The in vivo aspects of vector stability raise important biosafety issues for the future development of safe retroviral vector-based gene therapy.

Vectors derived from retroviruses are the most extensively used vehicles for gene transfer (reviewed by Hu and Pathak [1]). In this respect, retroviral vectors display a number of unique features including the absence of viral gene expression and the capacity to integrate into the host genome, a prerequisite for permanent gene transfer in a number of applications. The safety of retroviral-based systems, however, and the possible transmission of replication-competent virus to patients is a major concern associated with using retroviral vectors for gene therapy.

It is well documented that retroviruses display high recombination and mutation rates [2, 3]. Replication-competent viruses (RCR) can be generated through recombination between the vector and either the helper constructs required for vector production or endogenous retroviruses present in the host genome. Both the host cell derived RNA polymerase II and the virus-encoded reverse transcriptase are incapable of exerting exonucleolytic proofreading activity and therefore contribute to the generation of genetically divergent retroviruses. The dimeric nature of the genome moreover allows template switching of reverse transcriptase during the copying of copackaged RNA molecules, leading to the generation of recombinant proviruses harboring information derived from both parental RNAs. For further information regarding the basic concepts of recombination, examples of recombinational reassortment and their impact on retrovirus evolution, we refer the reader to the reviews of Negroni and Buc [4] and Mikkelsen and Pedersen [5].

Because of these safety concerns, screening assays have been elaborated for testing the presence of RCR during production of retroviral vectors, in *ex vivo*-transduced cells and during patient follow-up [6, 7]. Much effort has been put into the design of safe retroviral vector production methods for generating helper free recombinant vectors through the development of systems with reduced homology between retroviral vector and packaging components, self-inactivating vectors, or split-genome conditional packaging systems [8, 9, 10]. Cell culture studies have provided examples of retroviral genetic changes introduced by recombination and there is extensive data demonstrating the impact of recombination during vector preparation [5, 11, 12, 13]. However, in vitro approaches currently used to monitor structural changes introduced in the integrated vector post-transduction are limited by the absence of host-associated processes that govern genetic changes and therefore do not fully mimic the complex in vivo situation.

There is very little data evaluating the dynamics of provirus structural modifications in vivo, and the risks resulting from retroviral vector instability at post-transduction stages are largely ignored, especially following in vivo gene delivery. The generation of mosaic retroviruses has been described in mice and primates [14, 15, 16], but to our knowledge, there is poor direct evidence of in vivo recombination that involves retroviral vector sequences and either exogenous or endogenous retroviruses. Human endogenous retroviruses (HERVs) are the proviral remnants of previous retroviral infections and make up as much as 8% of the human genome [17]. Most importantly, some endogenous retroviruses are capable of interacting with exogenous counterparts, including retroviral

vectors, through different mechanisms including recombination and transactivation [18]. Endogenous retroelements thus represent a significant source of functional viral sequences, which may serve as donors in recombination. In addition, proteins-provided in trans by retroviral vectors may lead to the spread of otherwise inactive endogenous retroviruses. This phenomenon may challenge the safe usage of retroviral vector-based gene vehicles.

Although recombination between MLV-derived vectors and human endogenous retroviruses may thus potentially happen, there are no examples of in vivo cross-species recombination, probably because these events require coinfection by viruses which normally replicate in distinct host organisms. This hypothesis was further supported by data from patients and co-workers, indicating that human retroviruses may be inefficiently recognized and packaged by the MLV assembly machinery [19]. However, in contrast with previous studies, recent data from our laboratory revealed unexpected in vivo interactions between distinct retroviral structures. Hereafter, we briefly describe and discuss our observations in an in vivo ovine model, providing evidence for in vivo recombination between a gibbon ape leukemia virus (GaLV)-pseudotyped MLV-derived retroviral vector and bovine leukemia virus, a leukemogenic complex retrovirus that infects cattle and sheep. The ovine in vivo experimental approach described here relies on the possibility to easily detect novel acquired biological properties resulting from recombination, thus providing a unique tool for studying the changes in integrated retroviral structures. Our observations address the biosafety issue associated with retroviral vector stability in vivo, a key parameter that may impact the therapeutic potential of retroviral vector-based gene therapy.

We have developed a retroviral vector-mediated gene transfer strategy in a model that was initially established for investigating bovine leukemia virus (BLV)-associated leukemia in sheep. BLV is a complex retrovirus closely related to the HTLV family of human retroviruses and the ovine leukemia model provides an excellent means for studying aspects of human complex retrovirus-induced diseases (reviewed in [20, 21]). In this highly reproducible model of BLV-induced lymphoid tumors, viral infection can be monitored after one week postinoculation, following injection of either naked provirus or virus particles produced in tissue culture.

The lack of viral expression in the ovine tumors has made it difficult to identify the discrete mechanisms by which BLV can mediate leukemogenesis [22, 23]. Tax, the transactivating transcriptional activator of viral expression, is believed to initiate early events in the multistep process leading to full malignancy, but its expression is not required to maintain the transformed phenotype. To gain insight into the mechanisms that govern virus silencing in BLV-induced tumors, we have studied YR2 leukemic B cells that contain a single, monoclonally integrated, silent provirus, which cannot be reactivated by either in vitro stimulation, or in vivo injection of the tumor cells or the cloned proviral DNA in sheep. Sequence analysis of the YR2 proviral *tax* gene identified two G to A transitions responsible for E to K amino acid changes at positions 228 and 303 of the Tax protein (309 aa) resulting in a transactivation-deficient phenotype. (GaLV)-pseudotyped pLTaxSN retroviral vector-mediated transfer of wild-type *tax* into YR2 cells [24] resulted in the production of BLV transcripts characterized by the parental mutations in *tax*, thus potentially leading to a defective provirus. Injection of sheep with transduced cells was then assayed to investigate the in vivo infectious potential of BLV particles induced by LTaxSN. $YR2_{LTaxSN}$ inoculation resulted in rapid seroconversion and lead to the in vivo rescue of replication-competent proviruses with unique chimeric *tax* genes, which arose from recombination between the transduced LTaxSN vector-derived wild-type *tax* and the YR2-derived *tax* sequences [25].

We furthermore found that while BLV-specific antibody titers rose over time in the majority of these inoculated animals, a limited number of sheep displayed weak and transient antibody responses that were no longer detectable three months postinoculation. Most interestingly, one of those seronegative animals converted to high serological titers 18 months postinoculation, indicating that recombinant infectious proviruses generated by recombination with retroviral vector sequences may survive and emerge long after the initial injection. Finally, recent observations in a $YR2_{LTaxSN}$-injected leukemic sheep revealed that a unique chimeric Tax-mutated replication-deficient provirus was integrated in the malignant B cell clone while recombinant functional provirus had been consistently monitored over the aleukemic period (Van den Broeke, unpublished observations, 2001). This observation strongly supports the hypothesis that switching off expression of Tax, an essential contributor to the oncogenic potential of BLV, is linked with the onset of acute leukemia.

In terms of BLV-associated leukemogenesis, our data provided clear evidence for the role of strategically-located mutations in retrovirus tumor-associated silencing, stressing the relevance of viral immunosurveillance escape mechanisms, and thus recombination, in the onset of the tumor. Most importantly, in terms of viral vector biosafety, our in vivo experimental approach based on the inoculation of retroviral vector-transduced tumor cells ($YR2_{LTaxSN}$) in sheep raised important questions regarding the in vivo stability of retroviral vectors, providing clear evidence for the presence of chimeric proviruses, as well as the long-term storage and subsequent selection of recombinant retroviral genetic information. Of particular interest was the emergence, as monitored by the full seroconversion, of a functional BLV provirus after an 18-month period of seronegative carrier state in one of our experimental sheep. This suggests that the in vivo generation of mosaic viruses is a dynamic process and that virus variants, generated by retroviral vector-mediated

recombination, may be stored and persist in the infected individual prior to selection at the level of replication. Finally, our findings suggest that there is a potential link between the modification of an integrated proviral structure and the emergence of pathology. In our model, retroviruses face changing selective forces favoring either increased viral fitness during the productive stage of the infection, or virus silencing during the leukemic stage of the BLV-associated disease. Thus, the system is actively driving recombination, generating retroviruses with altered functional capacities to fulfil the novel in vivo requirements. Reactivation restores full virulence. Silencing is likely to facilitate escape from immune responses and leads to full-blown malignancy. To our knowledge, this is the first example of a direct deleterious in vivo effect associated with vector-mediated recombination. In this particular case, the mechanism by which the immune system targets and destroys developing malignancies, is evaded by the pre-malignant cell by downregulating its intrinsic immunogenicity through recombination-mediated virus silencing.

These in vivo observations using a sheep model break new ground in our understanding how retroviral vectors may have an impact on the course of a preestablished disease or reactivate dormant or endogenous viruses. Recombination may not only restore essential viral functions or provide selective advantages in a changing environment but also re-establish or enhance the pathogenic potential of the particular virus undergoing recombination, such as the silencing-associated leukemogenicity studied as a model in our project. Our study furthermore stresses the limitations associated with retroviral vector-mediated gene delivery using current vectors in a host population infected with retroviruses (HIV, HTLV-I) or genomes that harbor endogenous retroelements. Although the exact mechanisms leading to the generation of mosaic retroviruses remain uncertain, our observations in sheep illustrate the largely ignored potential risks associated with retroviral gene transfer in humans. Because of the enormous selective pressures exerted in vivo, even theoretically rare events may pose a realistic safety concern. Further advancement in retroviral vector-mediated gene therapy and development of safe strategies for gene delivery must deal with the risks of in vivo recombination, generation of mosaic retroviruses, and long-term storage of viral genetic information. Future research will probably need to focus more on the in vivo aspects of vector biosafety and stability, a major concern with possible serious consequences to the therapeutic potential of retroviral vector based gene therapy.

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PERSPECTIVE

Gene Therapy for Pediatric Cancer: State of the Art and Future Perspectives

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While modern treatments have led to a dramatic improvement in survival for pediatric malignancy, toxicities are high and a significant proportion of patients remain resistant. Gene transfer offers the prospect of highly specific therapies for childhood cancer. "Corrective" genes may be transferred to overcome the genetic abnormalities present in the precancerous cell. Alternatively, genes can be introduced to render the malignant cell sensitive to therapeutic drugs. The tumor can also be attacked by decreasing its blood supply with genes that inhibit vascular growth. Another possible approach is to modify normal tissues with genes that make them more resistant to conventional drugs and/or radiation, thereby increasing the therapeutic index. Finally, it may be possible to attack the tumor indirectly by using genes that modify the behavior of the immune system, either by making the tumor more immunogenic, or by rendering host effector cells more efficient. Several gene therapy applications have already been reported for pediatric cancer patients in preliminary Phase 1 studies. Although no major clinical success has yet been achieved, improvements in gene delivery technologies and a better understanding of mechanisms of tumor progression and immune escape have opened new perspectives for the cure of pediatric cancer by combining gene therapy with standard therapeutic available treatments.

INTRODUCTION

A multidisciplinary approach combining surgery, chemotherapy, and bone marrow transplantation has led to a dramatic improvement in survival for pediatric malignancy over the past 20 years. Currently, overall 5-year survival rates are more than 75% for children younger than 15 years of age and 77% for 15–19 year olds [1]. But despite these advances in the treatment of pediatric cancer, a significant proportion of patients remain resistant to the standard therapeutic procedures. Moreover, the price of the cure is often unacceptable, and includes not just acute and chronic organ toxicity but most troublingly, an increased risk of secondary malignancy. Hence, new strategies are required to improve the overall survival rate and decrease treatment-related morbidity.

Gene therapy offers the prospect of efficient and highly specific therapy for childhood cancer, and the concept was initially welcomed by investigators and clinicians alike with great enthusiasm mixed with unrealistic expectations. Unfortunately, it has become evident that the complexities of childhood malignancy and the limitations of current gene transfer vectors mean that the successful application of gene transfer technologies for the cure of pediatric malignancy will be a gradual and progressive process over many years. Nonetheless, as we will describe here, gene transfer technologies are already successfully being applied to the treatment of childhood cancer and should increasingly benefit this patient group in the coming years.

Genes may be transferred to cells in vitro with subsequent transfer of the gene-modified cells to the patient, or transfer may take place directly to the target cells in vivo. The gene delivery system, or vector, generally consists of a specific DNA sequence and promoter that drives the expression of the transgene of interest, as well as a polyadenylation signal that stabilizes the specific messenger RNA. The vector usually takes the form of a modified virus, but synthetic nonviral vectors are playing an increasingly important role [2, 3, 4].

There are a number of ways in which these transferred genes may be used for the treatment of cancer. The most obvious, and perhaps the most intellectually appealing, is to transfer *corrective* genes that will help overcome the genetic abnormalities that have arisen in the precancerous cell and led to the malignant process. Alternatively, it is possible to introduce genes that will render the malignant cell sensitive to small molecules to which it might otherwise be resistant. The third approach is to attack the tumor blood supply with genes that inhibit vascular growth or function. The fourth is to leave the tumor unmodified but to alter normal tissues instead, so that they

Table 1. Advantages and disadvantages of vector systems.

Vector	Advantages	Disadvantages	Current uses
Murine retrovirus	Stable integration into dividing cells Minimal immunogenicity Stable packaging system	Low titer Only integrates in dividing cells Limited insert size Risk of silencing Risk of insertional mutagenesis	Marker studies Gene therapy approaches using hemopoietic stem cells or T cells, for example, to treat immunodeficiency syndromes Transduction tumor cell lines
Lentivirus	Integrates into dividing cells Integrates into nondividing cells Larger insert size than murine retroviruses	No stable packaging system Complex safety issues	No approved trials as yet
Self-inactivating lentiviral vectors (SIN-Lenti)	Incapable of replication post transfection → ? increased safety Stable packaging system	Safety concerns remain	No approved trials as yet
Adenovirus	Infects wide range cell types Infects nondividing cells High titers High level of expression Accepts 12–15 kb DNA inserts	Highly immunogenic Nonintegrating	Direct in vivo applications Transduction tumor cells
Adeno-associated virus (AAV)	Integrates into dividing cells Infects wide-range cell types	No stable packaging cell line Very limited insert size	Gene therapy approaches using hemopoietic stem cells
Herpesvirus	High titers Transduces some target cells at high efficiency Accepts large DNA inserts	No packaging cell lines Nonintegrating May be cytotoxic to target cell	Transduction tumor cells Neurologic disorders
Liposomes and other physical methods using plasmid DNA	Easy to prepare in quantity Virtually unlimited size Limited immunogenicity	Inefficient entry into target cell Nonintegrating	Topical applications Transduction tumor cells

are more resistant to conventional drugs and/or radiation and thereby increase the therapeutic index. Finally, and perhaps most widely used of all, it may be possible to attack the tumor indirectly by using genes to modify the behavior of the immune system, either by making the tumor more immunogenic, or by rendering host effector cells more efficient.

While each of these approaches has its advantages and disadvantages, at the moment all must be tempered by an appreciation that none of the current vectors with which we work possess the desirable characteristics of high efficiency and specific targeting to the tumor and tumor cell DNA. For many gene therapy approaches, it would also be highly desirable to control the transgene product, something that is not yet readily achievable in humans, and it would also be helpful to further improve the safety of viral vectors. While each of the available vectors has advantages and disadvantages, at present none comes close to meeting the requirements for a truly effective gene therapy vector that could be used in all the approaches outlined above. Instead, the choice of a vector system is based on the "least bad" alternative for the proposed use. An outline of the advantages and disadvantages of each of the vector systems is given in Table 1, while a more detailed account of each of these vectors can be obtained from references [2, 5, 6, 7, 8].

TUMOR CORRECTION

Tumors are increasingly being characterized by their molecular aberrations. Many of these defects involve deletions in molecules important in regulating cell growth, survival, or differentiation, while others lead to the formation of mutant fusion products providing an unwanted gain of function affecting the same critical activities.

Gene therapy could in principle be used to replace an inactive gene with an active one, or to neutralize the behavior of a gain of function mutation. In adults, such efforts have been made, apparently with some success, in the treatment of p53 deficient head and neck tumors and carcinoma of the bronchus [9, 10, 11]. No equivalent pediatric trials have been reported, and formidable problems remain before this approach can be fully exploited. For example, it will be necessary to get a corrective gene into an extremely high proportion of malignant cells, although it has been suggested that there is some form of

uncharacterized *bystander* effect on nontransduced tumor. Secondly, targeting to metastatic tumor will usually be necessary. Thirdly, correction of a single defect may be inadequate to actually kill the tumor cells, leaving instead a collection of "$n-1$ cells" (where n is the number of mutations required for malignancy to occur) capable of undergoing another mutation to restart the malignant process. Finally, since many of the mutations observed are gain of function and/or have a dominant phenotype, introduction of a wild-type gene alone is insufficient. Instead, the mutant gene or its products must be neutralized using strategies that include introduction of ribozymes or antisense RNA or of siRNA that are proving troublesome to develop [12, 13].

Hence, exploitation of the tumor correction approach will require significant improvements in vector efficiency and targeting, and until these come to pass, the development of novel rationally targeted small molecules will likely dominate this approach.

PRODRUG-METABOLIZING ENZYME (PDME)

Introduction of a gene encoding an enzyme which metabolizes an otherwise inert molecule into a cytotoxic agent has frequently been used in tumor gene therapy. Although the herpes simplex thymidine kinase-ganciclovir system has been most widely applied, there are in fact more than 20 such PDME systems currently in various stages of development and/or clinical trials [14, 15]. For all of these, the concept is that the gene encoding the prodrug-metabolizing enzyme is expressed in the cancer cell, and metabolizes a small molecule to an active moiety which then kills the tumor cell directly. The molecule may also diffuse either through intercellular gap junctions or in the extracellular space and destroy adjacent tumor cells. In this way, transduction of even a small proportion of tumor cells can produce a large *bystander* effect on adjacent tumor tissue. This in turn compensates for the low efficiency of transduction achieved by currently available vectors and may help to destroy a large tumor burden.

Pediatric clinical studies of genetic transfer of PDME

Brain tumors were an attractive initial target for PDME gene therapy. Since the tumors seldom metastasize, the goal of the therapy is the local eradication of the tumor. Hence, the major limitation of PDME, that it requires local inoculation of a tumor with the vector encoding the gene, does not represent a major disadvantage. A number of adult studies have been performed using retroviral and, more recently, adenoviral vectors [16, 17, 18], but to date only one pediatric study has been reported in patients with recurrent or progressive supratentorial brain tumors [19]. Twelve children were enrolled and after tumor resection they were treated with instillation in the tumor bed of retroviral producer cells generating particles encoding HSV-*tk* followed by ganciclovir administration. Unfortunately, disease progression was seen in 10 of 11 patients, although one patient remained free of progression for 18 months.

More recently, a study has been performed on patients with retinoblastoma [20, 21], which is also a highly localized tumor that is conventionally, treated by enucleation and/or chemoradiotherapy. Enucleation is obviously disabling and deforming, and if the tumor is bilateral it leads to blindness. The alternatives of chemotherapy and radiotherapy are less mutilating but both are associated with secondary malignancies. In a study at Baylor College of Medicine, Hurwitz and colleagues are injecting bilateral retinoblastomas with adenovirus type 5 encoding the thymidine kinase gene, followed by administration of ganciclovir. Two of the first three patients have shown substantial tumor responses with the Ad-*tk* and both are disease free, with retained vision, at up-to-one-year post therapy. This trial is now accruing patients with monolateral retinoblastoma.

Future trends in PDME therapy

Other suicide gene therapies are being evaluated. Amongst the most developed of these is the cytosine deaminase system, which converts fluorocytosine to fluorourosil [22]. There are, however, concerns that this suicide system may be less potent than the *tk*-ganciclovir prodrug system. Other molecules which metabolize drugs or trigger apoptotic pathways within tumor cells are also being considered. Perhaps the most important future trend is to attempt to enhance the bystander effect. At present, this is mediated predominantly by transfer of the small molecule cytotoxic drug from cell to cell. However, it is apparent that at least part of the bystander effect is dependent on an immune response generated to the lysed tumor. Hence, the bystander effect in immunocompromised animals has been observed to be substantially less than in those with intact immune systems. Investigators are now attempting to combine PDME genes with sequences encoding a variety of immunostimulatory molecules (see section "Gene Modification of The Immune Response"), including but not limited to Interleukin 2, Interleukin 12, and GM-CSF [23, 24, 25, 26, 27, 28]. Data from these studies are yet to be evaluated. Efforts are also being made to generate vectors, which can selectively divide in malignant cells (conditionally replication-competent vectors) and may therefore spread their encoded PDME genes throughout the tumor bed [29].

PDME has also effectively been used as a means of controlling T cell therapies. For example, graft versus host disease may occur when donor T cells are given to patients after allogeneic stem cell transplantation in an effort to treat tumor relapse (graft versus tumor effect) or post-transplant infections. Several groups have infused donor T cells transduced with the HSV-*tk* gene and reported successful abrogation of GvHD after treatment with ganciclovir [30, 31]. More recently efforts have been made to induce expression of the death signal, Fas, in donor T cells. An inducible construct is used in which Fas expression

occurs only in the presence of an orally administered small molecule (rapamycin or its analogues) that dimerises two individually inactive components of a Fas transcriptional regulator, leading to expression of the Fas receptor and cell death on exposure to the ligand [32].

As T cell therapies for cancer become more widespread, these suicide mechanisms will become extremely important in ensuring that the regimens are acceptably safe.

ANTIANGIOGENESIS GENE THERAPY

Because angiogenesis is a prerequisite for the development of metastatic disease for solid tumors, and probably for leukemias and lymphomas as well, an attack on newly formed blood vessels may help impede the spread of the disease. A number of different large and small molecule inhibitors are currently under study and some of these are suitable for a gene therapy approach [33]. For example, endostatin, a 20- kilodalton fragment of collagen XV111 can efficiently block angiogenesis, but the recombinant protein is difficult and expensive to produce and is somewhat unstable. Delivery of endostatin in murine tumor models by several different vector systems has been able to overcome this limitation and has proved extremely promising [34, 35, 36]. Similarly, angiostatin, a fragment of plasminogen, also functions as a large molecule inhibitor of vessel growth and impedes metastastic tumors. This too can be transferred (eg, by adeno-associated virus vector) to produce benefit in animal models of malignant brain tumors [37, 38].

Much remains to be learned about the most appropriate route and cell of delivery of angiogenesis inhibition, but as with any protein-based therapeutic, gene transfer should allow a continual delivery of the drug rather than the peak and trough concentrations that result from most forms of injection, and may thereby produce a more sustained and effective response.

CYTOTOXIC DRUG RESISTANCE GENE TRANSFER

The concept of dose intensification has long been current in modern oncology, including pediatric oncology. In other words, it is believed that giving more of a cytotoxic drug over a longer period will cure a higher proportion of patients. While there are many obvious exceptions to this rule, for many pediatric malignancies it is clear that failure to tolerate chemotherapy in the intended doses correlates well with an increased risk of relapse. For that reason, there is an interest in using genes which will protect normal tissues while leaving malignant cells vulnerable to destruction. By increasing the therapeutic index in this way, it is hoped that more drug can be administered and a higher percentage of patients cured.

There are many different candidate drug resistance genes to be transferred, but perhaps the most widely studied is the human Multidrug Resistance-1 (*MDR*-1) gene. The gene product acts as a drug efflux pump and prevents accumulation of toxic small molecules, including a range of cytotoxic drugs such as mitoxantrone and daunorubicin. The primary toxicity of many of these cytotoxic drugs is on hematopoietic progenitor cells. Retroviral-mediated gene transfer of drug resistance genes into hematopoietic stem cells has, until recently, been difficult to accomplish. The incorporation of fibronectin together with hematopoietic growth factors into the transduction regimen, together with repeated cycles of gene transfer, has allowed a significant proportion of hematopoietic cells to be protected with expression levels adequate to reduce the sensitivity of these stem cells to chemotherapeutic agents [39]. Several other drug resistance genes are currently under study and may soon join *MDR*-1 in clinical trial. These include the bacterial nitroreductase gene (which protects against drugs such as thiotepa) [40] and dihydrofolate reductase mutants which protect against methotrexate/trimetrexate [41].

There are two major problems with using transfer of drug resistance genes. The lack of targeting of current vectors means that they may transduce malignant cells as well as normal cells, and therefore increase the resistance of both to the cytotoxic drug. Moreover, while it may be possible to protect a significant proportion of marrow stem cells, secondary toxicities to other organ systems such as skin, lung, and gut will rapidly become evident as doses are escalated because these tissues are much less readily protected than hematopoietic stem cells.

GENE MARKING AND PEDIATRIC MALIGNANCIES

The principle of gene marking is the transfer of a unique DNA sequence (eg, a nonhuman gene) into a host cell (eg, T cell, hematopoietic stem cell, etc) allowing the gene or the gene product to be easily detected, thereby serving as a marker for these labeled cells [42].

In all these studies, gene marking is not intended for direct therapeutic benefit, but rather to obtain information regarding the biology and function of adoptively transferred cells.

Gene marking for autologous stem cell transplantation

By marking stem cells prior to stem cell infusion, it has been possible to determine if contaminating malignant cells in the stem cell harvest contribute to relapse following autologous stem cell transplant [43]. The hematopoietic stem cell (HSC) product is marked at the time of harvest with murine retroviral vectors encoding the neomycin resistance gene. Then, at relapse, it is possible to detect whether the marker gene is present in the malignant cells. Since 1991, studies have been initiated using this approach in a variety of malignancies treated by autologous HSC transplantation [43, 44, 45, 46] including acute myeloid leukemia (AML), chronic myeloid

leukemia (CML), acute lymphoblastic leukemia (ALL), neuroblastoma, and lymphoma.

In pediatric patients, receiving autologous BMT as part of therapy for AML, four of twelve patients who received marked marrow relapsed. In three of the four patients, detection of both the transferred marker and the tumor-specific marker in the same cells at the time of relapse provided unequivocal evidence that the residual malignant cells in the marrow were a source of leukemic recurrence [42]. These marking studies also provided information on the transfer of marker genes to normal hematopoietic cells and showed that marrow autografts contribute to long-term hematopoietic reconstitution after transplant [47]. Long-term transfer for more than 10 years has been seen in the mature progeny of marrow precursor cells, including peripheral blood T and B cells and neutrophils [48].

Gene marking of T cells

Several studies have also shown the feasibility of gene marking cytotoxic T lymphocytes (CTL) to track their expansion, persistence, and homing potential to the sites of disease [49, 50, 51]. For example, gene marking of Epstein Barr virus (EBV)-specific CTL for the prophylaxis and treatment of lymphoproliferative disorder posthematopoietic stem cell transplant has demonstrated persistence of gene marked CTL to 78 months post-infusion [52]. In addition, as described below, gene-marked EBV-CTL given as treatment for relapsed Hodgkin disease have been shown to traffic to tumor sites [51].

GENE MODIFICATION OF THE IMMUNE RESPONSE

Rendering the tumor more immunogenic

One of the most striking observations of the past 10 years has been the demonstration that human tumors widely express tumor associated or tumor specific antigens. Moreover, even if these are internal antigens, they may be processed and presented by the tumor cell and become targets for the immune response. These antigens may be particularly prevalent on pediatric malignancies which frequently express oncofetal or developmental antigens not present in the *mature* child or which may express antigens directly relating to the genetic lesions that have caused the tumor [3, 53].

One of the most commonly used approaches to cancer gene therapy is the attempt to enhance the immunogenicity of these weak tumor antigens and to amplify the scanty T cell precursors capable of recognizing them. An immune response to any antigen has a number of different phases. These include antigen processing and presentation, chemoattraction of T cells to the site of the presented antigen, the costimulation of any T cell which engages the antigen with its specific receptor and the amplification of the immune response so generated. Each of these stages is the primary responsibility of one or more of a range of secreted chemokines and cytokines or of cell-bound receptor-ligand systems. It has become apparent that the forced expression of one or more of these agents within a tumor cell is capable of greatly enhancing the immune response to the weak tumor antigens that cell may express. The immune response so generated may then be effective elsewhere in the body against nontransduced cells. This immunologic bystander effect is an important consideration, since the inefficiency of vectors, currently available, makes the probability of transducing all tumor cells in a patient exceedingly remote. Hence, by transducing even a small proportion of cells, it may be possible to use the efficient targeting mechanisms of the immune system to ensure that the response affects the bulk of tumor cells, including those that were not genetically modified.

Genetic modification of tumor cells

A number of different agents have been successfully utilized in animals, including chemokines such as lymphotactin [54], agents which enhance antigen presentation such as GM-CSF [55, 56, 57], and cytokines that enhance CD4 cell activity (eg, TNF and Interleukin 7) [58, 59], increase expression of class I MHC antigens (eg, gamma Interferon) [60], or amplify T cell responses (eg, Interleukin 2) [61]. Additionally, efforts have been made to express costimulator molecules on tumor cells, including CD40 Ligand [62, 63, 64, 65, 66] and B7.1 [67], or intercellular adhesion molecules such as ICAM 1 and ICAM 3 [68].

Source of cells

The cells used for gene-modified tumor immunotherapy may be derived either from the patient themselves or from an allogeneic cell line grown in culture. Each of these approaches has reciprocal advantages and disadvantages. For example, an autologous cell line, unlike an allogeneic cell line, will almost certainly express the tumor specific antigens that are present elsewhere in the patient. The heterogeneity of human tumors means that this may not be so for an allogeneic tumor cell line. An autologous cell line will also express these tumor associated antigens in the context of the patient's own MHC molecules, and so will be recognized by the host immune system. An allogeneic cell line will likely only do this if the antigens on that cell line are taken up by host antigen presenting cells and subsequently presented to the host immune system (cross priming). The clear advantages of allogeneic tumors are that they are much more readily obtained in quantity than autologous tumors, which may be difficult to harvest in adequate numbers to generate a vaccine. Allogeneic cells are also much easier to standardize, since the level of transgene expression will be constant and will not vary from patient to patient. This makes the design and interpretation of clinical trials much simpler. Finally, from a practical point of view, should a tumor vaccine be promising in early phase clinical study, the development of an allogeneic vaccine would be substantially

facilitated, since the material could be manufactured, tested, and stored in bulk rather than generated as an individualized therapy for each patient in a large study. For the moment, it seems reasonable to study both autologous and allogeneic tumor cells and to decide which approach is optimal when more information is available.

Pediatric clinical studies with gene modified tumor cells

Neuroblastoma cells were transduced with adenoviral vectors so that they expressed the Interleukin 2 gene. Both autologous and allogeneic studies were instituted. In the autologous trial, patients received up to 8 injections of their own tumor cells subcutaneously. More than half the patients produced specific antibody and a specific cytotoxic T cell response directed against the autologous neuroblasts. Of 10 patients, 5 had clinical tumor responses including one complete and one very good partial response [69]. In the allogeneic study, however, the immunizing cell line induced no evident specific immunity and only one patient showed a partial response [70]. Of note, in both studies a significant number of children showed good tumor responses on subsequent treatment with low dose oral etoposide. This interaction between genetic immunotherapy and low dose chemotherapy has subsequently been observed in a number of adult tumor vaccine studies, and likely represents a genuine interactivity between these treatment modalities that may usefully be exploited for therapeutic benefit in the future.

A subsequent clinical study in neuroblastoma was based upon animal data showing that the combination of lymphotactin (Lptn), a T cell chemokine, and Interleukin 2 (IL-2), the T cell growth factor, accelerated and augmented the immune response to a neuroblastoma cell line [54]. Accordingly, patients received either an autologous vaccine or an allogeneic one expressing both IL-2 and Lptn [71]. In the allogeneic group, it was possible for the first time to observe specific antitumor immune responses to the immunizing cell line, and two patients entered complete remission, which was durable in one. In the autologous study, the results did not appear to be measurably superior to Interleukin 2 alone. Hence, in the allogeneic setting at least, there is preliminary evidence that the combination of two agents acting at different phases of the immune response may be superior to a single agent. If these results are confirmed they may increase the feasibility of utilizing allogeneic vaccines with the considerable simplification in protocol development that would result.

In hematologic malignancy, a Phase I study of autologous acute myeloblastic leukemia cells engineered to secrete GM-CSF has recently commenced and this study is now being extended to pediatric AML [57].

It has also proved feasible to express costimulator molecules such as CD40, CD40 Ligand, or B7.1 on primary tumor cells surfaces. We are currently using a combination of CD40 Ligand and IL-2 gene transfer into pediatric acute lymphoblastic leukemia cells in an effort to generate an antitumor immune response in patients with high-risk disease who have entered remission. To date, this study has proved to be safe and has generated antileukemia immune responses. Because these patients are treated in remission, we do not yet know whether there has been any antileukemia activity.

In conclusion, therefore, genetic modification of tumor cells appears safe and is capable of generating specific humoral and cellular antitumor cytotoxic responses. There have been at least some tumor regressions and the approach is now being evaluated in a wider range of tumors and in a larger number of patients.

PEDIATRIC CANCER THERAPY WITH GENE MODIFIED T CELLS

Prophylaxis and treatment of Epstein Barr virus (EBV)-associated posttransplant lymphoproliferative disorder (EBV-PTLD)

Several studies have suggested the feasibility and apparent clinical efficacy of adoptive transfer of cytotoxic T-cells (CTL) directed at viral or tumor antigens [50, 72, 73, 74]. By using gene-marked cells in these studies, it has not only been possible to determine the survival and homing of the infused T-cells, but also determine if they mediate adverse effects such as GvHD [75, 76].

For example, Epstein Barr virus (EBV)-associated posttransplant lymphoproliferative disorder (PTLD) is a complication due to proliferation of EBV-infected B cells and occurs in 5–30% of patients receiving T-depleted marrows from mismatched family or unrelated donors. Several groups have investigated the feasibility of generating donor-derived EBV-specific CTL to treat this disorder [77, 78, 79, 80]. Our group generated EBV-specific T cell lines from donor lymphocytes and used them as prophylaxis and treatment for EBV-PTLD in patients post HSCT [77]. Over a 7-year period, 56 pediatric patients who received a T cell-depleted HSCT were given EBV-CTL prophylactically. The first 26 patients enrolled on to the study received CTL marked with the neomycin resistance gene. None of the 56 patients who received the EBV-CTL developed PTLD compared with an incidence of 11.5% in a comparable group who did not receive CTL [81]. Using conventional PCR and real-time PCR, the marker gene was identified in the peripheral blood for at least 78 months post CTL [82]. Three patients who declined or were ineligible for our prophylaxis study were treated for established EBV lymphoma. The EBV-specific CTL therapy induced sustained remission in 2 patients, but the third patient treatment failed and was found to have an antigen-loss mutant in her EBV lymphoma cells [83].

These studies are now being extended to patients receiving solid organ transplants. Pediatric populations are particularly susceptible to PTLD after solid organ transplant, because children are more frequently EBV-seronegative at the time of transplant [84].

Adoptive immunotherapy for EBV-positive Hodgkin disease

EBV-positive lymphoma cells in posttransplant lymphoproliferative disease, express a wide range of EBV encoded antigens and are readily susceptible to immunotherapy. What of the malignant cells of Hodgkin disease and Nasopharyngeal cancer, which express a more restricted pattern of antigens? More than 80% of children with EBV-associated Hodgkin disease can be cured, but treatment for those who relapse is limited. Moreover, long-term follow-up studies of Hodgkin disease survivors show greatly increased risks of second malignancy [85]. Nonfatal sequelae of therapy, such as altered somatic growth, infertility, and restrictive lung disease can also seriously affect the quality of the life of the survivors [86]. It is therefore desirable to develop novel therapies that could improve disease-free survival in relapsed/refractory patients and reduce long-term complications.

We have treated 13 patients with EBV+ Hodgkin disease using EBV-specific CTL. Five patients with minimal residual disease postautologous bone marrow transplant remain well for 2–21 months post CTL infusion [87], and mixed tumor responses in 6 patients. Of 8 patients treated with active disease, injection of EBV-specific gene-marked CTL showed gene-marked CTL within tumor [51] and in peripheral blood for up to 9 months following infusion [87].

Future trends in the development of gene-modified CTL

Although these results have been promising and there have been tumor responses, these have been partial, or often transient, and no patient with aggressive relapsed Hodgkin disease has been cured. This may be due to a lack of specificity of the EBV-specific CTL for the immunosubdominant LMP1 and LMP2 antigens that are all present on the Hodgkin tumor. In addition, the tumor secretes immunosuppressive cytokines and chemokines which affect CTL function and antigen presenting cell activity [88]. Gene transfer can be used to overcome both types of problems. By using dendritic cells transduced with adenoviral vectors encoding LMP2, it has proved possible to generate CTL that have high cytolytic activity in vitro to LMP2-positive targets when compared to conventional EBV-CTL [89, 90].

Although such specific cells may be more effective, there is a concern that the CTL will remain vulnerable to the immunosuppressive cytokines secreted by the Hodgkin Reed-Sternberg cell. The cytokine, which has the most devastating effects on CTL proliferation and function, is transforming growth factor-beta (TGFβ) [91, 92]. This cytokine is secreted by a wide variety of childhood tumors, and allows the tumor to escape the immune response [93]. To overcome this capacity to inhibit the EBV-CTL, we transduced CTL from patients with relapsed EBV-positive Hodgkin disease with a retrovirus vector expressing a dominant-negative TGFβ type-II receptor (DNR). This prevents formation of the functional trimeric receptor. Cytotoxicity, proliferation, and cytokine release assays showed that exogenous TGFβ had minimal inhibitory effects on DNR-transduced CTLs [94]. This combination of tumor-specific and tumor-resistant CTL may prove highly effective for therapy.

Adoptive immunotherapy for EBV-positive Nasopharyngeal carcinoma

Despite the good overall survival rates following conventional therapy for this disease in children, follow-up reports have shown substantial longer-term treatment-related morbidity and mortality [95, 96], including growth hormone deficiency, hypothyroidism, pulmonary fibrosis, and secondary malignancies [96, 97]. Although EBV-CTL have been used in this disease, [98] with limited success, we are using the same approaches described for Hodgkin disease to treat these tumors as well.

Chimeric T cells for tumor therapy

Primary T cells genetically modified to express chimeric receptors derived from antibodies and specific for tumor or viral antigens have considerable therapeutic potential. Chimeric T cell receptors allow the recognition specificity of T lymphocytes to extend beyond classical T cell epitopes by transducing cells with genes that encode the variable domain of a tumor-specific monoclonal antibody (MAb) (ScFv) joined to a cytoplasmic signaling domain. This strategy can therefore be applied to every malignancy that expresses a tumor-associated antigen for which an MAb exists [99, 100]. Unlike conventional T cell receptors, these chimeric receptors will be active even if the tumor cells are class-1-MHC negative.

Neuroblastoma is the commonest extracranial solid tumor of children, and is often resistant to conventional treatments. A high proportion of tumors express tumor-associated antigens such as GD2, L1-CAM, and N-CAM. CD8+ve CTL clones genetically modified to express the CE7R chimeric immunoreceptor which consists of an extracellular L1-CAM-specific single-chain antibody, transmembrane CD4, and T cell CD3-complex zeta chain, is currently being investigated in a clinical trial [101]. However, chimeric receptor signaling produces only limited activation of the T cells, and we are currently exploring an alternative approach to increase the in vivo functionality of the cells [102, 103]. We have transduced EBV-specific (not primary) T cells with GD2-specific chimeric receptor genes. In in vitro, we have shown that these cells can be expanded and maintained long term in the presence of EBV-infected B cells. While they recognize EBV-infected targets through their conventional T cell receptor and thereby become activated, they are also able to recognize and lyse tumor targets through their chimeric receptors. Several cycles of virus target → tumor target → virus target can be demonstrated *ex vivo*, implying that EBV-specific T cells expressing chimeric antitumor receptors may represent a new source of effector cells that would

persist and function long term after their transfer to cancer patients [104].

CONCLUSION

We have far to go before gene therapy of pediatric malignancy can truly be said to have made a major impact on these diseases. Nonetheless, over the past decade, these new techniques have produced unequivocal tumor responses even in advanced disease. As we continue to make incremental advances in the application of these approaches, we can expect to see gene therapy increasingly supplement and perhaps even eventually supplant conventional cancer therapeutics.

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REVIEW ARTICLE

Current and Future Gene Therapy for Malignant Gliomas

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Malignant gliomas are the most common neoplasm in the central nervous system. When treated with conventional treatments including surgery, irradiation, and chemotherapy, the average life expectancy of the most malignant type, glioblastoma multiforme is usually less than 1 year. Therefore, gene therapy is expected to be an effective and possibly curative treatment. Many gene therapeutic approaches have demonstrated efficacy in experimental animal models. However, the current clinical trials are disappointing. This review focuses on current therapeutic genes/vectors/delivery systems/targeting strategies in order to introduce updated trends and hopefully indicate prospective gene therapy for malignant gliomas.

INTRODUCTION

Malignant gliomas are the most common primary tumors arising in the human brain [1]. The most malignant type of them, the glioblastoma multiforme, represents 29% of all primary brain tumors or 5,000 new cases per year in the United States [2]. Despite surgery, chemotherapy, and radiotherapy, glioblastomas are almost always fatal, with a median survival rate of less than a year and a 5-year survival rate of 5% or less [1, 2, 3]. No therapeutic modality has substantially changed the outcome of patients with glioblastoma [2, 3]. Therefore, it is no wonder that one of the earliest targets of cancer gene therapy was malignant glioma.

The epoch-making human trial of herpes simplex virus *thymidine kinase* gene/ganciclovir (HSV-*tk*/GCV) using retroviral vector started in early 1990s [4]. Although the antitumor effect of HSV-*tk*/GCV therapy had looked very promising in the animal model, the effect on human patients was disappointing. To augment the effect of gene therapy, adenoviral vectors were developed and advanced to human trials [5]. Adenoviral vectors significantly improved transduction efficacy but raised other problems, as discussed later in this review. Additionally, there was a death of a patient who received gene therapy using an adenoviral vector [6]. This incident raised a nationwide debate especially on the safety of gene therapy using viral vectors. Most of clinical trials were put on hold for several months to make sure that safety guidelines are strictly followed. However, hopes for gene therapy have not been quenched out. Researchers have been exploring many candidate genes, developing improved viral and nonviral vectors, trying different methods to deliver genes, and combining gene therapies with other modalities such as immunotherapy. In this review, we focus on animal and human studies of gene therapy for malignant gliomas. We have collected relatively recent references to introduce updated trends and hopefully to indicate future directions in this field.

THERAPEUTIC GENES

Many therapeutic genes have shown efficacy in experimental models and been divided into three categories. First, therapeutic genes are used to induce direct killing effect (Table 1). In this category, HSV-*tk*/GCV [5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16], toxin [17], tumor suppressor genes [18, 19, 20, 21, 22, 23, 24, 25, 26], apoptosis-inducers [27, 28, 29, 30, 31, 32], antisense against telomerase [25, 33, 34, 35], and oncolytic viruses [36, 37, 38, 39, 40] are included. Second, immunomodulation has been performed to elicit immune response against malignant gliomas [41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54]. Third, angiogenesis inhibitors [55, 56, 57, 58] or neural stem cells [59, 60, 61] are used to induce antitumor effect, although direct killing effect or direct immunoreaction may be unrelated.

Direct killing effect

HSV-tk/GCV

Herpes simplex virus *thymidine kinase* gene/ganciclovir (HSV-*tk*/GCV) therapy is a two-step strategy [4]. First, HSV-*tk* gene is transduced into tumor cells. Second, GCV is administered systematically. GCV is harmless to normal cells without HSV-*tk*. When tumor

Table 1. Therapeutic genes.

Direct killing effect	References
HSV-*tk*/GCV	[5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16]
Toxin	[17]
Tumor suppresor gene	[18, 19, 20, 21, 22, 23, 24, 25, 26]
Apoptosis inducer	[27, 28, 29, 30, 31, 32]
Antisense therapy for telomerase	[25, 33, 34, 35]
Oncolytic virus	[36, 37, 38, 39, 40]
Immunomodulation	**References**
IL-2	[41, 42, 43]
IL-4	[44, 45, 46]
IL-12	[47, 48]
IFN	[49, 50]
TNF-α	[51, 52, 53, 54]
Others	**References**
Angiogenesis inhibitor	[55, 56, 57, 58]
Neural stem cell	[59, 60, 61]

cells express HSV-*tk*, this enzyme converts GCV into a cytotoxic molecule, resulting in cell death. This HSV-*tk*/GCV therapy is also called suicide gene therapy. When HSV-*tk* gene is transduced with retroviral vectors, these vectors are selectively incorporated into dividing cells, predominantly into brain tumor cells. However, the original scheme using retroviral vectors turned out to be not potent enough. In vivo studies showed that effective tumor-cell killing depends on bystander effect and transduction efficacy [7, 13]. Bystander effect refers to killing cells that do not express HSV-*tk*, but closely located with the cells expressing the enzyme. On the other hand, to improve transduction efficacy, retroviral-vector producing cells or adenoviral vectors were developed. Many human trials of HSV-*tk*/GCV therapy were carried out using either retroviral-vector producing cells or adenoviral vectors. Some phase I/II studies reported significant therapeutic responses in small groups of patients [10, 14], but others claimed only marginal benefit [5, 11, 15]; overall, results were disappointing. The main reasons of failure are assumed to be low transduction efficacy and lack of bystander effect. Additionally, there was a report warning that adenoviral vector induced long-term active inflammation in the animal model [12]. More recently, HSV-*tk*/GCV therapy is used in in vivo experiments with adeno-associated virus [9] or combined with other therapy [16].

Toxin

Martin et al constructed retroviral vectors with a toxin gene (the Pseudomonas exotoxin or the Ricinus toxin, ricin) placed under the control of the thyroid hormone (T3) regulatable promoter of the myelin basic protein (MBP) [17]. They showed that malignant glioma cells, stably transducted with the vector, failed to establish a tumor or regressed the tumor mass in the rat brain.

Tumor suppressor genes

Tumor suppressor genes are often mutated or deleted in malignant gliomas and the lack of function of these genes is supposed to cause tumorigenicity. Therefore, it is quite reasonable to replace them. The most extensively studied is the p53 tumor suppressor gene. Since alterations in the p53 gene occur in 35–60% of human malignant glioma [62, 63], p53 gene therapy is logically appropriate for these tumors. Accumulating evidences show that replacement of p53 significantly inhibits tumor growth in the subcutaneous [19] or intracranial [18, 22, 24] tumor model. However, many gliomas are mixture of cells with mutated p53 and wild-type p53 (wt-p53), and p53 gene transfer is known to be ineffective for the cells with wt-p53 [23, 25]. That is, p53 gene therapy is supposed to be effective only for part of malignant gliomas. There is a conflicting report claiming that p53 gene therapy induces apoptosis in glioma cells with wt-p53 [24]. New studies show that the combination of p53 gene therapy and irradiation is effective for malignant gliomas with heterogenous p53 status [20, 21]. Other tumor suppressor genes (p21, p16, and p27) belong to cyclin-dependent kinase inhibitors (CDKIs) and they are involved in the regulation of cell cycle. Wang et al showed that retroviral transfer of p16 and p21 inhibited tumor growth [26].

Apoptosis-inducers

Apoptosis, also called programmed cell death, is a genetically-encoded program to get rid of unwanted cells. It is well known that apoptotic pathways are suppressed in malignancies including malignant gliomas [64]. The rationale to use apoptosis-inducers is to activate apoptotic pathways and induce cell death effectively in malignant glioma cells. The genes used in the studies so far are caspase-1/interleukin-1β-converting enzyme (ICE) [27], caspase-3/CPP32β [28], caspase-6 [32], caspase-8 [30], Fas associated protein with death domain (FADD) [29], and Bax [31]. The main concern of these strategies is about safety, what if apoptosis is also induced in normal brain cells surrounding the tumor? It is necessary to regulate the induction of apoptosis to occur only in malignant cells as described below.

Antisense therapy for telomerase

Our group developed a system to inhibit human telomerase RNA (hTER) with 2-5A-linked antisense [25, 33, 35]. 2-5A or $2'$, $5'$-oligoadenylate is a pathway of interferon actions [65, 66]. It activates RNase L that is ubiquitous in the cells and degrades RNA randomly. Chimeric combination of an antisense and 2-5A enables us to

degrade specific RNA [67]. We designed an antisense and synthesized 2-5A antisense molecule to target hTER (2-5A-anti-hTER) [33]. It degrades hTER specifically and effectively, resulting in the inhibition of telomerase. Interestingly, 2-5A-anti-hTER induced apoptosis massively and inhibited tumor growth in the subcutaneous and intracranial tumor models [33, 35]. 2-5A-anti-hTER induced apoptosis unexpectedly early (within 4 days, in vitro) compared to the treatment with the cDNA vector for hTER (about one month) [34].

Oncolytic viruses

Oncolytic viruses are designed to replicate selectively in and lyse tumor cells. These viruses are more effective in infecting tumor cells compared to the viruses that are constructed as replication-defective. Oncolytic viruses increase in number in tumor cells and lyse the cells directly, not by transducing specific genes. A recombinant herpes simplex virus with some deletions, designated DM33, inhibited growth of intracranial tumors and prolonged the survival of tumor-bearing animals [38]. The tumor-killing effect was even better with the following injection of ganciclovir [40]. Adenovirus ONYX-015 targets tumors with mutant p53 and its clinical studies are ongoing for head and neck cancer. Recently, it was shown that ONYX-015 is effective in malignant gliomas regardless of their p53 status [39]. Fueyo et al constructed Delta 24, a tumor-selective adenovirus with a deletion in the EIA region responsible for binding Rb protein [36]. This virus targets tumor cells with Rb alteration. They showed that Delta 24 inhibited the growth of glioma cells implanted subcutaneously. Ansardi et al constructed oncolytic RNA-based vectors derived from poliovirus and termed replicons [37]. They showed that replicons suppressed tumor growth and extended the survival of the animals with intracranial tumors.

Immunomodulation

Central nervous system (CNS) had been considered as immune-privileged site. The important factors of tolerance for activated host immune are the presence of the blood-brain barrier and the absence of a lymphatic drainage system [68]. However, lymphocytic infiltration has been observed frequently in malignant gliomas and the degree of infiltration seems to correlate with survival [69]. Moreover, disability to elicit the immune reaction implies that the oncogenesis of primary glioma cells occurs without interaction to immune cells. Therefore, glioma cells derived from brain parenchyma may be more immunogenic than tumors derived from peripheral system and the immunogene therapy is very attractive for therapy of malignant glioma.

IL-2

Interleukin (IL)-2, a cytokine produced by activated T cells, can promote immune reactions. Fibroblasts, genetically engineered to secrete IL-2, suppress tumor growth and induce antitumor immunity to murine gliomas in vivo [41]. Clinically, patients with malignant glioma were subcutaneously immunized with autologous glioma cells and received IL-2 secreting fibroblasts [42]. Posttreatment with MRI revealed the marked tumor necrosis, but the tumor did not disappear. As shown in vivo immunotherapy model using IL-2, the combination of immunization in peripheral tissues and intracerebral transplantation of IL-2-producing cells is necessary to eliminate established brain tumors [43].

IL-4

IL-4 is a multifunctional lymphokine produced by helper T cells and has a broad range of activities on both B and T lymphocytes. When IL-4-transduced glioma cells were injected into animals, eosinophil infiltration and inhibition of tumor angiogenesis were observed in athymic mice [45] and T-cell infiltration and humoral response were shown in immunocompetent rats [46]. Furthermore, retroviral packaging cells producing IL-4 were produced [44]. When these cells were injected into established intracranial tumors, tumors were completely eradicated and inhibition of tumor angiogenesis and infiltration of T cells and macrophages were revealed.

IL-12

IL-12 is secreted by antigen-presenting cells such as dendritic cells, macrophages or microglia [70]. Among cytokines, it has been demonstrated that IL-12 possesses particularly potent antitumor properties [71]. It is because IL-12 plays a critical role in mediating inflammatory and immune responses in host defense, IL-12 exerts a variety of biological effects on T cells and natural killer (NK) cells [72], including induction of interferon (IFN)-γ production [71], enhancement of proliferation and cytolytic function of T cells and NK cells [73], and promotion of the Th1-type immune response [74]. In addition to stimulatory effects on the immune system, IL-12 is also a potent antiangiogenic factor [75]. Local delivery of IL-12 by genetically engineered cells significantly prolongs the survival time in animals with brain tumor [47]. Moreover, a single intratumoral treatment of nude mice with a vaccinia virus (VV) expressing IL-12 induced significant tumor growth inhibition [48]. However, most animals injected with high doses of recombinant viruses (10^5 to 10^7 pfu) showed signs of cytokine toxicity.

Interferon (IFN)

IFNs are produced by activated immune cells including T cells, NK cells, and monocyte lineage cells. Glioma cells transfected with the human β-interferon gene by liposomes, elicit systemic immune reactions and inhibit the tumor growth [49]. In addition, IFNs directly stimulate cell differentiation and apoptosis via signals from interferon receptors on glioma cells [50].

TNF-α

Tumor necrosis factor (TNF)-α was initially supposed to be a promising cancer therapeutic reagent. However, recent investigation shows that TNF-α does not kill most types of cancer cells partly due to the activation of an anti-apoptotic gene, NF-κB [76]. Therefore, suppressing NF-κB is expected to potentiate TNF-α-induced apoptosis. Recently, it has been demonstrated that combination of TNF-α with other cytokines [51], radiation [52], chemotherapy [53], or hyperthermia [54] is more effective in therapy for glioma models than single treatments.

Others

Angiogenesis inhibitors

Based on the observation that gliomas are among the most angiogenic tumors, therapeutic strategies aimed to inhibit angiogenesis are theoretically attractive. Angiostatin, an internal peptide fragment of plasminogen, has recently been shown to potently inhibit endothelial proliferation in vitro and tumor growth in vivo [77]. The AAV (adeno-associated virus) vector has been able to deliver sustained and high-level gene expression in vivo. Intratumoral [55] or intramuscular [56] injection of a high-titer AAV-angiostatin vector has rendered efficacious tumor suppression and resulted in long-term survival. However, recombinant angiostatin, a peptide fragment, might be unstable in vivo. A tricistronic retroviral vector, expressing natural antiangiogenic factors, inhibits angiogenesis in vitro, but is not able to block tumor progression in vivo [57]. A sustained in vivo protein delivery is required to achieve the therapeutic effects. Endostatin, internal peptide fragment of 18 collagen, is also an angiogenic inhibitor. Engineered C6 cells that endogenously express mouse endostatin reduced tumor growth in vivo [58]. However, complete tumor inhibition was not observed in either the athymic or immunocompetent tumor models. Antiangiogenetic therapy using these peptides might be developed as an adjuvant gene therapy for the effective treatment of malignant gliomas.

Neural stem cells

Gene therapy of glioblastomas is limited because viral vectors usually are unable to survive for long time, continue to express proteins, and hardly reach glioblastoma cells infiltrating the brain parenchyma. Neural stem cells, implanted distant from brain tumor lesion into experimental intracranial gliomas in vivo, migrated brain parenchyma towards brain tumor site, chasing the infiltrating tumors [59]. This migratory cell delivery method has the potential to expand the range of delivery of HSV-1 vectors to tumor cells in the brain [60]. Genetically engineering neural stem cells expressing IL-4 elicited the systemic immune response and inhibited tumor growth [61]. Moreover, supernatant of neural stem cells inhibited the proliferation of glioma cells [61]. Therefore, neural stem cells may be an ideal vehicle to overcome the above difficulties in gene therapy of malignant gliomas.

Table 2. Vectors.

Viral vectors	References
Retrovirus	[8, 13, 15, 44, 57]
Adenovirus	[5, 14]
Adeno-associated virus	[9, 56]
Nonviral vectors	**References**
Antisense oligonucleotide	[25, 33, 34, 80, 81]
Naked DNA plasmid	[32, 49, 82, 83]

VECTORS

Vector development is an important field of study, because efficacy of gene transfer depends mostly on the ability of vectors to be incorporated into tumor cells. Vectors for gene therapy can be divided into classes of viral and nonviral systems (Table 2). Viruses are effective vehicles for gene delivery as they can enter human cells and express their genes specifically and efficiently. The main device for viral vector development is improving the targeting efficiency of viruses, while abrogating their ability to cause disease. Modifying the viral genome to remove sequences necessary for viral replication and pathogenecity makes it possible to achieve these goals. The removed viral coding sequence can be replaced with exogenous therapeutic genes. Such genetically engineered viruses theoretically keep wild-type viral cellular tropism and ensure transgene expression in the target cell population without causing harmful diseases. Efforts to alter the natural tropism of viruses by manipulating the viral components that mediate cell binding and internalization represent a means of leading viruses specifically to chosen target cells.

Viral vectors

Retrovirus

Retrovirus and adenovirus have been used for a wide variety of gene therapy applications. Retrovirus is a single strand RNA virus. Retrovirus enters cells by binding surface envelope protein, encoded by the *env* gene, to specific cellular receptors. After entering cells, the viral enzyme reverse transcriptase, encoded by the *pol* gene, transcribes the viral genome into a double-strand DNA copy. Double-strand DNA can enter the nucleus of dividing cells and become integrated randomly into the host genome. This event occurs preferentially in dividing cells, meaning the virus does not enter neurons or other normal brain cells. Retroviruses used in gene therapy protocols are designed to be replication-deficient by removing their *gag*, *pol*, and *env* genes. Therefore, infectious but replication-deficient retrovirus particles are produced in packaging cells that express retrovirus *gag*, *pol*, and *env* genes from plasmids lacking the packaging sequence. A great variety of in vivo

experiments [13, 44, 57] and clinical trials [8, 15] using retroviral vectors for gene therapy for malignant glioma have been performed. There are some drawbacks to use retroviruses as vectors. Retrovirus packaging cells produce relatively low titers. The retrovirus genome is small, which limits the size of genetic constructs they can carry. Random integration into the host genome may disrupt cellular genes by insertional mutagenesis.

Adenovirus

Adenovirus is a double-strand DNA virus. Adenovirus enters cells by binding to the adenoviral receptor, which promotes interaction of viral arginine-glycine-aspartate sequences with cellular integrins. After internalization, the virus escapes from cellular endosomes, partially disassembles and translocates to the nucleus, where viral gene expression begins. They can produce in high titers and infect non-dividing cells. Gene expression occurs without integration into the host genome and gutless adenovirus offers the opportunity to develop vectors with expanded capacity for therapeutic transgenes. Transduction efficiency of adenovirus is better than that of retrovirus. One of the drawbacks is that the induced genomes decrease by cell divisions. The administration is limited only once, as it possesses high antigenicity. Sandmair et al compared the effect of HSV-*tk* gene therapy combined with ganciclovir medication in malignant gliomas between retrovirus-packaging cell and adenovirus gene therapy [14]. Although the results are from a small number of patients and should be interpreted cautiously, adenoviral HSV-*tk* gene therapy seems to be beneficial as shown by magnetic resonance imaging (MRI)-determined tumor regrowth, 3 months after gene therapy, and the survival of patients [14].

Adeno-associated virus

Enhanced gene delivery has been demonstrated in preclinical studies using adeno-associated viruses (AAV) vectors targeting malignant glioma cells [9, 56]. AAV is a native human parvovirus that does not cause any known human disease. They enter cells after binding to heparan sulfate but need coinfection with a helper virus (adenovirus or herpes virus) to replicate. Without coinfection of helper virus, AAV infection leads to latency, in which the viral genome remains in an integrated form or as episomal DNA. Subsequent infection of the cell with a virus capable of providing the necessary helper functions allows replication to proceed. AAV vectors have a number of potential advantages over retroviral and adenoviral vectors. They can infect a wide range of host cells independent of their cell cycling status and are stably integrated and maintained in the host genome in which transient transgene expression may be adequate. The drawbacks of AAV as vectors for gene therapy are limited packaging capacity of approximately 5 kbp and gene expression that may be slow to reach its peak. In addition, production requires helper viruses, which may contaminate preparations for preclinical and clinical use.

Nonviral vectors

Gene transfer using nucleic acid therapeutics or nonviral vectors is much less immunogenic and cytotoxic than viral-vector systems. Although major weakness of nonviral vectors is that transduction efficiency is significantly lower than viral vectors, it can be overcome by frequent intratumoral injection or application of osmotic minipumps or polymer/microsphere system as described below.

Antisense oligonucleotide

Over the last two decades, cloning and sequencing of the critical genes in tumorigenecity have made progress remarkably. Many target genes attractive for antisense therapy have been identified. Antisense oligonucleotides are designed to bind to a certain sequence of specific mRNA and degrade it, providing a powerful tool for the cancer therapy. We have recently selected telomerase, a ribonucleoprotein enzyme, as a target for the therapy of malignant gliomas [25, 33, 34]. Telomerase is considered as a potential target of cancer therapy because malignant gliomas are predominantly telomerase-positive, while normal brain tissues do not express the enzyme [78, 79]. We have shown that a 19-mer antisense oligonucleotide against human telomerase RNA linked to a $2'$, $5'$-oligoadenylate (2-5A) inhibited malignant tumors growth in subcutaneous or brain tumor models in mice [25, 33, 34]. Interestingly, inhibition of telomerase activity resulted in apoptotic cell death. Angiogenic factors are also potentially optimal targets for antisense oligonucleotide because malignant gliomas are highly angiogenic. The antisense therapy against vascular endothelial growth factor (VEGF) was useful in down-regulation of VEGF expression, resulting in inhibition of growth of malignant glioma cells in vivo [80, 81].

Naked DNA plasmid

Gene transfer with naked DNA plasmid in the presence of the modified liposomes such as cationic liposome or the hemagglutinating virus of Japan (HVJ) liposome has been developed and applied to the treatment of malignant tumors. Multiple intratumoral administrations of liposomes containing the murine IFN-β gene resulted in the reduction of tumors in the brains of mice and elicited cytotoxic T lymphocytes without side effects [49]. Intramuscular injection of DNA plasmid encoding murine IFN-α leads to potent antitumor effects in mice bearing subcutaneous glioma cells [82]. Moreover, this gene transfer system has been applied to the treatment using the suicide gene [83], apoptosis inducible-genes [32], because this delivery system can be repeated.

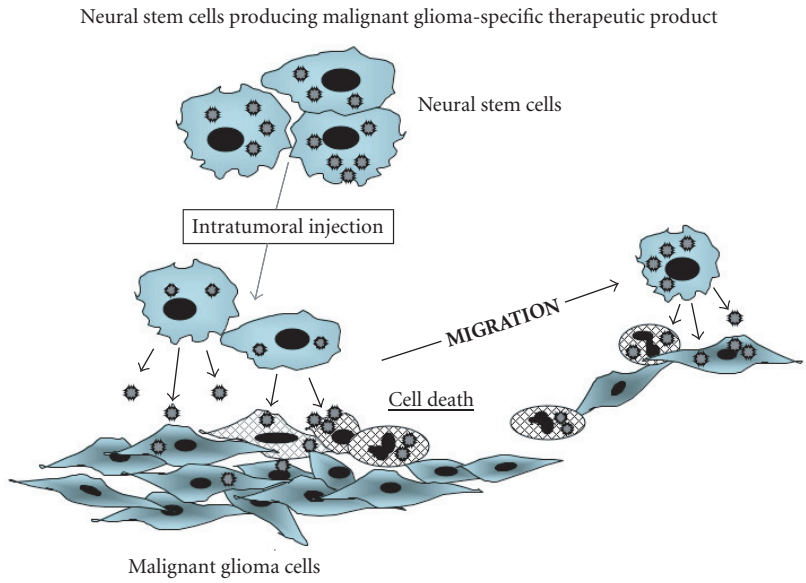

Figure 1. Prospective gene therapy for malignant gliomas.

Table 3. Delivery systems.

	References
Direct intratumoral injection	[5, 8, 9, 13, 14, 15, 25, 32, 33, 34, 36, 37, 38, 39, 40, 44, 47, 49, 56, 57, 80, 81, 82]
Osmotic minipump	[83]
Polymer/microsphere	[84, 85, 86]
Neural stem cell	[59, 60, 61]

Table 4. Targeting.

	References
HTERT	[32, 87, 88]
MBP	[30, 31]
EGFR	[90, 91]
Stress (hyperthermia, hypoxia)	[92, 93]

DELIVERY SYSTEMS

The transfer of therapeutic genes into malignant brain tumors is the subject of experimental models and clinical trial of gene therapy (Table 3). Most approaches have used direct intratumoral placement of a variety of vectors and genes, such as retroviruses [8, 13, 15, 44, 57], replication-defective adenoviruses [5, 14], replication-competent or-conditioned oncolytic viruses [36, 37, 38, 39, 40], antisense oligonucleotides [25, 33, 34, 80, 81], and naked plasmid vectors [32, 49, 82, 83]. These approaches have shown the efficiency of gene therapy. However, these approaches need to repeat injections or are unable to keep continuous gene expression.

Using a minipump combined with stereotaxic techniques allows continuous delivery of therapeutic genetic materials into the brain. Continuous intracerebral delivery of liposome-mediated HSV-*tk* gene complexes using an osmotic minipump led to tumor regression in the treated animal [83]. Polymer microspheres can encapsule antisense oligonucleotide, naked DNA plasmid, viral particle, or monoclonal antibody, and release them. This system not only decreases treatment frequency, but also reduces the potent immune response by sequestering the content from antibody exposure, leading to improvement of in vivo efficacy [84, 85, 86]. However, the above systems are unable to effectively distribute the genetic materials into the target cell population. As described above (Table 1), neural stem cells may have the potential to chase the infiltrating tumor cells [59, 60, 61].

TARGETING

Gene transfer vectors will dramatically increase the safety and effectiveness of cancer gene therapy, if they can restrict expression of the therapeutic products to the target tumors. Substances that are overexpressed in tumor cells but not in normal cells are good targets for gene therapy (Table 4). Although no specific marker is known for malignant gliomas, four targets including us came up with expression regulating system using specific promoters. First, we used the promoter of the human telomerase reverse transcriptase (hTERT) gene and developed expression vectors of caspase-6 [32], caspase-8 [87], or FADD [88] under the control of the promoter, respectively. The activity of telomerase is tightly regulated at the

transcriptional level of the hTERT gene [89]. Since about 75% of malignant gliomas have telomerase activity while normal brain tissues do not have the enzyme [78, 79], we can restrict the expression of apoptosis-inducing proteins to malignant glioma cells. We showed that the growth of subcutaneous tumors was inhibited due to induction of apoptosis after the treatment [32, 87, 88]. Second, Shinoura et al used the myelin basic protein (MBP) promoter to regulate the expression of Bax and caspase-8 [30, 31]. They showed that the growth of tumors in the animal model was suppressed. Third, epidermal growth factor receptor (EGFR) is often highly expressed in tumor but not in normal brain. EGFR may be a good target to increase the selectivity of delivering genes to tumor cells [90, 91]. Forth, stress can also be a target for tumor specific expression of therapeutic genes. The presence of hypoxic cells in human brain tumor is an important factor leading to resistance to radiation therapy. However, this physiological difference between tumor and normal tissues also provides the potential for designing cancer-specific gene therapy [92]. When the gene expression is triggered by heat stress, combined therapeutic effects of hyperthermia and gene therapy may be promising [93].

CONCLUSIONS

In recent years, many neuro-oncologists have focused on evolving gene therapy as a new therapeutic modality for malignant gliomas. However, clinical success has been limited due to insufficient gene transfer or limited spread of therapeutic genes. These obstacles may be overcome by neural stem cell-guided gene therapy (Figure 1). Neural stem cells are expected to migrate extensively into malignant gliomas in the brain, although further investigation is necessary. Therefore, the application of neural stem cells producing tumor-specific therapeutic product will offer a means of accessing invasive tumor cells. If we can engineer neural stem cells to produce oncolytic virus as tumor-specific therapeutic product, this approach may be significantly enhanced. It is expected that replication-conditional or competent oncolytic virus will significantly increase the extent of gene transfer into tumors compared to replication-defective virus. However, issues such as controlling virus replication and keeping normal cells intact should be confirmed. The use of tumor-specific promoter such as hTERT promoter system in the brain will increase the safety of oncolytic virus produced by neural stem cells.

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REVIEW ARTICLE

Gene Therapy Applications to Cancer Treatment

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Over the past ten years significant advances have been made in the fields of gene therapy and tumour immunology, such that there now exists a considerable body of evidence validating the proof in the principle of gene therapy based cancer vaccines. While clinical benefit has so far been marginal, data from preclinical and early clinical trials of gene therapy combined with standard therapies are strongly suggestive of additional benefit. Many reasons have been proposed to explain the paucity of clinical responses to single agent vaccination strategies including the poor antigenicity of tumour cells and the development of tolerance through down-regulation of MHC, costimulatory, signal transduction, and other molecules essential for the generation of strong immune responses. In addition, there is now evidence from animal models that the growing tumour may actively inhibit the host immune response. Removal of the primary tumour prior to T cell transfer from the spleen of cancer bearing animals, led to effective tumour cell line specific immunity in the recipient mouse suggesting that there is an ongoing tumour-host interaction. This model also illustrates the potential difficulties of clinical vaccine trials in patients with advanced stage disease.

INTRODUCTION

In spite of the slow clinical progress, efforts to develop specific nontoxic cancer therapies are increasing exponentially [1, 2, 3, 4, 5, 6, 7], with the result that over 500 gene therapy trials have been listed with the FDA to date [8]. A number of strategies are currently being pursued in cancer treatment, aiming to either

(i) enhance immunological rejection of the tumour by the host,

(ii) decrease tumour cell proliferation and increase cell cycle control by restoring functions such as p53 and RB,

(iii) specifically poison tumour cells according to a 2-step design; incorporation of an enzyme followed by administration of a prodrug to be specifically activated in tumour cells harbouring the enzyme, or

(iv) specifically lyse tumour cells defective in the p53 or RB pathways using oncolytic viruses which are able to invade the "defective" tumour cells.

VECTORS (TABLE 1)

Genetic material is optimally transported into host cells by naturally evolved vectors such as viruses or bacteria. Efforts are ongoing to improve on natures' designs with increasingly sophisticated vector systems aimed at allowing prolonged transgene expression at high titre in the desired cell type whilst remaining nontoxic to the host [9]. Ideally, vectors should also carry a low risk of recombination with wild-type pathogens. Currently, the most promising approaches are based on replication-competent agents that allow efficient tumour penetration. Exciting results are anticipated with poxviruses [10, 11] and with selectively replicating/targeted adenoviruses [12, 13, 14, 15, 16], although pre-clinical models suggest that significant response rates will only be achieved by combination with standard therapies.

Poxviruses

Vaccinia virus (VV)-based strategies have been brought to clinical fruition by a number of different sources [17, 18, 19]. The large potential size (25 kb) of the gene insert, the absence of viral integration into the host cellular genome, and the excellent immune stimulation induced by this virus all combine to make it an attractive candidate for immune based therapy in cancer. Vaccinia virus infects all cells, however the host immune response to the vector does not abrogate the tumour immune response even following repeated injections. The availability of attenuated virus (*tk*- modified *vaccinia ankara*) [10] allows the use of vaccinia in immuno-delicate cancer patients and there is evidence that this vector enhances immunological rejection of the tumour.

In preclinical studies, use of a diversified immunization scheme employing a recombinant vaccinia virus followed by recombinant avian pox virus was shown to be superior to the use of either vector alone in eliciting

Table 1. Gene therapy vectors.

Vector	Preexisting immunity	Proliferation needed	Genome integration	Pathogenicity	Viral persistence	Specificity	Limitations (Viral titres and safety)
Adenovirus	+	–	No	+ + +	No	CAR receptors	+
AAV	+	–	?	No	Yes		
Retrovirus	–	Yes	Yes	No	Yes		+
Lentivirus	–	–	Yes	No ?	Yes	CD4 +	+
Poxvirus	+/–	–	No	No	No		
Bacterial vvectors, eg, salmonella	?	–	No	Antibiotics	No	Inflammation	?
Liposomes	–	–	No	–	–		–
Naked DNA	–	–	?	No	No		

CEA-specific T-cell responses. Multiple boosts of ALVAC-CEA following rV-CEA priming further potentiated the antitumour effect and CEA specific T-cell response [20]. Using tetrameric-MHC complexes *ex vivo* as well as lytic assays, Estcourt et al [21] were able to show that "prime-boost" immunization with DNA vaccines and recombinant poxvirus vectors generates high frequencies of cytotoxic T lymphocytes (CTL) that recognize target cells expressing very low levels of the specific antigen. These cells persisted for at least 6 months [21]. Harrington et al [22] quantified the T-cell responses to both the viral vector and the insert following infection of mice with VV expressing a CTL epitope (NP118–126) from lymphocytic choriomeningitis virus and demonstrated potent and long-lasting CD8 and CD4 T-cell responses to the vector peaking at approximately 1 week. These numbers decreased to approximately 5×10^5 CD8 T cells (approximately 5% frequency) and approximately 10^5 CD4 T cells (approximately 0.5% frequency), respectively, by day 30, at which levels they were stably maintained for over 300 days. The CD8 T-cell response to the foreign gene (NP118-126 epitope) was correlated with the response to the vector during all three phases (expansion, contraction, and memory) of the T-cell response [22].

Clinical results are still limited to marginal benefit but the proof of concept is established. Responses to an intradermally administered live vaccinia virus HPV 16 and 18 E6/E7 gene construct (TA-HPV, Cantab Pharmaceuticals) were seen in 1/3 of the evaluable patients with advanced cervical cancer, in 3/12 CIN III volunteers, and in 4/29 patients with early invasive cervical cancer [19]. A HLA-A*O201 restricted CD8 T cell response has also been recorded in the single HLA-A*O201 patient whose tumour was shown to be HPV16 positive. Vaccination in breast cancer patients using a poxvirus vector, MUC1, and IL-2 was well tolerated [23] and did exhibit evidence of some clinical activity (unpublished results, 2002). Common toxicities included a local skin reaction at the site of the vaccine, usually of 4–5 days' duration, and mild flu-like symptoms of 1–2 days' duration. Cellular immune response did not correlate with clinical response. The presence of a strong immunogenic vector appears to be important, since vaccination in the absence of a viral vector (MUC1-KLH conjugate plus QS-21) while immunogenic (high IgM and IgG antibody titers against synthetic MUC1), did not result in a cellular immune response in breast cancer patients [24].

Adenoviral vectors and adeno associated vectors. [8, 12, 25, 26]

Adenoviral vectors also have a large transgene capacity, a high level of expression, and can infect a large variety of cell types, however limitations are the absence of adenoviral receptor expression in certain cell types and the strong preexisting immunity, which limits transgene expression. In this regard, a direct relationship between low susceptibility of tumours to adenovirus injections and the absence of CAR (Coxsackie adenovirus receptor) expression on tumour cells has been demonstrated.

Ongoing preclinical emphasis is on designing improved, better targeted, and infectivity-enhanced adenoviral vectors. Since CAR deficiency in tumours clearly limits current adenovirus-based therapies, the tropism has been altered through genetic modification of the adenovirus capsid by mutating critical residues in the fibre knob [1] such that tumour cells can be infected via CAR independent mechanisms [27]. Double mutant AdV additionally lacking the integrin-binding penton base RGD motif were shown to efficiently target epidermal growth factor receptor or epithelial cell adhesion molecules, depending on the choice of the bispecific linker, resulting in a relative glioma/normal brain transduction ratio of 60 times that achieved with native AdV. Adenovirus-mediated IFN-γR gene transfer was shown to be effective

in augmenting the biological activity of IFN-γ, a strategy which should be useful in studying other applications of cytokine receptor-based gene therapy for cancer [28]. Regarding the transfer of p53, Ad5CMV-p53-infected cells underwent apoptosis, and cell growth was greatly suppressed. Ad5CMV-p53 treatment significantly reduced the volumes of established subcutaneous tumors in vivo [29]. In another model using stably transfected mammary carcinoma cells, a dominant negative (DN) mutant of EGFR, (EGFR-CD533) could act as a potent inhibitor of EGFR (epithelial growth factor receptor) and its cytoprotective signaling after exposure to ionizing radiation. In a genetic approach, using replication-incompetent adenovirus-mediated transfer of EGFR-CD533, the vector was able to enhance the radiosensitivity in vitro of representative cell lines [30]. Adenovirus-mediated expression of dominant negative-estrogen receptor-induced apoptosis in breast cancer cells and regression of tumors in nude mice [31]. In a different approach, the antisense RNA transcript of the E6 and E7 genes of human papillomavirus (HPV) 16 were transfected into cervical cancer cells harbouring HPV 16, via a recombinant adenoviral vector, Ad5CMV-HPV 16 AS. Expression of these genes suppressed greatly the growth of the Ad5CMV-HPV 16 anti-sense infected cells [32]. A rapid induction of cytotoxic T-cell response against cervical cancer cells by human papillomavirus type 16 E6 antigen gene delivery into human dendritic cells was also demonstrated using an adeno-associated virus vector [33].

Clinical results

The majority of patients who have been treated with adenovirus vectors received them with the aim of replacing defective genes, in particular p53, however, thus far clinical efficacy has been limited [34]. Testing by PCR for adenovirus shedding in body fluids of NSCLC patients injected intratumorally with adenoviral vectors at doses of 10^7–10^9 plaque forming units, revealed detectable viral genome for up to 90 days after injection. Screening of the clinical staff proved consistently negative and did not provoke a rise in antivirus antibody titres. (Escudier B, Institut Gustave Roussy, personal communication, NDDO meeting, Valencia, 2001, oral presentation.) Novel strategies that exploit our knowledge of the function and regulation of p53 are being actively investigated [35, 36, 37]. Intravesical instillation of Adenovirus p53 (SCH 5850) combined with a transduction-enhancing agent is safe, feasible, and biologically active in patients with bladder cancer [38]. Direct bronchoscopic injection of Adp53 into endobronchial NSCLC is safe and with acceptable levels of toxicity. Initial clinical results demonstrating relief of airway obstruction warrant further clinical investigation [39].

Conditionally replicative adenovirus vectors with oncolytic potential [14, 15, 16, 40, 41, 42, 43]

While overall approximately 50% of tumour cells are defective in the p53 pathway, it is estimated that one hundred percent of tumour cells present one of several defects in the Rb pathway, the most prevalent being p16 mutations, cyclin D amplification, HPV E7 overexpression, or a defective Rb expression itself.

Preclinical studies

The cumulated deletions of two E1B-gene fragments (E1B 19K and E1B 55K) in Adl 118, engineered by Ramon and Cajal [42] resulted in clear cytopathic effects in most human cancer cell lines. Intravenous injection of this conditionally replicative adenovirus, in an adjuvant situation after excision of the primary tumour, reduced metastatic disease and could eventually be seen as a strategy to prevent tumour metastasis in high risk breast carcinomas. These results were improved on with concomitant use of chemotherapy. Another potent adenovirus, (ONYX 411, carrying an E1A mutation in the Rb binding domain) was significantly superior to ONYX 015 in all models. The E1A gene of ONYX 411 is not complexed by Rb (if Rb is still expressed) allowing the virus to replicate even in the presence of Rb. Tumour cells have high levels of free E2F and therefore genes that have E2F responsive elements (E1A, TS, TK, dhfr, E2F itself etc.) should be more highly expressed in tumour cells. High E2F levels in tumour cells will also drive viral E1A expression allowing effective tumour cell kill by the virus. Similar oncolytic adenoviruses with selectivity for Rb pathways but without the CR2 mutation are also under development. Another strategy is to utilize tumour selective promoters to control early viral gene expression. Insertion of the E3 region enhances selectivity in tumour cell killing. E3 is composed of a series of genes involved in evasion of immune cell control, decrease in host cell MHC, Fas, and TNF expression and gives a consistent better tumour cell to normal cell kill-ratio. The efficacy of these new vectors has been shown in xenograft models following intratumour injection. Another recombinant adenovirus vector in which p53-dependent expression of a fusion protein (E2F-Rb) selectively attenuated viral replication in normal cells, was further modified by insertion of the viral late promoter (MLP) in the E3 region with the aim of driving overexpression of Ad5-E3 11.6K protein, thereby increasing cytotoxicity in tumour cells, while decreasing cytotoxicity in normal cells. Selective targeting could be achieved by Ad5-Delta 24RGD, an adenovirus selectively replication-competent in cells defective in the Rb/p16 pathway, such as ovarian cancer cells. The fiber of Ad5-Delta 24RGD contains an integrin binding RGD-4C motif, allowing Coxsackie adenovirus receptor-independent infection of cancer cells [44].

Clinical results

Over 230 cancer patients have been treated to date with the dl-1520 (ONYX-015 [15]) a replication-selective adenovirus. Kirn recently confirmed excellent tolerance using various injection routes, and documented reproducible evidence of viral replication. Tumour regression

was seen following treatment with single agent therapy in H&N cancer patients (15–20%) but not in other tumours. An early clinical trial of intraperitoneal delivery, efficacious in nude mouse-human ovarian carcinomatosis xenografts, showed no major toxicity without clinical response [16].

Other vector systems

Reovirus is an ubiquitous and relatively benign virus which may infect cells of the upper respiratory and GI tracts of humans, but is usually asymptomatic. Based on the finding that cells become highly susceptible to reovirus upon transformation with oncogenes in the Ras signalling pathway, administration of reovirus in cancer bearing animals confirmed a specific antitumour activity which could be enhanced by combination with chemotherapy and immune suppressive drugs. In vivo studies of reovirus therapy revealed that viral administration caused tumour regression in an MDA-MB-435S mammary fat pad model in severe combined immunodeficient mice [45].

Evidence of antitumour activity of the G207 herpesvirus vector in a phase I study of malignant glioma was shown by MRI (magnetic resonance imaging). This vector was also shown to be nerve-sparing [46]. Preclinical evaluation showed increased efficacy when administered in association with either radiotherapy, Cisplatin, or cytokines such as IL-12, GM-CSF, or the costimulatory molecule B7.1 [47, 48, 49].

VNP 2009, an attenuated and genetically modified strain of *Salmonella typhimurium* showed tropism for tumour cells as well as antitumour activity in dogs with melanoma, rhabdo-myosarcoma or fibro-sarcoma 50. Shiga toxin B subunit has become a powerful tool to study retrograde transport between the plasma membrane and the endoplasmic reticulum and may be used for tumour antigen insertion and presentation by antigen presenting cells [50]. Retroviral vectors are often favoured for GPAT (gene prodrug-activated therapy), their advantages being their simple genome, the availability of AZT, and their mode of transmission which prevents epidemic outbreak. So far, tumour eradication has been obtained in vivo only when replicative, but not defective, vector systems were used to transfer a suicide gene 51. Both retroviral and lentiviral vectors were shown to be able to efficiently transduce cycling hepatocarcinoma cell lines in vitro. Following cell cycle arrest, transduction efficacy remained the same for lentiviral vectors while it decreased by 80% for retroviral vectors. The CMV promoter allowed a stronger transgene expression than the PGK promoter, but expression rapidly decreased with time due to promoter silencing [51]. Liver failure which occurred following TK expression in nontumour cells, emphasized the need to target the expression of the *tk* gene to tumor cells using a hepatoma-specific promoter such as AFP promoter.

RECOMBINANT STRATEGIES OF INTEREST

Tumour antigens

Many clinical trials in cancer are designed to enhance immune responsiveness of the host against the so-called tumour antigens. The advantage of using viral strategies to transfer tumour antigens is the potential to deliver the full length genetic information of a protein allowing it to be processed in accordance with the patients MHC type. Tumour antigens fall into three main categories.

The first are those coded by viral genomes [54, 55]. In principle, these are attractive targets for immunotherapeutic attack [56, 57, 58], since the cells capable of responding to these antigens should not have been removed from the repertoire by central tolerance-inducing mechanisms. The immune response to these exogenously coded antigens should be vigorous; therefore interference by other factors (such as peripheral tolerance or escape mechanisms) is theoretically minimal. The success of therapy directed at EBV in transplant patients and HPV in cervical cancer patients suggest that under ideal circumstances, this type of response can indeed be effective [33, 59].

The second category of antigens are self antigens altered by genetic changes and rendered more visible by overexpression. Most, if not all, tumours accumulate multiple mutations during the process of malignant transformation and provide treatment targets. Another type of altered self-antigen is exemplified by MUC1, where the altered pattern is caused by genetic changes affecting glycosylation. Just how distinct these neo-epitopes of MUC1 are, however, is called into question by evidence that most serologically detected epitopes on tumour mucins are equally seen in the lactating breast. In practice, there is a little firm evidence for the development of high frequencies of MUC1-reactive T cells in tumour bearing patients or even in those immunized with MUC1 [60]. Nevertheless, the overexpression of MUC1 by tumour cells and evidence of the generation of MUC1-specific T cells in response to vaccination [61, 62] suggest that this may be a good tumour antigen. Clinical activity has been seen with poxviral vectors carrying MUC1 (unpublished results, 2002). Poxvirus-based vaccines can reproducibly generate T-cell responses to tumours expressing CEA or PSA [63]. Disease stabilization has been seen in up to 37% of patients treated with these vaccines [64]. A phase III trial of ALVAC CEA B7.1 in colon cancer is under discussion [65]. Many clinical trials are ongoing in the prostate cancer field, the antigenic proteins to be expressed and presented to the immune system being PSA or PSMA [18] as well as MUC1. Selecting an appropriate therapeutic gene and vector system to carry the gene driven by a tissue specific promoter such as the PSA promoter (PSAP) in prostate cancer may be important [66, 67, 68]. Trials with complex designs, alternating vectors (prime-boost) [20, 21, 22, 69], and associating immune modulating agents with classical therapies are ongoing.

The remaining category of tumour antigens, originally described by Boon and colleagues, are unaltered self-antigens [70, 71] with an expression profile limited to specific tissues at certain times in development.

Immune modulatory agents

IL-12. In his introductory session at the NDDO meeting in Valencia, Woo [72] focused on preclinical models using various combinations of immuno-modulatory gene therapy for cancer. Following intrahepatic implantation of colon or breast carcinoma cells in syngeneic Balb/c mice, intratumour treatment with a recombinant adenovirus expressing murine IL-12 was followed by expression of very high IL-12 (25000 pg/ml) and Interferon gamma (6000 pg/ml) titres at the tumour site as well as tumour rejection and long term survival. This IL-12-dependent antitumour activity was shown to be mediated by NK cells, despite the fact that these tumours were MHC class-I-positive [73]. The NK antitumour response could be complemented through ligation of the 4-1BB receptor by an agonistic monoclonal antibody leading to long-term tumour-free survival in over 80% of the animals [74]. This in turn was associated with resolution of pre-established metastases in the lung (distant site) and was T cell-mediated [72]. A clinical trial using an IL-12 expression vector in patients with metastatic lesions from breast and colon cancer has been authorised by the FDA and is awaiting the GMP product. In animal models, the autoradiographic imaging of I [133]-labelled viral vector showed maximal bio-distribution in the injected tumour site with only low levels of activity in normal liver, possibly related to leakage to bile ducts through the needle puncture site.

IL-2 has a proven record of improving cancer vaccinations by expanding T cells [1]. DNA-lipid complex encoding the interleukin 2 (IL-2) gene (Leuvectin; Vical, San Diego, Calif) administered intraprostatically into the hypo-echogenic tumour lesion showed evidence of clinical efficiency based on an increase in the intensity of T-cell infiltration seen on immunohistochemical analysis of tissue samples from injected tumor sites and on increased proliferation rates of peripheral blood lymphocytes. Furthermore, transient decreases in serum prostate-specific antigen (PSA) were seen in 16 of 24 responding patients [75]. Established RM11-PSA tumors ranging in size from 500 to 1,000 mm^3 were efficiently eliminated if Ad5-PSA (adenovirus-5) priming was followed 7 days later by intratumoral injection of recombinant canarypox viruses (ALVAC) encoding interleukin-12 (IL-12), IL-2, and tumor necrosis factor-alpha. This data demonstrates the utility of an Ad5-PSA vaccine combined with cytokine gene delivery to eliminate large established tumours refractory to other intervention [76]. Intratumoral treatment of nude mice with vaccinia virus (VV) expressing interleukin 2 (IL-2) or IL-12 significantly inhibited tumour growth, however there was significant associated toxicity [77]. After four vaccinations with cytokine-transduced melanoma cells, antibodies (Abs) against vaccinating and autologous melanoma cells were generated in 62% of patients. These findings demonstrate that the identification and titration of alloreactive Ab helps to monitor the extent of immunization against cellular vaccines, while the induction of Ab reactive to antigens shared between vaccinating and autologous melanoma cells may contribute to their therapeutic efficacy [78]. The role of cytokines such as GM-CSF and IL-2 in the generation of antitumour immune responses was further demonstrated by their use in association with poxvirus vaccines. While rV-CEA was effective in priming the immune system, avipox-CEA could be given up to eight times with continued increases in CEA T-cell precursors, however further increases in CEA-specific T-cell precursors were seen when local granulocyte-macrophage colony-stimulating factor (GM-CSF) and low-dose interleukin (IL)-2 were given with subsequent vaccinations [79].

Targeted adenoviral transduction to activate cutaneous dendritic cells, was achieved by complexing virus to a bi-specific antibody, thereby neutralizing the virus receptor binding site as well as *agonistically binding to CD40* [80]. This resulted in a more selective insitu transduction of $CD1a^+$ dermal dendritic cells (DC) in a human skin explant model. DC's were shown to prime specific CTL more efficiently in vitro in an autologous restimulation protocol employing HER-2/neu as the model tumour target. However, with as little as 3–10% of tumour cell supernatant even CD40-targeted CTL had a reduced efficiency in the cancer situation. DC differentiation was hampered and cells retained the $CD14^+$ phenotype, an effect partially reversible by GM-CSF treatment. Similarly, in an orthotopic hepatocellular carcinoma model (HCC) in the rat, tumorigenicity could be abrogated by prior transfection with an adenoviral vector carrying the murine CD40 ligand [81, 82]. Tumour rejection was associated with a peak of IL-12 release on day 5 (> 700 pg/ml) and was $CD8^+$ T cell dependent. Animals developed protective immunity. Toxicity consisted of a mild increase in ALT levels with a minor infiltration of lymphocytes into normal liver.

IP10. Synergy between IL-12 and the interferon gamma inducible protein IP10 in cancer treatment was shown using a CT26 tumour model [83]. A one hundred percent eradication of both injected malignant hepatic nodules and distant tumour nodules could be achieved through co-injection of the adenoviral vectors carrying IL-12 and IP10. Antitumour activity was greatly diminished by simultaneous in vivo depletion of CD4 and $CD8^+$ T-cells. The use of the vector carrying IP10 alone or IP10 together with the IV adoptive transfer of antitumour T lymphocytes only eradicated tumour in 35% of cases.

Blockade of both the CD40-CD40L and CD80/CD86-CD28 costimulatory pathways represents a strategy to inhibit the immune response against Adenovirus vectors [84]. The CD80/CD86-CD28 costimulatory pathway can be effectively inhibited by a (stimulatory) CTLA4 fusion protein [84]. The opposite is desirable in cancer treatment

and the co-stimulatory pathway can be activated through blockade of CTLA4 and/or transfer of CD80/CD86 [85, 86]. In early stage clinical trials, the addition of B7.1 to virus-based vaccines showed some improvement in immunological response and in the number of patients with stable disease following vaccination against tumour-associated antigens [65]. ALVAC-CEA B7.1 alone (n = 30) or with GM-CSF (n = 30) was also administered to patients with advanced CEA-expressing tumors to determine whether the addition of the adjuvant GM-CSF could enhance induction of CEA-specific T cells [87]. All of the patients had evidence of leukocytic infiltration and CEA expression in vaccine biopsy sites. In the patients receiving GM-CSF, infiltration by leukocytes but not lymphocytes was greater. Designs of increasing complexity are being currently explored [88]. A diversified prime and boost strategy using a prime with a recombinant vaccinia vector expressing CEA and the triad of costimulatory molecules (designated rV-CEA/TRICOM) and a boost with rF-CEA/TRICOM was more potent in inducing CEA-specific T-cell responses than the repeated use of rF-CEA/TRICOM alone. The addition of GM-CSF-enhanced CEA-specific T-cell responses. These studies demonstrate that the use of cytokines and diversified prime and boost regimens can be combined with the use of recombinant vectors [89, 90].

Replacing defective genes (p53, BRCA1, RB, p16) [35, 38]

Genes that are mutated or deleted in cancer include the cancer susceptibility genes p53 and BRCA1 [91]. Both p53 and BRCA1 appear to inhibit cancer cells that lack mutations in these genes, suggesting that the so-called gene correction strategies may have broader potential than initially believed [92]. p16, also called *MTS1* (multiple tumor suppressive gene 1) is known to be an important tumour suppressor gene especially in nonsmall cell lung cancer [93]. Extensive effort may have been put prematurely into large scale phase III trials which in essence confirmed the excellent tolerance of these vectors, with little clinical activity as single agents, strongly suggesting a need for review of concept [94]. Over 900 patients have been treated by gene transfer products (nonreplication-selective AdV p53, Aventis Pharma) over a period of 5 years. Three phase II studies in patients with recurrent squamous cell carcinoma of the head & neck testing different schedules and doses of administration resulted in stable disease in 26% of patients (NDDO meeting report, Valencia, Spain). No replication competent adenovirus was detected.

Enzymes and prodrugs (TK) [95]

Genetic prodrug activation therapy depends on the conditional expression of a gene encoding an enzyme capable of converting a nontoxic prodrug into an active cytotoxic agent. An alternative strategy is to exploit the transcriptional regulatory elements of genes that display tumour selective patterns of expression [44, 96]. Examples of tissue specific patterns are those of MUC1, CEA, PSA, thyroglobulin, and calcitonin whereas tumour selective patterns include HER2 FGFR4 and VEGF [97]. In a phase I clinical trial of direct intratumour injection of an HER2-promoter-dependent cytosine deaminase (CD) plasmid in patients with skin nodules of recurrent breast cancer, restriction of cytosine deaminase expression to tumour cells was documented. Combination of the MUC1 enhancer and HER2 promoters in pancreatic cancer that expressed both MUC1 and HER2 enhanced the level of expression as shown by cDNA microarray analysis. An adenoviral vector encoding the enzyme *E.coli* nitro-reductase (NR) which reduces the prodrug CB1954 to a powerful alkylating agent under the control of the CMV promoter in primary and secondary liver cancer had some activity in tumour cells which were resistent to Cisplatin. Synergy was shown with Doxorubicin, Cisplatin and Topotecan [98].

TK gene expression has been placed under the control of the alpha-fetoprotein promoter to enhance specificity for HCC cells and to diminish tk/gancyclovir toxicity to normal cells. While 80% of animals died and 20% were cured with the original vector, this modification dramatically increased survival and reduced treatment-related toxicity.

PITFALLS IN GENE THERAPY / IMMUNOTHERAPY OF CANCER

Difficulties encountered in clinical trial design using biologicals are manifold, including the definition of optimal dose, the absence of a correlation between maximally tolerated dose (MTD) and maximal efficacy, and the sequence and frequency of injections over time among others. In addition, the frequently advanced disease stage of patients under consideration means a vast heterogeneity of tumour cells is to be expected with a highly variable expression of tumour antigens by subclones. Moreover, the heterogeneity of the genetic background in a patient population may affect the outcome and the usefulness of a particular product may be difficult to define in particular since clinical benefit is achieved only in a small fraction of patients. Prospective statistical methodologies based on MTD and clinical response are not optimal for making decisions as to whether to develop or reject the gene therapy product. Combinations with reference treatments appear to give added benefit, but synchronising the timing of injection of live viruses with potentially immune suppressive chemotherapy, as well as uncertainty surrounding how to assess the relative contribution of each product separately renders such combinations problematic. It is also well documented that the immune system in late stage disease is compromized, as evidenced by lymphopenia, low circulating $CD4^+$ T lymphocytes, and a Th2 bias in cytokine secretion, resulting in a less efficacious immune response.

T cell dysfunction, defective dendritic cell maturation, and inflammation in cancer patients

T-cell dysfunction in cancer patients has been classified by 120 experts in the field as the number-1 criteria to be evaluated against clinical response. Hallmarks of T-cell dysfunction are absent IFN-γ production, defective T cell proliferative response, low and nonstimulable TCR z chain expression, decreased signalling in T cells (Lck), and low expression of nuclear transcription factors. Dysfunctional T cells appear to be provoked, at least in part, through inadequate stimulation by immature DC [99], lacking costimulatory molecule and CD40 ligand expression. It has been repeatedly demonstrated that tumour culture supernatants contain elements which can inhibit the functional maturation of DCs [1, 100, 101], and that dendritic cells taken from patients with a variety of solid tumours, including breast cancer, have an impaired ability to stimulate allogeneic T-lymphocytes. A number of cytokines, such as IL-10 [102], IL-6 [103, 104], MCS-F (CSF-1) [105] VEGF [106, 107, 108], and soluble IL-2 receptor [109], have been associated with immunosuppression and/or poor patient survival. Ménétrier-Caux et al [1] in a comparative study demonstrated that CSF-1 (macrophage colony stimulating factor) was the dominant immuno-suppressive cytokine in renal cell cancer cell lines. In particular, CSF-1 produced by renal cell carcinoma cell lines inhibited the differentiation of DCs from $CD34^+$ progenitor cells, resulting instead in monocytic cells with a potent phagocytic activity but lacking antigen presenting function. We were further able to show that the CSF-1 induced reduction in allostimulatory function may be mediated through an effect on class-II traffic [110]. Clearly this has implications for immune based therapies. Given its physiological role, CSF-1 is an obvious candidate in the generation of these effects. CSF-1 expression by tumours is associated with extensive macrophage infiltration both in animal, and human models. In a recent publication, Lin et al [111] reported that CSF-1 is a critical factor in tumour progression and metastasis, an effect mediated through recruitment of inflammatory macrophages to the tumour site. In a clinical gene therapy trial, using VV-MUC1-IL-2 to treat patients with breast cancer, 2 out of 4 patients with low CSF-1 serum levels and high $CD4^+$ numbers at the start of treatment responded to therapy, whereas none of the patients with high CSF-1 titers and low $CD4^+$ responded (submitted).

Anti-inflammatory agents in cancer prevention and treatment

The link between chronic inflammation and the subsequent development of cancer is well established, and there is increasing evidence that these effects are mediated, at least in part, through the production of proinflammatory cytokines and other mediators of inflammation [112]. Tumour cells, tumor associated macrophages, tumour infiltrating lymphocytes, and the tumour stroma itself, secrete factors such as TNF, VEGF, GM-CSF, IL-6, and IL-10 which promote tumour progression. Effects include angiogenesis, DNA damage, induction of T cell anergy, production of proteases, and bypass of the tumour suppressor protein p53 [113]. It is because of these deleterious effects of inflammation on cancer pathogenesis that researchers are increasingly looking for ways to modify inflammation as part of cancer treatment. Breaking this cycle of chronic inflammation and immune suppression could thereby render existing therapies more efficacious.

Mediators of inflammation implicated to date include cyclo-oxygenase-2 (COX-2), which is highly induced in many solid tumours [114, 115, 116, 117, 118, 119, 120]. A role for this enzyme in tumour progression, angiogenesis, and the inhibition of apoptosis has been established in animal models [121, 122]. Moreover, epidemiological studies have established that long-term intake of nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit the enzymatic activity of COX-2, reduces the relative risk of developing colorectal cancer [123]. As a result their use as adjuvant therapeutic agents in cancer clinical trials is currently under assessment.

NSAIDs also inhibit the expression of the nuclear transcription factor NF-κB, which regulates activation of specific genes encoding for diverse proteins involved in the inflammatory response and the host immune response. These include many different cytokines and chemokines, proteins involved in immune recognition, proteins involved in the control of cellular proliferation and apoptosis (c-IAP1, cIAP-2), and cell adhesion proteins (ICAM-1). Through the regulation of genes encoding for matrix metalloproteinase 9, tissue plasminogen activator, and ICAM-1, NF-κB may also play a role in tumour metastasis. High levels of NF-κB have been demonstrated in both haematological and solid tumours, including breast, ovarian, prostate, and colon cancers [124]. In addition, preliminary results suggest that inhibition of NF-κB in association with chemotherapy may be beneficial [125, 126].

FUTURE STRATEGIES FOR CANCER TREATMENT IN PATIENTS

The need to develop adequate trial designs, to choose precisely defined endpoints, and to use methodological strategies which compare favourably with established reference treatments were recently emphasized by M. Papaluca-Amati from the preauthorization unit at the Agency for the Evaluation of Medicinal products for human use in Europe (EMEA). A major obstacle to the pan European development of clinical gene therapy protocols is the multitude of national regulatory bodies and the frequent requirement for translation into at least one other language. Furthermore, according to Dr Papaluca-Amati, common legislation is sometimes rendered problematic by the clash between Saxon and Latin cultures, exemplified in the contrasting attitudes according to which "what's not forbidden is allowed for one, whilst what is not allowed is forbidden for the other."

Future clinical trial design and evaluation of genetic therapies

Gene therapy is still in its infancy, but significant accomplishments have been achieved. The ability to transfer genes safely and successfully into animals and patients has been established and rapidly expanding preclinical evidence suggests that gene therapy will yet deliver on its promise. So far clinical response to cancer vaccines has been infrequent, but the ability to target tumour cells specifically [127] together with interesting results using a variety of vectors and transgenes in early tumour models are intriguing.

The future of cancer treatment could lie in customized treatment [128], based on the molecular properties of the tumour, utilizing combinations of novel and conventional agents. The revolution in molecular methods has allowed the development of approaches whereby cancer-specific changes can be targeted, including mutation compensation for correction of cancer-associated defects and molecular chemotherapy for delivering toxic substances and specific small molecular inhibitors of abnormally activated pathways.

The choice of vector will depend on the result to be achieved. If the expected result is increased immunogenicity, then poxvirus or adenovirus vectors will be favoured. If durable gene transfer is the goal, lentiviral vectors or liposomal vectors are ideally suited. If selective targeting for molecular chemotherapy or viral lytic agents are to be used, selectively replicating adenoviruses are optimally used. Tissue-specific promoters can be engineered into the vector such that they will be expressed in the target tissue.

The choice of the insert will depend on whether correction of cancer-associated defects is molecular chemotherapy for delivering toxic substances or an enhanced immune response against one or several specific tumour antigens is to be engineered. In the latter case, it would be important to know whether tumour MHC class-I expression is adequate or low (suggesting for instance the need for IFN-γ transfer) and whether inflammatory macrophages predominate over dendritic cells (suggesting strategies to decrease inflammation). Synergy of viral vector-based approaches with standard therapies has been documented by a number of authors and diagnosis and correction of cancer associated molecular defects can enhance the effectiveness of standard treatments. Because p53 status influences the expression of microtubule-associated proteins and hence the sensitivity of a tumour to taxanes, it is likely that p53 gene transfer could be useful in taxane refractory patients [129]. Combinations of standard therapies are extremely interesting in preclinical studies and should find their way into early clinical studies [3, 130]. Ad-p53 transfer and Cisplatin administration to GLC-82 cells exerted substantially greater therapeutic effects than the single agent treatment alone [5]. Data from Nishizaki et al suggests that a combination of gene therapy, chemotherapy, and radiation therapy may be an effective strategy for human cancer treatment [131].

Methodological aspects remain to be addressed; while single agent phase I and phase II designs appear not to be productive, the tolerance and the toxicity profile of combinations still need to be evaluated in the first instance. While the MTD is unlikely to be the most active dose, it seems reasonable to test extremes of potentially effective dosages based on preclinical studies. A flexible design allowing progressive association with standard or third biological agents, based on preclinical results, should allow tolerance assessment and a subsequent increase in the number of patients creating a phase II study if a real advantage is suggested. Multiple point surveys of molecular markers at baseline and following therapeutic interventions should shed light on the dynamic aspects of the tumour-host interactions. Finally, the development of tools to evaluate tumour-induced immune escape or drug resistance should be helpful in curbing more advanced disease. A continuous interaction with basic scientists involved in preclinical studies should permit us to define RNA expression profiles predictive of a clinical response. Statistical innovations for clinical trials include the minimax design [132] which assures the patients safety while allowing flexibility in the study.

Immunological monitoring has recently been reviewed by a group of 120 experts in the field [133]. The frequent discrepancy between clinical and immunological response in past trials was underlined and the advantages and disadvantages of the different methods (ease of assay, precision of the test, reliability of the measure) were commented upon. It is evident that immunological response documentation is most relevant at the tumour site as opposed to in peripheral PBMC and to this end, noninvasive imaging of vectors and immune competent cells might not be as futuristic as it first sounds. In vaccine based therapies, a better definition of the patients' genetic polymorphisms and immunological background should narrow the predictive window for an effective immune response.

CONCLUSIONS

Rapid clinical advances in gene therapy of cancer are to be expected. Progress will be achieved through the selection of the most likely effective therapy combinations based both on the molecular analysis of tumours as well as on preclinical studies aiming to correct given biological defects. There is little doubt that we are at the beginning of a new era in cancer treatment.

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REVIEW ARTICLE

Prodrugs for Gene-Directed Enzyme-Prodrug Therapy (Suicide Gene Therapy)

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This review focuses on the prodrugs used in suicide gene therapy. These prodrugs need to satisfy a number of criteria. They must be efficient and selective substrates for the activating enzyme, and be metabolized to potent cytotoxins preferably able to kill cells at all stages of the cell cycle. Both prodrugs and their activated species should have good distributive properties, so that the resulting bystander effects can maximize the effectiveness of the therapy, since gene transduction efficiencies are generally low. A total of 42 prodrugs explored for use in suicide gene therapy with 12 different enzymes are discussed, particularly in terms of their physiocochemical properties. An important parameter in determining bystander effects generated by passive diffusion is the lipophilicity of the activated form, a property conveniently compared by diffusion coefficients (log P for nonionizable compounds and log D_7 for compounds containing an ionizable centre). Many of the early antimetabolite-based prodrugs provide very polar activated forms that have limited abilities to diffuse across cell membranes, and rely on gap junctions between cells for their bystander effects. Several later studies have shown that more lipophilic, neutral compounds have superior diffusion-based bystander effects. Prodrugs of DNA alkylating agents, that are less cell cycle-specific than antimetabolites and more effective against noncycling tumor cells, appear in general to be more active prodrugs, requiring less prolonged dosing schedules to be effective. It is expected that continued studies to optimize the bystander effects and other properties of prodrugs and the activated species they generate will contribute to improvements in the effectiveness of suicide gene therapy.

INTRODUCTION

Gene-directed enzyme-prodrug therapy (GDEPT) or suicide gene therapy is comprised of three components; the prodrug to be activated, the enzyme (usually nonhuman) used for activation, and the delivery system for the corresponding gene [1]. Most attention to date has been directed towards development of the vector systems, on which there is a large literature. This review focuses on the small molecule prodrugs used in such therapies. Prodrugs can be considered as comprised of two major domains; a "trigger" unit that is the substrate for the activating enzyme, and an "effector" unit that is activated or released by this metabolic process, sometimes joined by a definable linker [2] (Figure 1).

As well as being an efficient and selective substrate for the activating enzyme, the prodrug itself needs to be a systemic agent, metabolically stable and able to diffuse efficiently by paracellular and/or transcellular routes to the areas in the tumor where the activating enzyme is being generated. The effector that is activated or released from the prodrug must be an effective cytotoxin (preferably able to kill cells at all stages of the cell cycle), but must also have good bystander effects (an ability to diffuse to and kill neighbouring tumor cells). Most delivery systems still have very low efficiencies of gene transduction, and the bystander effect of the effector species is important in compensating for this [3]. The physicochemical properties that govern these desirable pharmacokinetic characteristics of both prodrugs and effectors are beginning to be understood, and include molecular size, overall lipophilicity, charge, rate of metabolism, and the propensity to form reversible or irreversible complexes with cellular macromolecules.

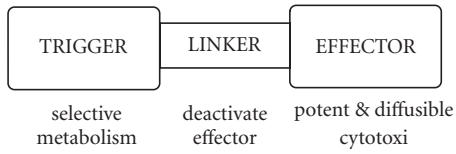


Figure 1. Trigger-linker-effector concept for prodrug design.

The next section provides a brief overview of prodrugs that have been explored for use in GDEPT, and their corresponding effectors. These are grouped according to the activating enzyme, and do not provide extensive details of relative levels of use but focus on the chemistry of the prodrugs. Table 1 provides a broad categorization of the DNA binding of the prodrugs (and their activated forms)

Table 1. Estimated physicochemical properties governing the bystander effects of GDEPT prodrugs and their principal effectors.

Prodrug			Effector		
No	log P[a]	DNA[b]	No	log P[a]	DNA[b]
1a (GCV)	−2.07	low	**1b**	−6.70[c]	low
2a (E-GCV)	7.0	low	**1b**	−6.70[c]	low
3a (PCV)	−2.03	low	**3b**	−6.71[c]	low
4a (ACV)	−1.76	low	**4b**	−6.34[c]	low
5a (VCV)	−0.78	low	**4b**	−6.34[c]	low
6a (BVDU)	−0.43	low	**6b**	−5.25[c]	low
7a (AZT)	−0.58	low	**7b**	−5.46	low
8a (MCT)	−1.77	low	**8b**	−6.49[c]	low
9a (5-FC)	−1.79[c]	low	**9b** (5-FU)	−2.17[c]	low
10a (MEP)	−0.35	low	**10b**	0.09[c]	low
11a (FAMP)	−2.32	low	**11b**	−1.26[c]	low
12a (CPA)	0.23	low	**12b**	−3.95[c]	bond[d]
13a (IFO)	0.50	low	**12b**	−3.95[c]	bond[d]
14a	0.34	low	**14b** (NABQI)	0.38	bond[d]
15a (4-IM)	0.70	low	unknown	—	bond[d]
16a (CMDA)	−4.67[c]	low	**16b**	0.05	bond[d]
17a	−3.09[c]	low	**17b**	1.66	bond[d]
18a	−1.62[c]	low	**18b**	3.73	bond[d]
19a	∼ 3.0	high	**19b** (DOX)	0.34[c]	high
20a (MTX-Phe)	2.74	low	**20b** (MTX)	−0.28	low
21a (IRT)	1.54[c]	low	**21b** (SN-38)	2.02	low
22a	2.04[c]	high	**22b**	1.51[c]	bond[d]
23a (CB1954)	1.28	low	**23b**	−0.34	bond[d]
24a (SN23862)	2.06	low	**24b**	2.50	bond[d]
29a	4.40	medium	**29b**	4.13	medium
32a	4.55	medium	**32b**	1.87	bond[d]
35a (IAA)	−0.81[c]	low	**35b**	1.35	high
36a (FIAA)	−1.09[c]	low	**36b**	1.80	bond[d]
37a (6-TX)	−0.41[c]	low	**37b**	−5.52[c]	low
38a (HM1826)	1.53	high	**19b**	0.36[c]	high
39a	0.78	high	**22b**	1.51	bond[d]
40a	0.78	high	**22b**	1.51	bond[d]
41a (5′-DFUR)	−0.82	low	**9b**	−2.17[c]	low
42a (SeMET)	−3.15[c]	low	**42b**	0.72[d,e]	low

a Calculated using Advanced Chemistry Development (ACD) Software 5.0 package.
b Estimate (based on structure only) of the level of reversible binding to DNA.
c log D at pH 7 for compounds with ionizable groups.
d Covalent alkylation of DNA.
e Calculated for S analogue.

discussed here, based on the knowledge of their structure. Most have little or no ability to bind reversibly to DNA, some are likely to have significant binding constants, and many (especially the activated forms) can covalently react with DNA. Since both reversible and irreversible binding lower the free drug concentration that drives diffusion, the level of DNA binding can significantly influence the bystander effect. The lipophilicity of both the prodrug and the activated drug also has an important influence on bystander abilities [4]. Table 1 contains log P values

(n-octanol/water partition coefficents) for the compounds, calculated using the Advanced Chemistry Development (ACD) 5.0 Software package. These calculated values are approximations, and can differ substantially from measured values in some series, but in the absence of measured values for most of the compounds serve to at least rank the compounds overall. For those possessing ionizable centres, log D_7 values are used; these take into account the log P values of the neutral and ionized forms and the relative proportions of each at pH 7.

PRODRUGS FOR THYMIDINE KINASE (TK)

Introduction

The most prominent GDEPT therapy has been the use of the herpes simplex type-1 thymidine kinase enzyme (HSV-Tk) in conjunction with a variety of guanosine-based prodrugs, compounds originally developed as antiviral (anti-herpes) agents [5, 6]. The enzyme converts these prodrugs very efficiently (much more efficiently than the corresponding endogenous kinases) to the monophosphates, which are then converted by cellular enzymes to the toxic triphosphates (Figure 2). These cause cell death by inhibition of incorporation of dGTP into DNA, and also by prevention of chain elongation [7]. Fusion proteins of HSV-Tk with green fluorescent protein showed that enzyme expression was predominantly in the nucleus of both transduced human and rodent glioma cells [8]. A study using positron-emission tomography (PET) showed that the extent of gene expression correlated with therapeutic response in glioma [9].

Ganciclovir (GCV; 1a)

This is the most widely used prodrug for HSV-Tk, and is well known in its own right as an antiviral agent [6]. It is a very hydrophilic compound (calculated log P – 2.07; see Table 1). The HSV-Tk enzyme converts this to the monophosphate (**1b**) which can then be converted by cellular enzymes to the toxic triphosphate (Figure 2). The high cytotoxicity shown by GCV in HSV-Tk transduced cells is suggested to be due to its enhanced ability, compared to related prodrugs, to incorporate into DNA without inhibiting progression through the S-phase [10]. In CHO cells, GCV is a potent inducer of chromosome breaks and sister chromatid exchange at concentrations well below those required for its gene therapy activity [11].

The monophosphate effector **1b** is more polar than GCV even as the neutral form (log P = −2.6), but is also partially ionized to an anionic species, giving a calculated partition coefficient at pH 7 (log D_7) of −6.7 (Table 1). Although this species has very limited ability to cross cell membranes by passive diffusion, HSV-Tk/GCV therapy demonstrates bystander effects in a number of models. Numerous studies have shown this is mediated primarily by gap junctional intercellular communication (GJIC) [7, 12, 13]. Because extensive gap junction networks are not common in tumors, a variety of methods to augment HSV-Tk/GCV therapy have been explored [7]. There is also extensive evidence of an immunological component to the bystander effect in vivo, in that along with the regression of HSV-*tk* transduced tumors, distant nontransduced tumors also show significant effects [14]. A T_H1-based immune response was seen in rat prostate cancer models undergoing HSV-Tk/GCV therapy [15], and a clinical study also showed elevated numbers of peripheral T and B cells, enhanced T-cell activation, and elevated serum levels of interleukin 12 during intracranial GCV treatment [16].

The major clinical use of GCV in GDEPT has been in glioblastoma, using direct injection of the tumor with the vector, followed by systemic administration of prodrug. The drug is well-tolerated, but there have been varied reports of its utility, including a recent large multicenter stage-III trial on 248 patients with newly diagnosed, previously untreated glioblastoma multiforme [17]. This trial found no benefit in either time to tumor progression nor overall survival time compared to best available therapy (surgical resection and radiotherapy), attributed to the poor rate of delivery of the HSV-*tk* gene, and possibly poor delivery of the prodrug across the blood-brain and blood-tumor barriers.

Another approach to improving therapy with GCV is the use of mutant enzymes. Several studies have showed that random [18] or semirandom [19] mutagenesis of the wild-type enzyme can provide substantial increases in both in vitro and in vivo activity, allowing lower and less immunosuppressive doses of GCV to be used. A number of mutation sites (eg, at Gln-125 and Ala-168) result in higher Km values for thymidine binding due to loss of H-bond contacts, and unaltered or even improved binding for GCV [20, 21]. This results in reduced competition between prodrug and thymidine at the active site, providing improved kinetics of conversion of GCV.

Combination therapy with HSV-Tk/GCV and other agents have also been explored. Some of these, like proteases such as trypsin or collagenase/dispase [22] are designed to improve gene delivery. Others are designed to complement the effects of the activated drug, such as stimulation of the immune system with GM-CSF in [23], which showed increased cure rates in animal models. Radiation therapy has been used both to upregulate promotors to increase gene expression [24] and to enhance the cytotoxicity of the activated drug [25]. In HSV-Tk transduced mouse RM-1 prostate cancer cells, the combination of GCV and radiation was additive at low doses of radiation, and possibly synergistic above doses of 8 Gy [26]. Retinoids increase GJIC by induction of connexin expression, and augment the efficiency of cell killing by GCV in HSV-YK transduced cell lines [27].

A variety of thymidylate synthase inhibitors, including Tomudex, 5-fluorouracil (5-FU) and (E)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) show synergistic

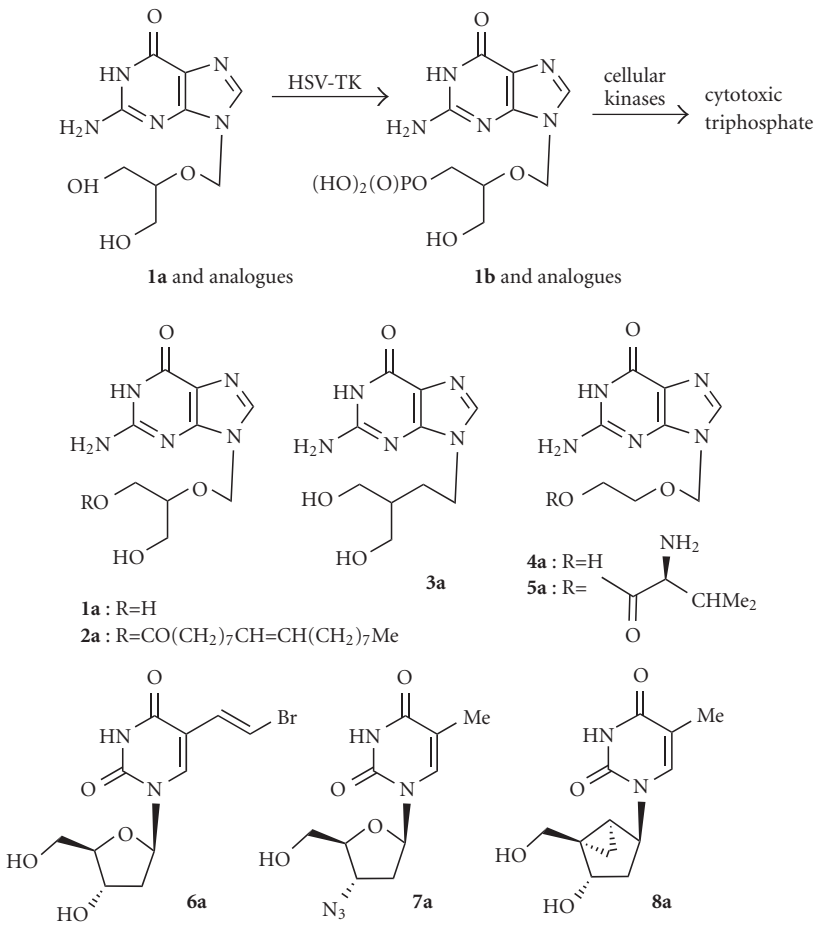


Figure 2. Activation of ganciclovir and analogues by HSV-Tk.

cell killing when used with GCV in clonogenic assay studies, while a combination of GCV and 5-FU provided significantly-enhanced survival rates in an sc HT-29 STK tumor xenograft model in mice [28]. Combinations of GCV and the topoisomerase I inhibitor topotecan also showed synergistic cell killing in HSV-Tk transduced murine MC38 and human HT-29 colon carcinoma cell lines in a clonogenic assay, and were superior to GCV alone in sc tumor xenograft models using the same cell lines in athymic nude mice [29].

Ganciclovir elaidic acid ester (E-GCV; 2a)

This very lipophilic (log P + 7.0) preprodrug of GCV is more potent than GCV itself in cell cultures (EC_{50} for E-GVC around 2 nmol), with a selectivity index (IC_{50} ratio) in FM3Atk-/HSV-1tk+ cells of > 2000 fold. Nonspecific hydrolysis of the ester gives GCV, and both GCV and E-GCV were converted to the mono-, di-, and triphosphates of GCV. However, the half-lives of both GCV and its phosphate metabolites were much longer (about 50 hours) in cells treated with E-GCV rather than GCV, suggesting the possible utility of lipophilic preprodrugs for modulating pharmacokinetics [30].

Penciclovir (PCV; 3a)

This prodrug is closely related to GVC, with the ether replaced by a CH_2 group, and has similarly low lipophilicity (log P − 2.03). PCV is less genoxic than GCV or ACV, inducing sister chromatid exchanges only at cytotoxic/apoptotic concentrations, and is only weakly clastogenic [11]. Treatment of HSV-Tk transformed baby hamster kidney cells with PCV and GCV, but not ACV, induced rapid accumulation of cells in the S-phase and apoptotic death as measured by the TUNEL assay [31], and this property, together with the low genotoxicity of PCV, makes it a relatively safe alternative drug for suicide gene therapy [32].

Acyclovir (ACV; 4a)

As Zovirax, ACV is the most frequently used antiherpes drug. It is closely-related chemically to GCV, but is slightly more lipophilic (log P − 1.76) due to one less alcohol group. ACV is a moderate genotoxin in CHO cells, inducing chromosomal aberrations and sister chromatid exchange but only at concentrations much higher than those achieved in blood plasma in vivo [11]. Comparative studies have generally shown it to be an effective prodrug

for HSV-Tk, both less toxic and less potent and (perhaps surprisingly in view of its higher lipophilicity) with lower bystander effects. However, in a study using ovarian cancer cell lines and comparing ACV and GCV at equal concentrations, the former showed equal or higher cell killing efficacy and bystander effects [33]. In other studies using U251tk human glioblastoma cells stably expressing HSV-Tk, ACV gave a lesser cell kill than did GCV (< 1.5 log compared to > 4 log) [10]. As with GCV, ACV sensitizes HSV-Tk transduced cells to radiation [34]. Clinical studies of patients with recurrent ovarian cancer treated with a replication-deficient HSV-*tk* gene vector, ACV and the topoisomerase I inhibitor topotecan showed median overall survival comparable to that of patients treated with conventional chemotherapy [35].

Valacyclovir (VCV; 5a)

Valacyclovir is a valine ester preprodrug form of ACV, which can be given orally. It is relatively lipophilic, although partial ionization of the amine counteracts this to some extent ($\log D_7 = -0.78$). In a clinical study of recurrent ovarian cancer treated with an IP-injected HSV-*tk* vector followed by topotecan and either IV ACV or oral VCV, the levels of ACV and VCV (measured as released ACV) were similar, suggesting the use of VCV as a cost-effective alternative in outpatient therapy [36]. Clinical trials of VCV and a locally-injected replication-deficient HSV-*tk* vector in prostate cancer, with [37] or without [38] concomitant radiation therapy, are in progress.

(E)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU; 6a)

This is a potent antiherpes agent recently also used as a prodrug in gene therapy, and is considerably more lipophilic than GCV ($\log P = -0.43$). An efficient synthesis has been reported [39]. BVDU exerts its cytotoxic effect not only by incorporation into replicating DNA, but also through inhibition of thymidylate synthase. It enhances the GCV-induced killing of HSV-Tk transduced glioma cells [40], although BVDU itself is reported to have a poor bystander effect, both in vitro [41, 42] and in vivo [43]. It is a particularly good substrate for the varicella zoster virus thymidine kinase (VSV-Tk). While GCV was not toxic in VSV-Tk transduced MCF7, T-47D, and MDA-MB-435 breast cancer cells, BVDU showed high cytotoxicity (IC_{50}s 600, 100, and 60 nmol, respectively) and selectivity indices (IC_{50} ratios of wild-type to VZV-Tk cells of 400, 750, and 2000, respectively). Bystander effects were not observed in vitro in MDA-MB-435 cells, but were seen in 9L rat gliosarcoma cells [44]. BVDU showed radiosensitizing activity in U-251 human glioma cells transduced with HSV-Tk, giving a sensitization enhancement ratio of 1.9.

Zidovudine (AZT; 7a)

While the anti-AIDS drug AZT is a relatively poor substrate for HSV-Tk, its antimetabolite-type mechanism of action is similar to that of the guanosine analogues antiherpes drugs discussed above, and its lipophilicity is ($\log P - 0.58$) similar to that of BVDU. Genetic modification of HSV-Tk produced mutants with reduced specificity for thymidine and much greater ability to phosphorylate AZT, due to active site mutations that better accommodate the azido group of AZT at the expense of thymidine [45].

2′-Exo-methanocarbathymidine (MCT; 8a)

The potent antiviral drug MCT is a substrate for HSV1-Tk [46], but a better one (12-fold increase in K_M) for the Y101F mutant. Crystal structure studies [47] of MCT with both enzymes show that the thymine moiety of MCT binds similarly to deoxythymidine, with the conformationally restricted bicyclo[3.1.0]hexane mimicking the sugar moiety. In MC38/HSV1-Tk murine colon cancer cells, MCT had an IC_{50} of 2.9 μmol (comparable to that of GCV), and was metabolized to the mono-, di-, and triphosphates. Treatment of MC38/HSV1-Tk tumors in C57/BL6 mice at 100 mg/kg twice daily caused complete inhibition of tumor growth [48].

PRODRUGS FOR CYTOSINE DEAMINASE (CD)

Introduction

After HSV-Tk, the cytosine deaminase gene is the next most widely studied for GDEPT. The enzyme (CD) encoded by this gene catalyzes the conversion of cytosine to uracil, and is an important member of the pyrimidine salvage pathway in prokaryotes and fungi, but is not present in multicellular eukaryotes. The crystal structure of *E. coli* CD has an $\alpha\beta$-8 barrel structure with similarity (structurally not sequence) to adenosine deaminase, but not to cytidine deaminase [49]. The enzyme is a hexamer, stabilized by domain swapping between enzyme subunits, and containing the active site in the mouth of the enzyme barrel. GDEPT therapy using CD has focused almost entirely on one prodrug, the clinically used antifungal agent 5-fluorocytosine.

5-Fluorocytosine (5-FC; 9a)

5-Fluorocytosine is a relatively hydrophilic ($\log P - 1.79$) antifungal agent with low toxicity in humans, who lack an endogenous enzyme that can activate it efficiently. However, bacterial and yeast CD enzymes convert it efficiently to 5-fluorouracil (5-FU) (**9b**). Studies with tritiated-5-FC in a human glioblastoma cell line stably transfected with the *E. coli* gene for CD showed it entered the cells relatively slowly by passive diffusion, and showed rapid efflux, suggesting that transport of this quite hydrophilic prodrug may be a limiting factor [50]. The active form 5-FC is also quite polar ($\log D_7 - 2.29$) but is a diffusible species that is itself the single most effective drug for colon cancer, being converted by cellular enzymes to the ribosyl monophosphate 5-FdUMP (Figure 3), which is an irreversible inhibitor of thymidylate synthetase. An NMR study of 5-FC in sc yeast CD-transfected human

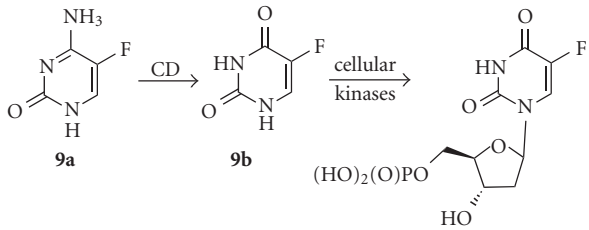


Figure 3. Activation of 5-FC by CD.

colorectal carcinoma xenografts in nude mice derived rate constants of 0.49 min^{-1} for CD-catalyzed prodrug conversion and 0.77 min^{-1} for 5-FU efflux from the tumor volume [51]. In contrast to GCV, the bystander effects of CD/5-FC therapy do not depend on gap junctional intercellular communication (GJIC), and very large effects are seen with both communication-competent and -incompetent cells [52], mediated by the diffusion of 5-FU. It has been suggested that CD/5-FU therapy in solid tumor models can generate complete cures if only 4% of the tumor cell mass express the enzyme [53].

CD/5-FC therapy has been studied, usually with the *E. coli* enzyme, in a wide variety of cancers, perhaps most notably colon using the carcinoembryonic antigen (CEA) promoter [53, 54]. MDA-MB-231 breast carcinoma cells transfected with *E. coli* CD were sensitized 1000 fold to 5-FC in culture, with only 10% of the infected cells needed to induce complete cytotoxicity of in cocultures with noninfected cells. Sc MDA-MB-231 breast carcinoma xenografts in nude mice [55] and intracranial human glioma xenografts in scid mice [56] were controlled by an intratumoral dose of an adenovirus encoding *E. coli* CD and systemic 5-FU. Similar studies have shown the utility of CD/5-FC in hepatic metastases of colon carcinoma [57] and prostate cancer [58]. Studies using Saccharomyces cerevisiae CD, which has a K_M for 5-FC about 22-fold lower than that of *E. coli* CD show that this enzyme also shows superior results in sc HT29 human colon carcinoma xenografts [59], producing about 15-fold more 5-FU in tumors at the same dose of 5-FC and greater radiosensitization [60]. When 5-FC/CD and HSV-Tk/GCV therapies were compared in a variety of in vivo models, both appeared of similar efficacy in hepatocellular carcinoma [61], but CD/5-FC was clearly superior in EBV-associated lymphomas [62], renal cell carcinoma [63], and colorectal carcinoma [64], attributed to its superior bystander effect.

Coexpression of CD together with *E. coli* uracil phosphoribosyltransferase (UPRT), which is absent in mammalian cells and which directly converts 5-FU to the 5′-monophosphate (5′-FdUMP), is synergistic. 9L glioma cells expressing both enzymes were 375-fold more sensitive to 5-FC than cells transduced with CD alone [65]. Cells expressing both enzymes showed large increases in 5-FdUMP, 5-fluorouridine triphosphate, incorporation into RNA and inhibition of thymidylate synthase [66]. Coexpression of both CD and HSV-Tk enzymes was also shown to be synergistic, both in vitro [68] and in sc rat 9L glioma tumors in nude mice [67] treated with 5-FC and GCV. The mechanism appears to be an enhancement of GCV phosphorylation by HSV-Tk following 5-FC treatment [68].

Combination studies of 5-FC with radiotherapy in CD-transfected tumors also show sensitization of sc xenografts of squamous cell carcinoma [69], cholangiocarcinoma [70] and colon carcinoma [71], using a dose of 800 mg/kg/day of 5-FC and from 10–50 Gy of tumor irradiation. Dose-modifying factors of up to 1.5 were observed. A Phase I clinical trial of CD linked to a tumor-specific erbB2 promotor in breast cancer patients showed significant levels of expression of the CD gene, restricted to erbB-2-positive tumor cells, in about 90% of cases [72].

PRODRUGS FOR PURINE NUCLEOSIDE PHOSPHORYLASE (PNP)

Introduction

The *E. coli* purine nucleoside phosphorylase (PNP) is a hexameric enzyme that catalyzes the reversible phosphorolysis of 6-amino-2′-deoxyribonucleosides to the free base and 2′-deoxyribose-1-phosphates, whereas the corresponding (trimeric) human enzyme only hydrolyses 6-oxopurine nucleosides. Comparative crystal structures show that the active-site location and its overall geometry are similar for the *E. coli* and human enzymes, but the subunit interactions are quite different, with the *E. coli* enzyme having more hydrophobic purine- and ribose-binding sites [73].

6-Methylpurine deoxyriboside (MEP; 10a)

The most widely used prodrug for PNP in GDEPT is 6-methylpurine deoxyriboside (MEP) ($\log D_7 - 0.35$), which is converted by PNP to more lipophilic ($\log D_7 - 0.09$) and highly diffusible metabolite **10b** (Figure 4). This has the potential advantages of being less cell cycle-specific than most antimetabolites and having excellent bystander effects. Human ovarian tumors transfected with *E. coli* PNP controlled by an SV40 promoter and implanted IP were shown to express PNP in only 0.1% of the cells after 5 days, yet treatment of these with MEP resulted in an average 49% reduction in tumor size and 30% increase in life span compared with control tumors [74]. A comparison of MEP/PNP and GCV/HSV-Tk therapy in a PC-3 human androgen-independent prostate cancer cell line showed that MEP/PNP caused more rapid cell killing at a 5–10-fold lower input of virus [75]. Against the same cells as sc tumors in nude mice, both systems showed comparable activity, holding tumor growth to about 75% of that of controls after 52 days, and providing about 20% of long-term survivors [76].

Fludarabine (FAMP; 11a)

Fludarabine (arabinofuranosyl-2-fluoroadenine mono phosphate), has also been studied as a possible prodrug

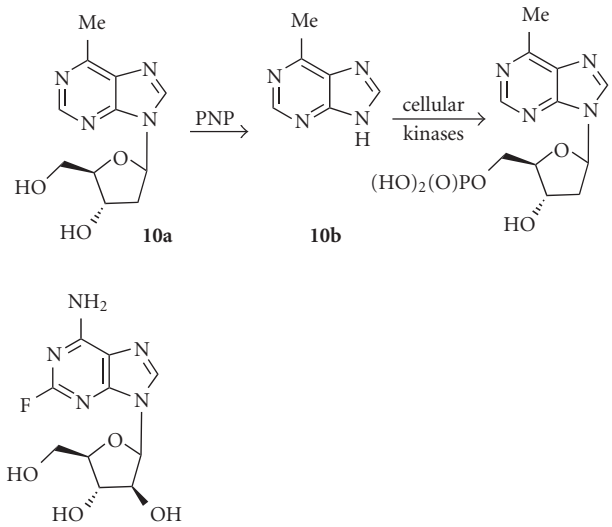


Figure 4. Activation of MEP by PNP.

for PNP gene therapy. FAMP showed good activity against sc D54MG glioma tumor xenografts expressing *E. coli* PNP, but was not quite as effective as MEP [77]. Human hepatocellular carcinoma cell lines (HepG2, Hep3B, and HuH-7) expressing PNP were highly sensitized to FAMP, showing IC_{50}s of < 1 μmol, with excellent bystander effects (better than those for GCV) [78]. Treatment of PNP-transduced HepG2 and Hep3B cells with FAMP induced p53 accumulation and the rapid onset of apoptosis, and caused similar levels of killing in both p53-positive and negative lines. This independence of FAMP-induced killing on p53 status suggests that FAMP/PNP may be superior to GCV/HSV-Tk for gene therapy of human hepatocellular carcinoma [79].

PRODRUGS FOR CYP ENZYMES

Introduction

Prodrugs for gene therapy based on NADH cytochrome P450 (CYP) enzymes are compounds that are normally activated primarily by one or more of the many CYP isozymes [80]. The large number of different isozymes, and the fact that many drugs and other xenobiotics are metabolized by them, makes the potential choice of prodrugs quite wide. Many of these enzymes are expressed to greater extent in liver than in tumor cells, so the primary goal of this strategy is to selectively increase tumor cell exposure to cytotoxic drug metabolites by targeting expression of the enzymes to tumor cells by gene vectors. To date, this area has been dominated by only two prodrugs, the alkylating agents cyclophosphamide and ifosfamide, but the range of potential compounds is expanding.

Cyclophosphamide (CPA; 12a)

This is the most widely used alkylating agent in conventional cancer chemotherapy. It is much more lipophilic than the majority of the antimetabolites discussed above (log P + 0.23) and works by a different mechanism. It is converted to the active alkylating agent phosphoramide mustard (**12b**) via initial hydroxylation to 4-hydroxycyclophosphamide (hydroxy-CPA) by CYP enzymes (primarily CYP2B1) in the liver (tumor cells generally contain only low levels of enzyme). Hydroxy-CPA is in equilibrium with the open-chain aldophosphamide, which undergoes spontaneous elimination to give acrolein and phosphoramide mustard (Figure 5a). Phosphoramide mustard is a DNA cross-linking agent that kills cells in a largely cell cycle-independent manner, and thus CPA has potential advantages over GCV and 5-FC, which are cell cycle-specific agents. While phosphoramide mustard itself is very polar ($\log D_7$ – 3.95), the more lipophilic intermediate CPA metabolites (primarily hydroxy-CPA) do not require cell-cell contact for a bystander effect, distributing by passive diffusion [81, 82]. Early studies with CPA in gene therapy utilized both retroviral- and adenoviral-mediated transduction of glioma cells with CYP2B1, and showed that this sensitized them to CPA both in vitro [83] and in vivo [81]. Protection of transduced cells from CPA-induced cytotoxicity with the CYP2B1 enzyme inhibitor metyrapone showed that this sensitization was a direct consequence of intracellular prodrug activation [81]. Since CYP-catalyzed drug metabolism is dependent on electron transfer from the flavoenzyme NADPH-P450 reductase, double transduction of rat 9L glioma cells with both enzymes substantially increased tumor cell killing [84], and inhibitors of NADPH-P450 reductase inhibited the activation of CPA by CYP enzymes [85, 86].

The use of other CYP isozymes to activate CPA has been explored. AHH-1 human lymphoblastoid cells transfected with CYP2C9 were 5-fold sensitized to CPA compared with the wild-type line (IC_{50}s 0.80 and 4.1 mmol, respectively), and showed a bystander killing effect. This sensitivity was blocked by the CYP2C9-specific inhibitor sulfaphenazole. While the sensitivity enhancement is not large, CYP2C9 and CPA may possibly be useful for GDEPT [87]. In a comparative study, 9L-rat glioma cells were transfected with six different CYP enzymes; 2B6, 2C8, 2C9, 2C18 (Met385 and Thr385 alleles), 2C19, and 3A4 [88]. Greatest sensitization to CPA was seen with CYP2B6, but CYP2C18-Met was also effective, despite a very low level of CYP protein expression (> 60-fold lower than that of 2B6), with substantial further increases upon additional transduction with NADH P450 reductase. In 9L glioma sc tumors transduced with 2B6 or 2C18-Met in immunodeficient mice, the best effects were seen with concomitant expression of NADH P450 reductase (growth delays of 25–50 days, compared with 5–6 days). Transduction of HT29 and T47D human colon carcinoma cells with CYP2B6 using a retroviral vector (MetXia-P450) sensitized these cells to CPA, and produced a substantial bystander effect in 3-D multicellular spheroid models [89]. Treatment of scid mice bearing 400 mm^3 sc tumors from 9L glioma cells transduced with

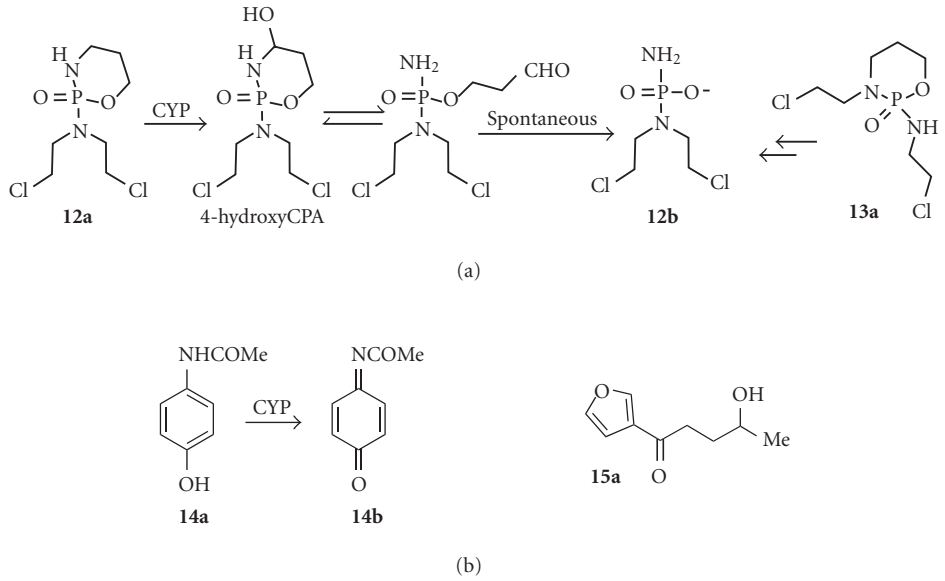


Figure 5. (a) Activation of cyclophosphamide and ifosfamide of CYP. (b) Activation of acetaminophen by CYP.

CYP2B6 and NADHP450 reductase with CPA (140 mg/kg every 6 days) achieved eradication of 6 out of 8 tumors. With larger tumors, some resistance to CPA was seen, involving loss of expression of the genes [90].

Ifosfamide (IFO; 13a)

Ifosfamide is also used clinically as a conventional anticancer drug. It is closely related both chemically and mechanistically to cyclophosphamide, having similar lipophilicity (log P + 0.50) and releasing the same ultimate metabolite, phosphoramide mustard (**12b**) (Figure 5a). Feline kidney cells transfected with CYP2B1 were sensitized to IFO, and killed via necrotic rather than apoptotic mechanisms [91]. When these cells also expressed cytosine deaminase, additive killing was seen with combinations of both IFO and 5-FU [92]. CYP2B1-transduced human embryonic epithelial cells implanted in mice bearing human PaCa-44 pancreatic tumor xenografts sensitized these to IP IFO, resulting in partial or even complete tumor ablation [93]. 9L glioma cells transduced with CYP2B6 and NADPH P450 reductase were sensitized to both CPA and the bioreductive drug tirapazamine (TPZ) to the same extent under both normoxic (20% O_2) and hypoxic (1% O_2) conditions [94]. Under hypoxia, both CPA and TPZ showed bystander effects, which is surprizingly given the nature of the highly reactive radical intermediate thought to be the active species of TPZ.

Acetaminophen (14a)

The widely used and relatively lipophilic (log P + 0.34) anti-inflammatory drug acetaminophen is oxidized by the human CYP1A2 enzyme to the cytotoxic metabolite N-acetylbenzoquinoneimine (NABQI; **14b**) (Figure 5b), which is the major source of toxicity of this drug. Transfection of H1A2 MZ cells with human CYP1A2 sensitized them to treatment with acetaminophen, with the generation of a substantial bystander effect (complete killing of V79 cells in a mixture containing 5% transduced H1A2 MZ cells) [95]. Similar bystander effects were seen with transduced SK-OV-3 human ovarian tumor cells and HCT116 human colon tumor cells, but not with MDA-MB-361 breast tumor cells. Acetaminophen is thus a possible prodrug for GDEPT in conjunction with CYP1A2.

4-Ipomeanol (4-IM; 15a)

4-Ipomeanol is a relatively lipophilic furoketone (log P + 0.70) that is efficiently converted by the rabbit CYP4B1 enzyme, but not by the corresponding human isozyme, into toxic DNA-alkylating metabolites of unreported structure [96]. Both rat (9L) and human (U87) glioma cell lines transduced with rabbit CYP4B1 were sensitized about 20 fold to 4-IM (IC_{50}s about 2.5 μmol), with an efficient bystander effect. Cell killing was associated with DNA fragmentation (TUNEL assays) and extensive protein-DNA crosslinking and single-strand breaks. In 9L sc tumor xenografts in nude mice, 4-IM induced significant growth delays. Similar results were seen in a range of human hepatocellular carcinoma cell lines (Hep3B, HuH-7, and HepG2) transduced with rabbit CYP4B1 and treated with 4-IM, except that bystander effects seemed to be cell-specific [97]. In a further comparative study [98], 4-IM treatment of CYP4B1-transduced 9L glioma cells showed very little bystander effect (much less than that shown by GVC/HSV-Tk). Thus the utility of 4-IM for GDEPT needs further clarification.

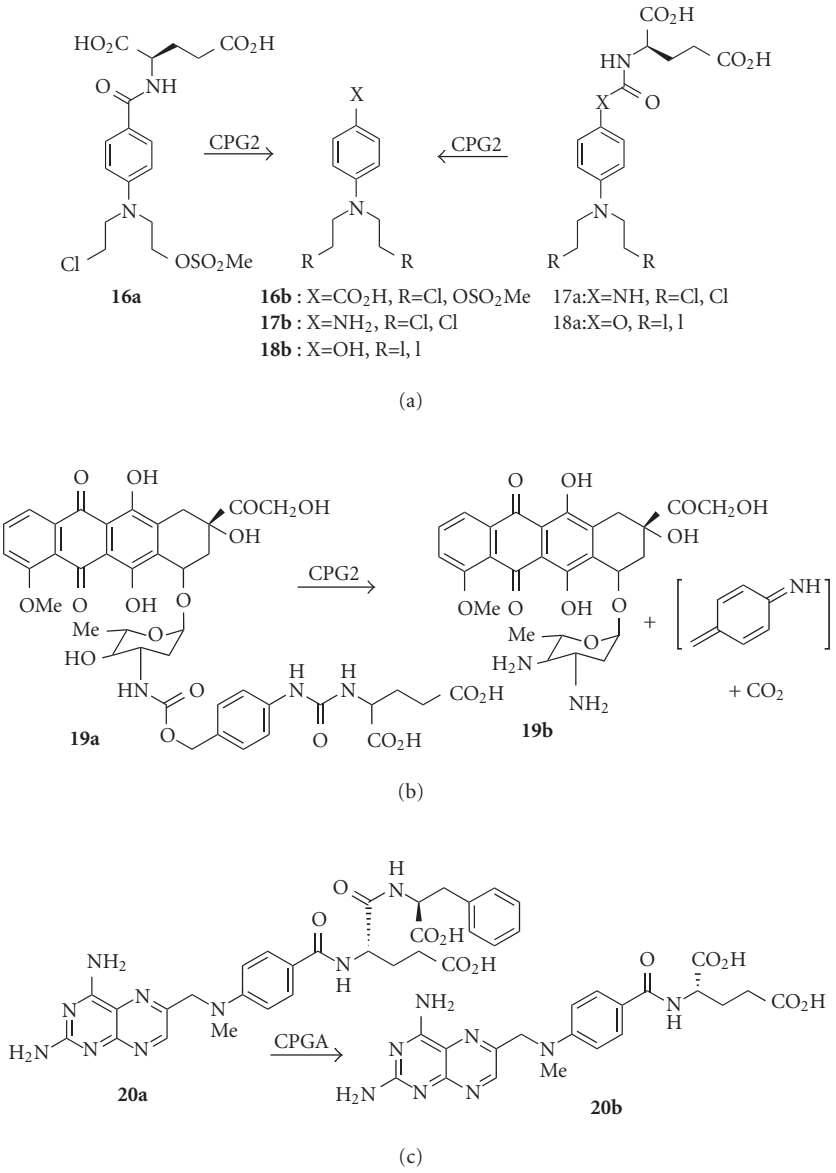


Figure 6. (a) Activation of mustards by CPG2. (b) Activation of anthracycline glutamates by CPG2. (c) Activation of methotrexate-α-peptides by CPGA.

PRODRUGS FOR CARBOXYPEPTIDASES (CP)

Introduction

This enzyme, from various species of *Pseudomonas* bacteria, and for which there is no mammalian counterpart, has been investigated for use in gene therapy because of its ability to cleave glutamate moieties [99]. Because the substrates for this enzyme are necessarily diacids, it has been used primarily in ADEPT protocols, where cell exclusion of very polar prodrugs until activation is an added benefit. However, more recently it has been adapted for use in GDEPT by being engineered for surface expression on the cells [100].

4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid (CMDA; 16a)

The very polar (log D_7 – 4.67) "mixed" chloromesylate mustard CMDA was first studied as a prodrug for ADEPT, in conjunction with the enzyme carboxypeptidase G2 (CPG2) derived from the bacterium *Pseudomonas putida*, which cleaves the glutamate to generate the active carboxylic acid species **16b** (Figure 6a) [99]. However, studies with human adenocarcinoma cell lines (A2780 and SK-OV-3) and human colon carcinoma cell lines (LS174T and WiDr) that expressed CPG2 internally showed enhanced sensitivity to CMDA over control lines (11–16 fold for WiDr and SK-OV-3, 95 fold for A2780 and LS174T),

showing these diacids do enter cells [100]. Complete cell killing was achieved with 4–12% of cells expressing the enzyme, indicating a substantial bystander effect from the much more lipophilic ($\log P + 0.05$) released aniline mustard effector **16b**. Nevertheless, later work comparing CMDA with internally-expressed and surface-tethered CPG2 enzymes in the A2780, SK-OV-3, and WiDr cell lines showed the latter was superior for activating CMDA [101]. Lower levels of enzyme and shorter exposures to prodrug were required for cell killing with surface-tethered compared to internally-expressed CPG2. The activity of CMDA against human MDA-MB-361 breast carcinoma xenografts in nude mice correlated with the percentage of CPG2-expressing cells, with some cures seen even with tumors containing 50% wild-type cells, confirming a substantial bystander effect [102].

Hydroxy- and amino-aniline mustards (eg, 17a and 18a)

Because the effector **16b** released from CMDA is only a moderately potent cytotoxin (IC_{50} 65 μm in LS174T human colon carcinoma cells [103]), more potent effectors have been sought. Thus the prodrugs **17a** and **18a** release effectors (**17b** and **18b**; Figure 6a) that are up to 70-fold more potent than **16b** (IC_{50}s in LoVo colon carcinoma cells of 1.8 μmol and 0.34 μmol, respectively) [103, 104]. These prodrugs showed IC_{50} differentials of 12–14 fold between wild-type and surface CPG2-expressing WiDr human colon tumor cell lines in culture. The iodomustard glutamate prodrug **18a**, already in clinical trial as a prodrug for ADEPT [105], also showed much greater IC_{50} differentials in surface CPG2-expressing MDA-MB-361 and WiDr cells than did CMDA (70–450 fold compared to 19–27 fold), and was highly effective against the MDA-MB-361 xenografts in nude mice [106].

Anthracycline glutamates

Prodrugs of anthracycline topoisomerase inhibitors such as doxorubin with glutamate residues directly attached to the glycoside nitrogen were not substrates for CPG2, so analogues with a 4-benzylcarbamate spacer group were investigated [107]. These prodrugs (eg, **19a**) undergo 1,6-elimination following cleavage of the glutamate by CPG2 to release doxorubicin (**19b**) (Figure 6b). MDA-MB-361 breast cancer cell lines expressing CPG2 intracellularly or tethered to the outer cell membrane showed about a 10-fold IC_{50} differential compared to wild-type cells [107].

Methotrexate α-peptides (MTX-Phe; 20a)

Methotrexate-α-peptides (eg, MTX-Phe; **20a**) are prodrugs of methotrexate (MTX; **20b**), a potent inhibitor of dihydrofolate reductase, and a widely used anticancer drug. Unlike MTX, the prodrugs are poor substrates for the reduced folate carrier, and thus not taken up by cells, but can be cleaved to methotrexate by carboxypeptidase A1 (CPGA) (Figure 6c) [108]. This endogenous enzyme is normally inactive until trypsin proteolysis of its propeptide, but a mutant form (CPAST3), activated by ubiquitously expressed intracellular propeptidases, has similar prodrug activating properties [109]. Tumor cells retrovirally transfected with a cell surface tethered version of CPAST3 were highly sensitized to MTX-Phe, which also showed an efficient bystander effect (complete cell kill with < 10% of the cells transduced).

PRODRUGS FOR CARBOXYLESTERASE (CE)

Introduction

The group of enzymes known as carboxylesterases hydrolyse esters and some carbamates to their component acids and alcohols, and are known to activate some clinical anticancer drugs, notably irinotecan [110] and KW-2189 [111].

Irinotecan (IRT; 21a)

Irinotecan is a lipophilic ($\log D_7 = 1.54$) topo I inhibitor that has been used widely clinically as an anticancer drug. It is converted to the active form SN-38 (7-ethyl-10-hydroxycamptothecin, **21b**) in humans primarily by carboxylesterase (Figure 7a). However, human CE is relatively poor at activating IRT, with rabbit liver CE being 100–1000-fold more efficient [110]. A 549 human lung adenocarcinoma cells transfected with the human carboxylesterase gene driven by the CMV promoter were able to convert IRT to the active metabolite SN-38, which showed a substantial bystander effect in cell culture [112]. When the construct was injected directly into established sc A549 tumors in nude mice, treatment with IRT resulted in 30–40% reductions in tumor size compared with controls. A panel of human tumor cell lines transduced with rabbit liver CE showed high levels of CE activity, and were sensitized by 11–127 fold to IRT [110]. This protocol has been studied as a potential purging method in autologous stem cell rescue for neuroblastoma. Transfection of neuroblastoma cell lines (SJNB-1, NB-1691, and SK-N-SH) by a replication-deficient adenoviral construct containing rabbit CE resulted in 100% transfection, and sensitized the cells to IRT by 20–50 fold [113].

Anthracycline acetals (22a)

The lipophilic doxorubicin acetal (**22a**) ($\log D_7 + 2.04$) is a substrate for CE, which hydrolyses it to an intermediate hemiacetal that undergoes immediate cyclization to the cyclic carbinolamine (**22b**) (Figure 7b) [114]. These anthracycline carbinolamines (or more likely the resultant iminium ion; Figure 7b) are known to be extremely cytotoxic agents (100–1000-fold more potent than doxorubicin itself), probably due to covalent interaction with DNA. However, the system does not appear to have been evaluated in CE-transfected cell lines, and it is likely that compounds like **22a** are too unstable to act as tumor-specific prodrugs.

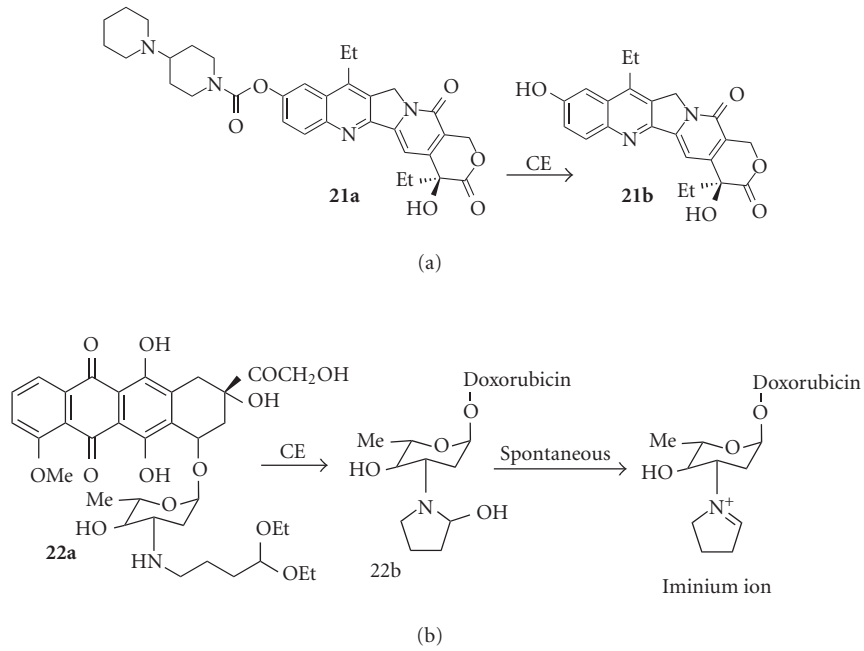


Figure 7. (a) Activation of irinotecan by CE. (b) Activation of doxorubicin acetal by CE.

PRODRUGS FOR NITROREDUCTASE (NTR)

Introduction

Enzymes that metabolize aromatic nitro groups are attractive for GDEPT because of the very large electronic change that this metabolism generates. Metabolism of a nitro group (Hammett σ_p electronic parameter = 0.78) to the corresponding hydroxylamine 4-electron reduction product ($\sigma_p = -0.34$) that is normally the major metabolite, or the possible amine 6-electron reduction product ($\sigma_p = -0.66$) (Figure 1). These are among the largest possible increases in the electronic effect ($\Delta\sigma = 1.12$ and 1.44) that can be achieved in a single metabolic step [115], and this substantial change in electron distribution in the prodrug can be exploited in a variety of ways to activate the effector. Nearly, all GDEPT studies with nitroreductases have used the nfsB gene product of *E. coli*, an oxygen-insensitive flavin mononucleotide (FMN)-containing nitroreductase (NTR) [116], with a close sequence homology to the classical nitroreductase of *S. typhimurium* [117]. Crystal structure determinations of NTR show it to be a homodimer with one FMN per monomer, with two channels leading into the active site [118]. There are relatively few contacts made with the ligand, which may contribute to its observed broad substrate specificity [119]. NTR efficiently reduces aromatic nitro groups to the hydroxylamines in a two-step ping pong bi-bi mechanism [118]. Four broad classes of prodrugs for NTR have been studied; dinitroaziridinylbenzamides, dinitrobenzamide mustards, 4-nitrobenzylcarbamates, and nitroindolines, and most work has been done with the dinitroaziridinylbenzamide CB1954.

CB1954 (23a)

The dinitroaziridinylbenzamide CB1954 (**23a**) is a relatively lipophilic (log P + 1.54) prodrug that is efficiently reduced by NTR ($k_{cat} = 360\,min^{-1}$) [117], effecting reduction of either the 4- or 2-nitro groups to the corresponding hydroxylamines at about equal rates (Figure 8a). Modelling suggests that the small aziridine residue allows the drug far enough into the binding pocket that both the 2- and 4-nitro groups have access to the FMN [118]. The 4-hydroxylamine (**23b**) is then further metabolized by cellular acetylation pathways to a cytotoxic DNA interstrand-crosslinking agent [120] (Figure 8a). CB1954 demonstrates substantial bystander effects [121], due to the cell-permeable hydroxylamine metabolite (log P – 0.34) [122]. CB1954 shows high selectivity (100–2000 fold) in a variety of NTR-transfected cell lines, including human ovarian (SKOV-3) [123], colorectal (LS174T), and pancreatic (SUIT2, BxPC3) [124], with sensitivity correlating closely with the level of NTR enzyme expression [123].

B1954 also showed excellent bystander effects in vivo, inducing long growth delays of human hepatocellular carcinoma and squamous carcinoma xenografts, even when only a minority of the tumor cells expressed the enzyme [125]. Treatment of scid mice with Burkitt lymphoma (Jijoye) tumors containing 30% NTR-expressing cells with CB1954 at 20 mg/kg/day for 10 days also gave growth inhibition, suggesting a substantial bystander effect [126]. However, expression of NTR in the luminal cells of the mammary gland using the ovine β-lactoglobulin promoter resulted in rapid and selective killing of this cell population by CB1954 with minimal effects

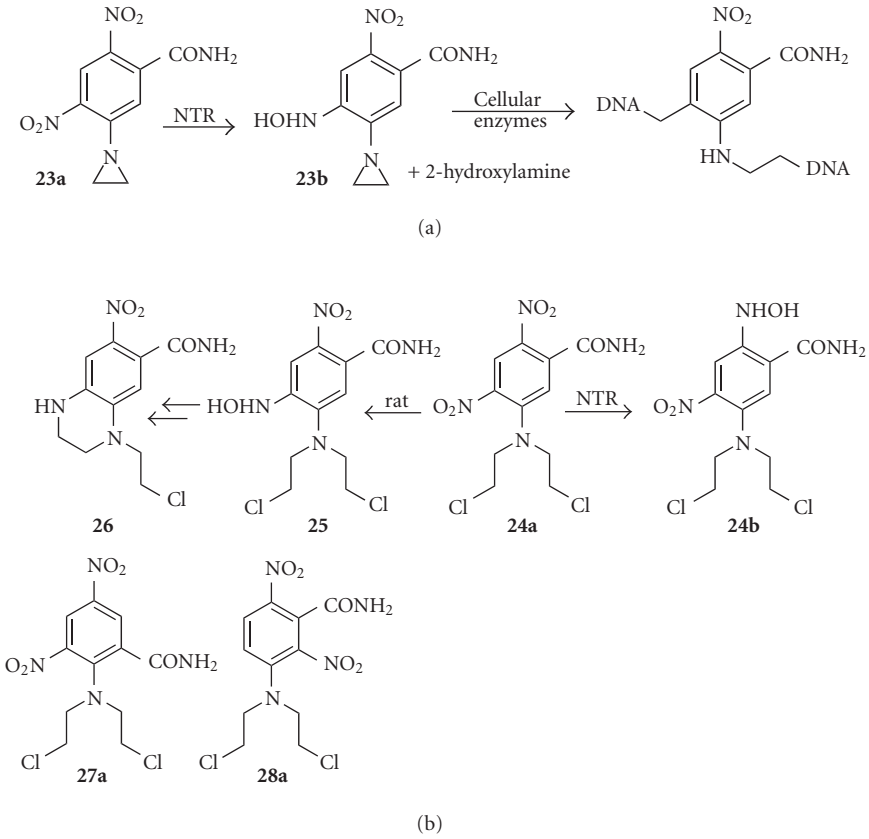


Figure 8. (a) Activation of CB1954 by NTR. (b) Activation of SN23862 by NTR.

neighbouring myoepithelial cells, suggesting that the toxic metabolite does have a finite diffusion range [127].

A Phase I clinical trial of CB1954 without NTR, administered IV on a 3-weekly cycle, determined a recommended IV dose of 24 mg/m^2. Dose-limiting toxicities at 37.5 mg/m^2 were diarrhoea and elevation of liver transaminases, but no marrow suppression or nephrotoxicity. A Phase I clinical trial of CB1954 in combination with adenovirus-delivered NTR is in progress in patients with primary and secondary liver cancer [128].

SN23862 (24a) and analogues

The dinitrobenzamide mustard SN23862 is more lipophilic than CB1954 (log P = 2.06), and a better substrate for NTR (K_{cat}1580 min^{-1}) [129]. Unlike CB1954, only the 2-nitro group is reduced (possibly because the larger mustard unit restricts drug entry to the active site of NTR) to give the lipophilic (log P = 2.50) 2-hydroxylamine (**24b**) (Figure 8b) [130]. The initial reductive step fully activates the mustard for DNA crosslinking without further metabolism. While mammalian enzymes with nitoreductase activities exist, in rats, the major endogenous reductive pathway is via the 4-nitro group, giving the 4-hydroxylamine (**25**) and ultimately the relatively nonpotent tetrahydroquinoxaline (**26**) as the major metabolite (Figure 8b) [131]. SN23862 [132] and other mustard analogues [133] are also not substrates for endogenous DT diaphorase, increasing their attractiveness as NTR prodrugs.

A recent study [4] of a series of analogues of **23a** and **24a** demonstrated good correlations between the lipophilicities of the parent prodrugs (measured log P values) and their bystander effects, measured in both 3-D cell multilayer cocultures of NTR+ve and NTR-ve V79 and WiDR cells, and also in mixed NTR+ve/NTR-ve WiDr tumors in nude mice. This study provides quantitative evidence, in a series of close analogues, of the importance of lipophilicity in determining the level of bystander effect; analogues with log P values lower than that of CB1954 showed relatively poor bystander effects.

In a series of analogues of SN23862 where the 4-nitro group was replaced by other substituents of varying electronic properties, cytotoxicity in UV4 cell cultures, from added NTR enzyme, correlated roughly with the electron-withdrawing properties of the 4-substituent [134]. The 2-amino-3, 5-dinitrobenzamide regioisomer (**27a**) of SN23862 was also a substrate for NTR, and was in fact superior, with a k_{cat} of 4540 min^{-1} [129]. Comparative cell line studies with SN23862 and the regioisomers **27a** and **28a** in NTR-transfected Chinese hamster-derived

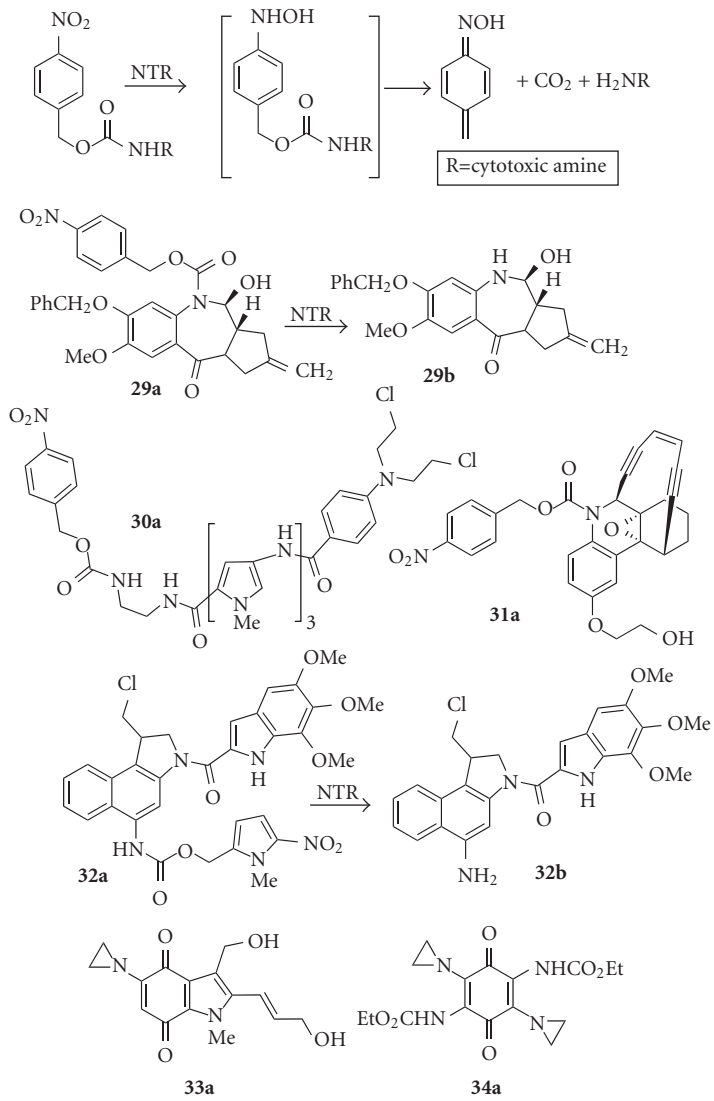


Figure 9. Activation of 4-nitrobenzyl carbamates by NTR.

cell lines showed that regioisomeric changes are permitted, with the all three retaining selective cytotoxicity. Finally, analogues with other leaving groups (bromo- and iodo-mustards) retain good selectivity for the NTR-transfected line together with higher potency [135].

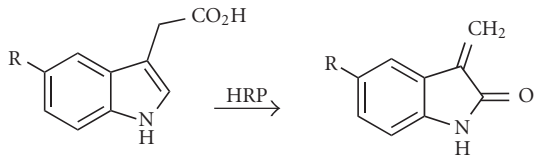
4-Nitrobenzyl carbamates

Despite their low reduction potential of around −490 mV [136], 4-nitrobenzyl carbamates are substrates for NTR, being reduced to the hydroxylamines which then undergo spontaneous fragmentation to release an amine-based effector (Figure 9) [137]. Electron-donating substituents on the benzyl ring accelerate the fragmentation step [138]. This system is theoretically very flexible, and 4-nitrobenzyl carbamates of a number of different classes of cytotoxic amines have been explored as potential GDEPT prodrugs [139].

4-Nitrobenzyl carbamate prodrugs (**29a, 30a**) of DNA major groove alkylating pyrrolo[2,1-c]benzodiazepines (**29b**) [140], and of DNA minor groove alkylating tallimustine mustards (**30b**) [141] showed differential cytotoxicity towards cells in culture when cotreated with NTR plus cofactor NADH, but studies have not been reported in NTR-transfected cell lines. The enediyne prodrug (**31a**) showed moderately selective cytotoxicity (135 fold) in the NTR-transfected WiDr human colon carcinoma cell line [142]. Nitroheterocyclic carbamate trigger units have also been reported. The 2-nitroimidazole carbamate prodrug (**32a**) of an amino-duocarmycin effector (**32b**) was 20-fold more toxic to NTR-transfected SKOV-3 human ovarian carcinoma cell line over the wild-type [143], extending the types of trigger units that can be used for this purpose.

Quinones

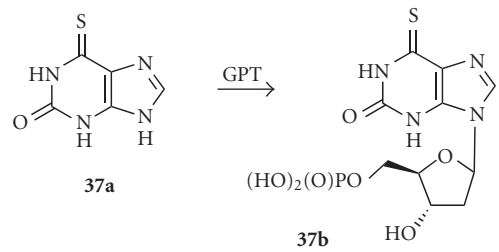
Quinones can be excellent substrates for NTR (the k_{cat} for menadione is 4.2×10^4 min^{-1} compared with 360 min^{-1} for CB1954) [117], but this is not universal; mitomycin



35a : R=H
36a : R=F

35b : R=H
36b : R=F

Figure 10. Activation of IAA and FIAA by HRP.



37a

37b

Figure 11. Activation of 6-thioxanthine by GPT.

C is a poor substrate [129]. The quinones EO9 (**33a**) and AZQ (**34a**) (Figure 10) generate DNA-reactive species when reduced, but were less than three-fold more cytotoxic in NTR-transfected V79 cells compared to wild-type cells [144].

PRODRUGS FOR HORSE-RADISH PEROXIDASE (HRP)

Introduction

HRP is an iron-containing heme peroxidase that is well known to oxidize a variety of phenols and amines, including indole-3-acetic acid, without requiring added hydrogen peroxide as a cofactor. The mechanism of oxidation by HRP has been widely studied, and shown to involve a series of free radical intermediates of varying iron oxidation levels [145].

Indole-3-acetic acid (IAA; 35a)

Indole-3-acetic acid, a catabolite of tryptophan and a plant growth hormone, is relatively nontoxic to mammalian cells. It is oxidized by HRP, initially to a nitrogen-centred radical-cation species that rapidly fragments via a carbon-centred benzyl radical. However, both of these radical species are extremely short-lived, and unlikely to account for the observed bystander effects of IAA. The 3-methylene-2-oxindole (**35b**) (Figure 10), derived from the hydroperoxide of the benzyl radical by an unclear pathway, has been suggested as the active diffusing species, able to react with DNA [145]. This is sufficiently long-lived to generate a bystander effect, and sufficiently lipophilic ($\log P + 1.35$) to diffuse rapidly by passive diffusion [146]. HRP transfection effectively sensitized human T24 bladder carcinoma cells to IAA under both normoxic and anoxic conditions. IAA also elicted a significant, selective enhancement of radiation-mediated cytotoxicity in T24 cells transiently transfected with the HRP, showing sensitizer enhancement ratios (SER) ranging from 2.6 to 5.4 [147].

5-Fluoroindole-3-acetic acid (FIAA; 36a)

This prodrug is related to IAA, and despite being less rapidly oxidized by HRP to the corresponding effector (**36b**) (rate constant $3.8 \times 10^2\,\mathrm{mol}^{-1}\mathrm{sec}^{-1}$ compared to $3.8\times10^3\,\mathrm{mol}^{-1}\mathrm{sec}^{-1}$ for IAA) is more cytotoxic than IAA in a range of HRP-transfected human and rodent tumor cell lines [148]. Plasma levels of 1 mmol and tumor levels of 200 μmol of FIAA were seen in mice bearing murine carcinoma NT tumors, after IP administration of 50 mg/kg FIAA.

PRODRUGS FOR GUANINE RIBOSYLTRANSFERASE (XGRPT)

Introduction

The *E. coli gpt* gene encodes a xanthine-guanine phosphoribosyl transferase (XGRPT) that has various xanthine and guanine analogues as substrates [149].

6-Thioxanthine (6-TX; 37a)

6-Thioxanthine (**37a**) is a relatively nontoxic compound (it is a catabolite of the clinically used antimetabolite 6-thioguanine), and is a substrate for *E. coli* XGRPT, being converted to the toxic ribophosphate (**37b**) (Figure 11). Rat C6 glioma cells retrovirally transduced with *E. coli* XGRPT were more than 20-fold sensitized to 6-TX (IC_{50} of 2.5 μmol compared to > 50 μmol) [149]. It showed only a weak bystander effect in mixed cocultures [150], which is not unexpected in view of the very hydrophilic ($\log D_7 - 5.52$), charged nature of the phosphorylated active species **37b**. The prodrug was moderately active in intracerebral C6 glioma xenografts in nude mice, with an 80% reduction in intracerebral tumor volume and a 28% increase in mean survival, following intratumoral injection of 6-TX [150].

PRODRUGS FOR GLYCOSIDASE ENZYMES

Introduction

Glycosidase enzymes have been used mostly in ADEPT, because their very hydrophilic sugar-bearing substrates show slow cell uptake. However, as with the case of the CPG2 enzyme, studies with secreted forms of β-glucuronidase and β-galactosidase show that these enzymes can also be used for GDEPT, in conjunction with anthracycline-based prodrugs. Both human and *E. coli* enzymes have been used.

HM1826 (38a)

OVCAR-3 cells transfected (using a cationic lipid transfecting agent) with plasmids encoding *E. coli*- or human β-glucuronidase were equally sensitized to both the

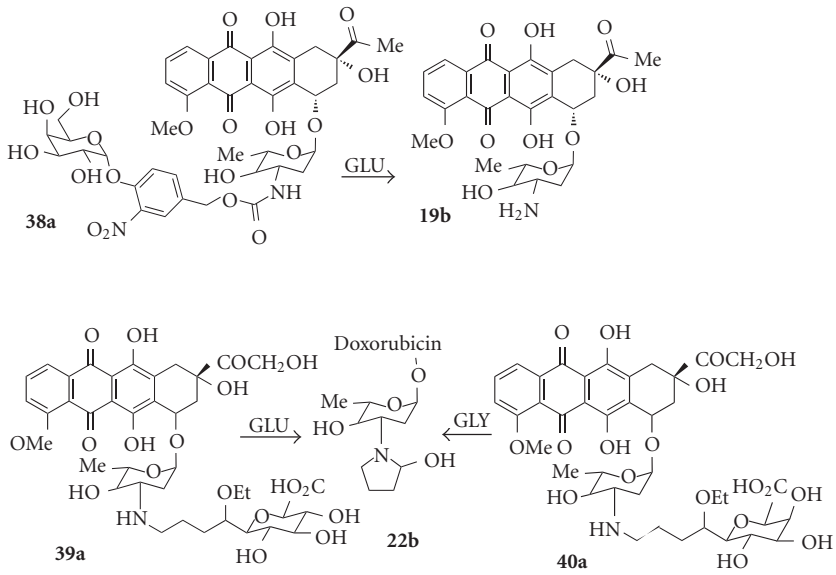


Figure 12. Activation of anthracycline by glycosidases.

glucuronide prodrug DOX-GA3 and the effector doxorubicin produced from it (Figure 12), suggesting good uptake and conversion of the prodrug, but the bacterial enzyme was more efficient. Studies with mixed cell cultures showed a strong bystander effect [151]. However, most work has been done using secreted [152] or surface-tethered [153] forms of the enzymes, with prodrugs such as HMR1826 (**38a**). Despite the high DNA binding of the released doxorubicin effector **19b**, moderate to good bystander effects were reported in culture and in vivo.

Anthracycline acetals

As noted in "Anthracycline acetals", anthracycline carbinolamines such as **22b** are extremely potent cytotoxins, probably because of their spontaneous conversion to iminium ions that can alkylate DNA. The glucuronide and galactosyl prodrugs (**39a** and **40a**, respectively) are substrates for *E. coli* β-glucuronidase and β-galactosidase, from which that release the carbinolamine **22b** (see Figure 7). In cell culture they were about 10^4- and 10^6-fold more toxic respectively to human A375 melanoma cells in the presence of the enzymes [154, 155] (see also "Anthracycline acetals"). While this is only a simple assay (the activating enzymes would need to be surface-tethered in a GDEPT application because of the very hydrophilic prodrugs), the very large differential cytotoxicity is noteworthy.

PRODRUGS FOR THYMIDINE PHOSPHORYLASE (TP)

Introduction

The endogenous enzyme thymidine phosphorylase (TP), which is reported to occur to a greater extent in tumor cells than in normal tissue, is a monomeric 55-kd enzyme that dephosphorylates thymidine and related pyrimidine antimetabolites such as 5′-deoxy-5-fluorouridine [156].

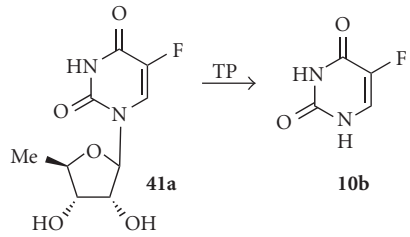


Figure 13. Activation of 5′-DFUR by TP.

5′-Deoxy-5-fluorouridine (5′-DFUR; 41a)

LS174T human colon carcinoma cells transfected with the human TP gene were up to 40-fold sensitized to 5′-DFUR, which is converted to 5-FU (**10b**) by TP (Figure 13). The degree of sensitization correlated with the extent of expression of TP, and in the cocultures a bystander effect was seen that did not require cell-cell contact [156]. Transfection of PC-9 human lung adenocarcinoma cells with platelet-derived endothelial cell growth factor (identical to human TP) resulted in a 50-fold increase in levels of enzyme expression, and sensitized them to both 5′-DFUR and tegafur [157]. MCF-7 breast cancer cells transfected with the TP had up to 165-fold increased sensitivity to 5′-DFUR (but not to 5-FU) in culture, and substantial bystander effects [158].

PRODRUGS FOR METHIONINE-α, γ-LYASE (MET)

Introduction

Many tumors are methionine-dependent, and show elevated levels of methionine synthesis [159]. Thus

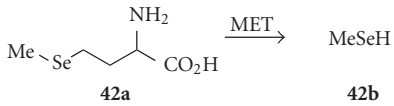


Figure 14. Activation of selenomethionine by MET.

the enzyme methionine-α, γ-lyase, which degrades the amino acid methionine to ketobutyrate, ammonia, and methylthiol, has been of therapeutic interest, and xenografts expressing the MET gene from *Pseudomonas putida* grow more slowly than wild-type tumors [160].

Selenomethionine (SeMET; 42a)

Selenomethionine (SeMET) is a relatively toxic and very polar ($\log D_7 - 3.15$) analogue of the natural amino acid methionine, and is a substrate for MET which converts it to ketobutyrate, ammonia, and the moe lipophilic methylselenol (**42b**) (Figure 14). Cells transduced by the adenovirus-delivered MET gene from *Pseudomonas putida* showed up to a 1000-fold increase in sensitivity to SeMET, and showed a strong bystander effect [160]. The enzyme converts SeMET to methylselenol, which damages mitochondria by oxidative stress (production of superoxide and other reactive oxgyen species), resulting in the release of cytochrome c and activation of the caspase cascade and apoptosis. SeMET inhibited the growth of MET-transfected rat hepatoma N1S1 ascites in nude mice, showing substantial delays in body weight gain due to growth of the tumor ascites [160].

CONCLUSIONS

There are many issues to be considered in the choice of prodrugs for a particular activating enzyme. While the most obvious are the high turnover by the enzyme and the large differential cytotoxicity between the prodrug and the activated form, another important early consideration was to select compounds (eg, GCV, 5-FU, CPA, IRT) that were already in clinical use. This avoided the regulatory difficulties associated with the combination of a new therapeutic agent and a new activating technology.

Also of importance is the nature of the cytoxicity of the activated drug. Most of the early prodrugs released antimetabolites that act only on cycling cells. However, clinical solid tumors are slow-growing and contain hypoxic regions, resulting in considerable proportions of noncycling tumor cells. This requires prolonged dosing with cell cycle-specific agents to ensure high tumor cell killing. Thus there has been increasing interest ("Prodrugs for CYP enzymes," "Prodrugs for Carboxypeptidases (CP)", and "Prodrugs for Nitroreductase (NTR)" sections) in prodrugs that release DNA cross-linking agents, a class of cytotoxins that are less cell cycle-specific. The potential advantage of these compounds is shown by the fact that single dose protocols can provide curative effects in tumor xenograft models [4].

Later studies have also drawn attention to the importance of the maximizing bystander effects, and this review has focused on the importance of the lipophilicity, charge, and macromolecular binding of both prodrugs and their effectors (Table 1). Suicide gene therapy is a promising but very complex technology, and to be broadly clinically useful will require maximization of the therapeutic properties of all of the components. We are slowly learning how to do this for the prodrugs.

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REVIEW ARTICLE

Taking Gene Therapy into the Clinic

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Gene therapy represents a promising novel treatment strategy for colorectal cancer. Preclinical data has been encouraging and several clinical trials are underway. Many phase 1 trials have proven the safety of the reagents but have yet to demonstrate significant therapeutic benefit. Ongoing efforts are being made to improve the efficiency of gene delivery and accuracy of gene targeting with the aim of enhancing antitumor potency. It is envisaged that gene therapy will be used in combination with other therapies including surgery, chemotherapy, and radiotherapy to facilitate the improvements in cancer treatments in the future.

INTRODUCTION

Currently, 600 gene therapy clinical trial protocols have been activated in the U.S., 60% of which pertain to cancer gene therapy. Nearly 3500 patients have been treated within these protocols, of which approximately 2400 were patients with cancer [1]. Similarly in the U.K., of approximately 70 gene therapy protocols approved or under review by GTAC, 70% relates to cancer gene therapy [2].

This increase in clinical trial activity is underpinned by the expansion of the number of therapeutic vectors in preclinical development and scientific innovation with respect to novel mechanistic approaches to tumour-cell kill. This article aims to provide an overview of the current clinical state of gene therapy, especially focusing on the trials, for colorectal cancer.

IMMUNE STIMULATION

The aim of immune stimulation is to activate a tumour-specific immune response, which may be either cell-mediated or antibody dependent, against the tumour cells. Several approaches to stimulate the key mediators of immune function have been tested in preclinical experiments and have now entered clinical trials, including the following approaches.

Utilization of human leukocyte antigen (HLA) to stimulate T-cell response

HLA class-I molecules are down regulated in up to 60% of colorectal cancers. Animal studies have demonstrated that the expression of foreign MHC (the analogue of HLA in humans) on tumours can induce a T cell-dependent antitumour immune response, not only to the foreign MHC but also to previously unrecognised tumour associated antigens [3].

On the basis of preclinical models, gene transfer of the HLA class-I molecule, HLA-B7, has been examined in clinical trials. In one trial, an allogeneic HLA-B7 plasmid in a lipid vector was administered via direct intratumoural injection to HLA-B7-negative patients with melanoma. Gene transfer rate was 93% when measured by polymerase chain reaction (PCR) and HLA-B7 protein was found in 50% of biopsied tumours by immunohistochemistry (IHC). Eight of 15 evaluated patients developed anti-HLA-B7 $CD8^+$ cytotoxic T cells (CTLs), and 7 patients had tumour reduction (4 partial responses) [4]. A phase II trial reported a response rate in evaluable patients approaching 15%, including two complete responses, demonstrating this to be a safe and active treatment against melanoma [5].

This trial has been extended to include patients with hepatic colorectal metastases, in which the vector was injected intratumourally under ultrasound guidance. Of 15 patients evaluated, 14 had detectable transgenic DNA by PCR, and HLA-B7 protein was detected by immunohistochemistry (IHC) in 63% of biopsied lesions. A biological response was evident as induction of B7-specific CTLs in peripheral blood of 8 patients and also infiltration of $CD8^+$ T cells into some tumours on IHC. However, no objective responses were seen [6]. This creates an interesting tension as to whether induction of a CTL response is sufficient to encourage further development of this immunogenetic approach, in the absence of bona fide reduction in tumour volume. The most obvious clinical test-bed for

this sort of treatment would be as an adjuvant following resection of the primary tumour leaving a minimal residual volume. Although logical, clinical trials of this sort require thousands of patients, an enormous commitment of resource on the basis of an immune assay which may not correlate with efficacy.

Utilization of cytokines to stimulate T cell response

Cytokines play a key role in coordinating the immune response. Therefore, the insertion of genes encoding cytokines presents a potential strategy to increase the immunogenicity of tumours and overcome immune tolerance. Preclinical models have tested a range of cytokines including interleukins 2, 4, and 12 (IL-2, -4, -12), granulocyte macrophage colony-stimulating factor (GM-CSF), and interferon gamma (IFN-γ). In general, in vivo models confirm that tumour-specific immunity can be generated by cytokine-transduced tumour cells. However, while this is often strong enough to prevent tumour formation/growth when rechallenged with new untransduced tumour cells, it is less efficient in eradicating established tumours.

Interleukin 2 as an effector

Autologous fibroblasts from 10 colorectal-cancer patients (used for their ease of growth in tissue culture and transducibility by retrovirus vectors expressing cytokines) were transduced with a retrovirus carrying the IL-2 gene and mixed with autologous irradiated tumour cells prior to subcutaneous reinjection [7]. In two of six evaluable patients, there was a successful induction of tumour-specific CTL precursors, however, no objective responses were demonstrated. Another approach has been to transfect autologous immune effector cells with the IL-2 gene. Preclinical studies have shown that cytokine-induced killer cells (CIKs non-MHC restricted cytotoxic lymphocytes) can eradicate tumours in nude mice. In a phase I study, 10 patients with a range of different malignancies were treated with autologous CIKs derived from peripheral blood mononuclear cells, PBMCs, transfected ex vivo by electroporation with an IL-2 plasmid before reinfusion intravenously. There was an increase in serum IFN-γ, GM-CSF, and TGF-β during treatment and also an increase in the cytotoxic activity of circulating lymphocytes tested against a range of HLA-matched carcinoma cell lines. One patient with follicular B cell lymphoma achieved a complete response [8] and the associated side effect profile comprised mainly of fever and myalgia.

Two further phase I studies treating patients with a range of advanced cancers have utilized either allogeneic fibroblasts secreting IL-2, or an IL-2 DNA/lipid complex (leuvectin) delivered by direct intratumoural injection. Both approaches were well tolerated with evidence of biological activity (detection of IL-2 on tumour biopsy and tumour infiltration by T cells) in vivo as well as clinical objective responses in some patients (with melanoma or renal carcinoma).

In summary, cytokine gene therapy appears to be safe when mediated via a number of different vectors but despite evidence of biological activity objective responses have been rare.

Vaccination against tumour-specific antigens

Tumour-associated antigens have been identified for a range of human tumours including viral antigens (eg, HPV E6, E7), mutated oncogenes (eg, ras), and nonmutated onco-fetal proteins (eg, CEA). Since T cell epitopes to these antigens have been identified, they may serve as targets for CTLs under appropriate conditions. Molecular characterization of tumour-associated antigens and identification of their genes has allowed the development of recombinant vaccines in which a vector is used to introduce DNA encoding tumour-associated antigens into patients. Viruses (especially, poxviruses) present antigen and induce both humoral and cell-mediated responses. Therefore, copresentation of tumour-associated protein with the vaccinia vector may enhance immunogenicity and increase the possibility of tumour rejection.

Carcinoembryonic antigen (CEA) is a cell surface glycoprotein over expressed on the majority of colorectal cancer cells, and is expressed at low levels in normal colon and biliary epithelium. On the basis of differential expression levels, CEA has been selected as a potential target for immunotherapy approaches. Different methods have been utilized in clinical trials.

Vaccinia vector

Several phase I trials have now tested recombinant vaccinia vectors encoding full length CEA administered subcutaneously or intradermally at doses between 10^7–10^8 pfu to patients with metastatic colorectal cancer [9]. Side effects include low-grade fever, fatigue, and inflammation at the injection site. The vaccine was able to induce a CTL response to CEA epitopes but no objective tumour responses.

A potential problem with vaccinia vectors is the generation of neutralizing antibodies, which may limit efficacy. Canarypox viruses, are not pathogenic in humans, do not replicate in human cells and may therefore be given repeatedly without neutralization by antibodies. A recombinant canarypox virus containing the human CEA gene has demonstrated antitumour efficacy in mice. In a phase I trial of this vector (Avipox), patients with advanced CEA positive tumours were treated with three monthly intramuscular injections. In seven of nine evaluated patients, CEA-specific CTL responses were induced without objective tumour shrinkage [10]. A novel approach to generate an immune response to CEA is the development of anti-CEA "designer T cells." In a phase I trial, T cells from patients were transduced by retrovirus delivery of chimaeric Ig-T-Cell receptor genes to generate immune effector cells which bind specifically to CEA positive cells before reinfusion. This treatment was well-tolerated up to doses of

10^{11} T cells although no objective reductions in tumour volume were documented.

Most tumours do not express costimulatory molecules and therefore, expression of such molecules on tumours may enable presentation of tumour antigens directly to T cells, reducing the need for professional antigen presenting cells (APCs). Further, systemic immunity against unmodified tumour cells (distant bystander effect) may be evoked. Since T cell activation requires both a specific antigen epitope and a costimulatory signal, a canarypox vector expressing human CEA and B7.1 has been constructed. A phase I study of 18 patients with CEA-expressing adenocarcinoma showed it to be well-tolerated up to 4.5×10^8 pfu with no autoimmune reactions. Two of thirteen patients with colorectal cancer achieved stable disease, correlating with an increase in CEA-specific precursor T cells.

Overall, immunogene therapy approaches are attractive and constitute about two thirds of the ongoing clinical trials for cancer treatment. In the main, clinical trials described here have so far failed to demonstrate clinically significant responses, despite clear biological activation in the form of antibody and cell-mediated response. The discrepancy may be due, at least in part, to the dynamic evolution of tumour antigens as a result of negative selection. However, it may also highlight the limitations of traditional clinical trial design whereby efficacy must first be demonstrated in the setting of advanced disease. This may not be appropriate for immunogene therapy, which is likely to be most effective against minimal residual disease.

Mutant gene correction

The phenotypic correction of key genetic aberrations in malignant cells has shown the potential to trigger the induction of apoptosis in a range of preclinical models. Different strategies including tumour suppressor gene correction (eg, p53) or oncogene suppression (eg, K-*ras*) have shown antitumoural effects in animal models of colorectal cancer. p53 gene correction delivered in adenoviral vectors is being tested in clinical trials in combination with conventional chemotherapy.

About 50% of colorectal cancers harbour p53 mutations. It has been shown that reexpression of wild-type-p53 in mutated colon cancer xenografts expressing the mutant variant can lead to inhibition of tumour growth and increased animal survival. A number of clinical trials using a replication-deficient adenoviral vector to deliver wild-type p53 to a range of human tumours have been carried out. Initial studies demonstrated the safety of direct intratumoural injection of these vectors and confirmed p53 gene expression even in the presence of an antiadenovirus immune response. A phase I study has assessed the safety and efficacy of a single dose of adenovirus-delivered p53 (SCH58500) administered via the hepatic artery to patients with hepatic colorectal metastases with the aim of maximizing tumour cell exposure and minimizing systemic exposure. Treatment was well-tolerated up to the maximum dose of 2.5×10^{12} virus particles with toxicity comprizing flu-like symptoms and in four out of sixteen patients, a transient asymptomatic rise in liver transaminases. Of twelve patients who went on to receive intrahepatic chemotherapy with 5-fluorodeoxyuridine, eleven achieved a partial response [12]. This compares favourably to partial response rates of about 50% in historical controls.

Mutation of K-*ras* is common to many malignancies of the gastrointestinal tract. This provides a potential target for antisense oligonucleotide therapy. The introduction of synthetic oligonucleotides, capable of hybridization to specific complementary messenger RNAs, can block the expression of a single protein that plays a critical role in tumour growth. Preclinical studies of K-*ras* antisense therapy suggest this to be a safe, relatively non-toxic treatment.

Virus-directed enzyme prodrug therapy

Enzyme prodrug systems, also called suicide gene therapy or gene-directed enzyme prodrug system (GDEPT), are alternatives to systemic chemotherapy. This involves gene transfer, for example, via a viral vector (virus-directed enzyme prodrug therapy, VDEPT) to express viral, bacterial, or fungal enzymes in tumour cells. The enzyme can convert an inactive prodrug into a toxic metabolite, leading to tumour cell death. Compared to systemic chemotherapy, the merit of this approach is of confining generation of a short-lived cytotoxic species to the tumour, reducing systemic metabolite concentrations, and therefore limiting the potential for toxicity to normal cells, such as bone marrow and gastrointestinal tract. The main obstacle of this method is the limited gene transfer efficiency at the tumour site by vectors currently available. However, this hurdle may be partially overcome by the *bystander effect*. The bystander effect refers to the observation that only a fraction of the total cancer cell population needs to be transfected by the vector to lead to significant degrees of cell kill. This may be a local effect mediated by passage of the toxic metabolite (or other apoptotic factors from dying cells) to neighbouring cells either by passive diffusion, via gap junctions or via apoptotic vesicles. Alternatively, there may be an immune-mediated response that could induce a distant bystander effect. This has been observed in murine GDEPT models in which regression of distant tumour deposits is seen in immunocompetent mice but is less marked in athymic animals [13]. A logical extension of this phenomenon may be to combine GDEPT with cytokine gene therapy in order to maximize this effect.

A number of bacterial/viral enzymes have been cloned which have the capacity to catalyze the conversion of a range of prodrugs to mechanistically diverse cytotoxics. Phosphorylation of ganciclovir produces the toxic metabolite GCV-triphosphate, which competes with dGTP and inhibits DNA synthesis. This reaction

is catalysed by herpes simplex virus (HSV)-Thymidine Kinase (*tk*) 1000-fold more efficiently than the human nucleoside kinase, making this an attractive model for GDEPT. In a syngeneic murine model of colorectal cancer, the HSV-*tk*/GCV system could achieve complete tumour regression when only 9% of cells express the *tk* gene. The lipid-insoluble GCV metabolite cannot diffuse into adjacent cells suggesting that the bystander effect may be mediated by gap-junction transport or via an immune response [14]. This system has been shown to be well-tolerated and has achieved clinical responses in phase I trials of brain and prostate cancer. Similar studies have been undertaken using a replication-deficient adenovirus vector to deliver RSV-*tk* with similar preclinical results. A phase I trial utilizing direct intratumoural injection of this vector followed by a fixed dose of GCV in patients with hepatic metastatic colorectal cancer has been undertaken. Sixteen patients were treated with escalating doses of virus up to 1×10^{13} particles. This treatment was well-tolerated with no dose-limiting toxicity observed, confirming the safety of the adenovirus vector delivered by the intratumoural route [15].

Bacterial or fungal cytosine deaminase (CD) is able to convert the antifungal agent, 5-fluorocytosine (5-FC), into 5-fluorouracil (5-FU), one of the most effective chemotherapeutic agents for colorectal cancer. There is some evidence suggesting that the fungal CD is superior to its bacterial counterpart, and a profound bystander effect is seen in vivo. Significant tumour regression has been achieved when only 2% of cells in xenografts expressed cytosine deaminase [16]. Furthermore, the combination of the pyrimidine salvage pathway enzyme, uracil phosphoribosyl transferase (UPRT), with CD has been shown to enhance the antitumoral effect possibly by increasing the conversion of 5-FU to 5-fluoro-deoxyuracil monophosphate, thereby accelerating a rate-limiting step in conversion of 5-FU to its cytotoxic metabolites [17]. A phase I trial of a replication-deficient adenovirus carrying the *E. coli* CD gene (Ad-GVCD-10) given intratumourally followed by oral 5-FC to patients with hepatic metastatic colorectal cancer is underway. Dose escalation to a maximum of 2×10^9 pfu is planned. The trial comprises two arms, one of which is treated with the vector and the prodrug only, in the other, the tumour is removed after treatment so that histological and molecular analysis can be undertaken.

Nitroreductase can convert the prodrug CB1954 to a highly toxic bifunctional alkylating agent, which can cause interstrand DNA crosslinks, leading to cell death. This effect is cell cycle independent. In cell-mixing experiments, a significant bystander effect was seen when only 10% of pancreatic cancer cells expressing nitroreductase were treated with CB1954 [18]. A phase I dose-escalating study of the prodrug, CB1954 has already been completed, establishing the dose of CB1954 that can be delivered safely by the intravenous (IV) and intraperitoneal (IP) routes. Pharmacokinetic analysis showed that venous levels sufficient for clinically significant prodrug activation

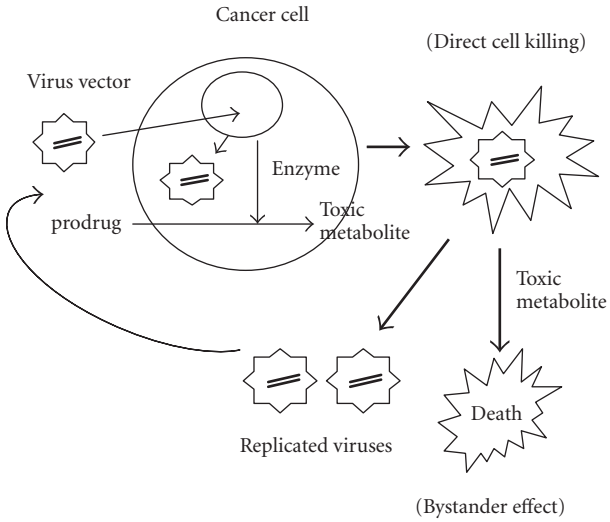

Figure 1. The use of replication-competent viral vector as a carrier for therapeutic gene delivery. The potential advantages are: (1) the dual killing effect caused by viral oncolysis and the therapeutic gene system, for example, enzyme prodrug system; (2) the continuing infection by the replicated viruses to surrounding tumor cells which were not infected initially.

could be achieved, based on data from preclinical models with IP concentrations of around 100 μmol [19]. Meanwhile, in the other arm of this ongoing phase I study, an E1- and E3-deleted adenovirus containing the nitroreductase gene, under the control of a cytomegalovirus promoter, is given to patients with hepatic colorectal metastasis or hepatocellular carcinoma by ultrasound-guided intratumoral injection. CB1954 will be given intravenously 48 hours after viral injection, once an adequate level of nitroreductase gene expression has been detected in resected hepatic tumours from operable patients. So far, preliminary data from this trial have demonstrated the safety of intratumoural vector administration up to 5×10^{11} virus particles. Immunohistochemical analysis of resected tumours has confirmed nitroreductase expression which increases in a dose-dependent manner. Generally, the viral vectors used in these trials were delivered by intratumoral injection directly to the tumour site. This approach secures accurate tumour targeting but may limit the use to solitary tumours rather than to systemic disease. Clinical efficacy has not yet been shown.

A further level of specificity for these systems is the insertion of a tumour specific promotor, for example, the CEA tumour promoter, to regulate gene expression so that even if normal cells were infected, the enzyme would not be transcribed. It is envisaged that this would allow regional administration of the vector to the liver in patients undergoing resection of a primary colorectal tumour. This would be followed by systemic administration of prodrug. Given the favourable growth kinetics of microscopic metastases following resection of the primary tumour, GDEPT may play a useful role in this

adjuvant setting. A further extension of the targeted approach would be to consider intrahepatic arterial (IHA) administration of the vector. The liver is a common site of metastasis for many cancer types and, once established, draw their blood supply from the hepatic artery. It is possible to cannulate the artery and implant a catheter which allows repeated administration of virus and/or drug, generating high local concentrations of the relevant therapeutic agent and perhaps increasing the opportunity for transfection and prodrug activation. In the adjuvant setting, where micrometastases are more likely to be served by the portal vein, it is possible to deliver the VDEPT components by an intraportal catheter inserted at the time of resection of the primary colorectal cancer.

Oncolytic virus therapy

In recent years, genetically modified oncolytic viruses including adenovirus, herpes simplex virus, and reovirus have been developed and tested. These viruses require key tumourigenic pathways to be mutated for viral replication and hence can selectively replicate in and lyse tumour cells while sparing normal cells [20]. Among them, mutants of adenovirus and herpes simplex viruses are already in trials for various cancers.

The development of replication-competent viral vectors may have the potential to improve the relatively low levels of transgene expression in many gene therapy protocols. It is envisaged that conditionally replicating vectors may be able to overcome these hurdles and, further, may have the potential to reach disseminated metastases.

Oncolytic adenovirus

The wild-type adenovirus was first used in the treatment of cervical cancer. Recently, an E1B-attenuated adenovirus, dl1520 (ONYX-015) has been tested on more than 200 patients with head and neck cancer, hepatic colorectal metastases, ovarian cancer, and pancreatic cancer. This mutant virus was engineered not to express the E1B-55-kDa virus protein and, therefore, was initially reported to replicate specifically in cancer cells lacking functional p53, leading to cell lysis. However, it was subsequently found that this virus could also replicate efficiently in several tumour cell lines with wild-type p53. These contradictory results raised the doubt about the specificity of dl1520-mediated killing effect in p53-mutated cells. Nevertheless, recent data has shown that the loss of p14-mediated Mdm2 inhibition plays an important role in supporting the replication of this virus in tumour cells with wild-type p53.

In a phase I/II trial, ONYX-015 was administered via hepatic artery infusion for patients with metastatic hepatic tumours. Two courses of a five-day infusion of 5-FU/folinic acid were given concurrently. The virus was well-tolerated at the dose of 10^{11} pfu/infusion without dose-limiting toxicities, although most of the patients developed grade-I/II fever and a few patients developed rigors after viral injection. Preliminary data showed partial responses in two of the four evaluable patients, which is comparable to the standard chemotherapeutic treatment [21].

Another trial using the same virus administered via hepatic artery infusion, intravenous infusion, or intratumoral injection, without chemotherapy, was conducted in 16 patients with primary or metastatic hepatic tumour (mainly from colorectal primaries). Tumour necrosis after viral injection was seen on CT scanning and histological analysis in all patients. No severe side effects were observed at a dose of 3×10^{11} pfu [22].

In summary, ONYX-015 has been investigated in several clinical trials, treating a range of tumours. Doses up to 2×10^{12} virus particles have been well-tolerated when administered intratumourally, via the hepatic artery, intraperitoneally, and intravenously. No dose-limiting toxicity has been observed. Virus replication has been observed after administration by all routes, but to a variable extent depending on tumour type. There is also a confirmation that distant tumours can be infected following systemic delivery. Interestingly, ONYX-015 has demonstrated very little efficacy as a single agent in head and neck cancer (0–14% objective response rate). However, clinical benefit has been seen where combined with chemotherapeutic agents. ONYX-015, in combination with chemotherapy (5-FU and cisplatin), has also shown promising results in a trial of head and neck tumours, with a complete response rate of 27% and a partial response rate of 36% [23]. The combination of oncolytic virus with chemotherapeutic agents seemed synergistic but randomised, properly, powered trials comparing chemotherapy versus chemotherapy plus virus are required.

The original concept that genetically engineered viruses could specifically target tumour cells derived from the observation that deletion of *tk* gene from HSV allowed viral replication exclusively in mitotic cells. The current oncolytic HSV's are engineered with mutations in one or both of two other genes; viral ribonucleotide reductase (ICP6), the loss of which restricts lytic virus replication to the dividing cells that retain sufficient ribonucleotide reductase activity to support the replication of the virus; and viral ICP34.4, mutation of which allows continued protein synthesis by blocking the shutdown of host cell protein synthesis normally associated with HSV infection thereby enhancing the generation of virus progeny. Such attenuated HSV's have been engineered and exploited in cancer trials for brain tumours and prostate cancer [24].

Replicating viruses have the potential to infect a greater proportion of tumour cells and to increase transgene expression. It is attractive, therefore, to combine these viruses with gene therapy strategies such as prodrug activation or cytokine expression by inserting the appropriate cDNA into the vector (Figure 1). Preclinical studies using the combination of a replication-competent HSV vector encoding the *tk* gene have been performed. In a colorectal cancer model, the addition of GCV actually

reduced the cytotoxic effect of the oncolytic vector, probably because of the antiviral activity of the activated prodrug. More encouraging results have been reported with E1B-attenuated adenovirus vectors encoding a combination of CD and *tk*. Data showed that the vector alone was cytotoxic, but the addition of both 5-FU and GCV further enhanced cell killing [25]. Interesting data regarding a potentially synergistic interaction between oncolytic viruses and radiotherapy is also emerging, with evidence that radiotherapy may enhance viral replication within tumours [25].

Overall, progress is being made in the clinical development of oncolytic viruses. Future clinical studies will address the optimum combination of replicating vector, gene insert, chemotherapy, and radiotherapy to maximize their therapeutic potential.

FUTURE DEVELOPMENTS

Gene therapy is now no longer in its basic scientific or clinical infancy. It is a toddler, and the initial expressions and gasps of delight that surrounded its conceptual birth have somewhat given way to the recognition that there will now be a longer haul through school days to adolescence before we can see, through properly powered randomized clinical trials, its true worth. In the meantime, several challenges remain.

(i) Improving the efficiency of gene transfer:

The recent development of replication-competent vectors for cancer gene therapy may improve gene transfer efficiency.

(ii) Improving the potency of antitumor effect:

Approaches to improve the potency of antitumor efficacy include, combination of gene therapy with conventional chemotherapy, inclusion of different enzyme activating genes within a single vector, and utilization of cytotoxic and immune effector systems.

(iii) Specific gene expression at tumour sites to avoid toxicity to normal tissues:

To limit specific gene expression to the site of tumours, tumour- or tissue-specific promoters such as CEA, Prostate Specific Antigen, and Alpha-fetoprotein can be used or retargeted viruses incorporating novel ligands in cell binding domains.

(iv) Safety of viral vectors:

Safety is still a concern, especially when viral vectors are used. For herpes simplex virus, the infection of this virus can be eradicated by antiviral drug (acyclovir or ganciclovir). However, for adenovirus, viral clearance is dependent on the immune system. Therefore, screening should rule out the immunocompromised patients from adenovirus-mediated gene transfer protocols. Safety can be potentially improved by the development of gutless virus, chimaeric virus, minivirus, or complementary oncolytic virus.

(v) Noninvasive monitoring of transgene expression:

It is critical that noninvasive imaging systems are developed, that can detect transgene expression in vivo and evaluate the pharmacokinetics of genetic medicines to allow thorough pharmacological studies.

It is likely that gene therapies will be integrated with existing treatment modalities. The envisaged future practice for cancer treatment may involve a multimodality approach, integrating curative or debulking resection, followed by adjuvant therapies including concurrent or sequential gene therapy, chemotherapy, and radiotherapy.

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