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CANCER GENE THERAPY: 'DELIVERY, DELIVERY, DELIVERY'.

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1. ABSTRACT

Gene therapy for cancer treatment represents a promising approach that has shown selectivity and efficacy in experimental systems as well as clinical trials. Some major problems remain to be solved before this strategy becomes routinely adopted in the clinic, one of the main challenges being the improvement of gene delivery. Namely, the development of DNA vectors characterized by maximum efficiency and minimal toxicity will define the success of gene therapy and its chances of being accepted by public and clinicians. A number of issues need to be considered. The "magic" vector should be targeted, protected from degradation and immune attack, and safe for the recipient and the environment. Moreover, it should express the therapeutic gene for as long as required, in an appropriately regulated fashion. Vehicles such as retroviruses, adenoviruses and liposomes have been adopted in clinical studies, with varying results. New therapeutic modalities are also being explored in order to overcome the limitation of poor gene transfer and patient toxicity, including bacteria, adeno-associated and herpes simplex viruses, lentiviruses, cationic polymer-DNA complexes and electroporation. Some of the delivery systems tested in preclinical and clinical models are reviewed in this article, with particular attention to the targeting of the tumor environment.

2. INTRODUCTION

Gene therapy can be described as the transfer of DNA or RNA to transiently or permanently modify the genetic repertoire of target cells for therapeutic purposes. Initially designed to treat genetic diseases, the first clinical gene therapy protocol was approved in 1989 (1). Since then mre than 400 clinical trials have been undertaken

Table 1. Common viral and non-viral DNA delivery systems

Viral vectors	Non-viral vectors
Retrovirus	Calcium phosphate precipitation
Adenovirus	Nanoparticle
Lentivirus	Liposome-DNA complex (lipoplex)
Herpes Simplex virus	Gene gun
Adeno-associated virus	DNA injection
Chimeric (e.g. retro/adeno) virus	DNA electroporation
Poxvirus	Immunoporation
Vaccinia virus	Ultrasound
Parvovirus	Cationic agents-DNA complex (polyplex)

worldwide, of which over half relate to cancer (2). As an antitumor approach, gene therapy has the potential to target tumor cells selectively and specifically, by combining highly specific gene delivery with highly specific gene expression and therapeutic application.

In order to engineer cancer-targeted gene expression, transcription regulation specific to certain tissues, diseases (3), conditions or stimuli, such as hypoxia (reviewed in ref. 4) or ionizing radiation (5), can be exploited. For instance, DNA-regulatory sequences from several cancer-selective genes, including α-fetoprotein (hepatoma), tyrosinase (melanoma), *c-erbB2* and *DF3/MUC1* (breast carcinoma), carcinoembryonic antigen (epithelial cancers) and prostate-specific antigen (PSA), have been shown to direct expression of therapeutic genes in the indicated tumor cell types (6). In the design of therapeutic genes for cancer gene therapy, genetic immunopotentiation, mutation compensation and molecular chemotherapy represent the three major approaches. In the first case, tumor immunogenicity is enhanced by the delivery of genes that encode immunomodulators, such as cytokines (7-9) or co-stimulatory molecules (10). Mutation compensation aims to inactivate oncogenes or induce tumor suppressor gene expression (11-13), while in molecular chemotherapy, or gene-directed enzyme/prodrug therapy (GDEPT; ref 14) a "suicide" gene is delivered to the tumor cells. In this latter approach, the enzyme encoded by the therapeutic gene is non-toxic *per se*, but is able to convert a prodrug into a potent cytotoxin.

A growing body of evidence indicates that the success of gene therapy is mainly dependent on the development of vectors characterized by maximum efficiency and minimal toxicity. Aim of this review is to briefly outline some of the delivery systems tested in preclinical and clinical models.

3. GENE DELIVERY

The administration of gene therapy vectors requires that they be not only targeted, but also protected from degradation, sequestration or immune attack, in order to reach the appropriate sites for transfection. Although some success has been reported with naked DNA (15), efficient delivery has been restricted to intratumoral injection. The ideal DNA vehicle should be of the size needed for the clinical application, capable of hosting an expression cassette carrying one or more genes, and should be easy to produce and purify in large quantities and high concentrations. It should not induce inflammation and be safe for the recipient and the environment and it should express the gene for as long as required, in an appropriately regulated fashion. Finally, clinicians and patients should find it acceptable to administer in their therapy schedule.

The efficient delivery of DNA to tumor sites remains a formidable task, but progress has been made in recent years using both viral and non-viral methods. Vehicles such as retro- and adenoviruses, liposomes and naked DNA injection have been adopted in clinical studies (Table 1). New therapeutic modalities are also being explored in order to overcome the limitation of poor gene transfer and patient toxicity, including immunoporation (16), macrophages, bacteria, adeno-associated and herpes simplex viruses, lentiviruses, cationic polymer-DNA complexes and electroporation (Table 1; details below).

4. VIRAL DELIVERY SYSTEMS

Viral vectors capitalize on the ability of the viruses to efficiently enter the cells through specific receptors and transfer their genome. Some viral genes need to be removed to permit the insertion of the expression cassette and make the virus non-toxic. In order to achieve higher levels of infection and antitumor activity, it is currently becoming more common to adopt replication-competent rather than non-replicative viral vectors (17). In this case, tumor-specific replication is mandatory.

Retroviruses are small RNA viruses, which replicate through a DNA intermediate, consisting of the 5' and 3' long terminal repeats (LTRs) and the *gag*, *pol* and *env* genes. Viruses deleted of one or more of their structural genes can infect packaging cells that express the missing genes. Transfection of these producer cells with a minimal vector containing the desired therapeutic gene results in the production of defective recombinant retrovirus, able to transfer the transgene but not to establish a productive infection in the host. Tissue specificity may be achieved by fusing part of the *env* gene to a coding sequence for the ligand to a specific receptor (e.g., erythropoietin; 18). Retroviral vectors can host 9-10 kbp of foreign genetic material and integrate the therapeutic gene into the genome of the infected cells, allowing it to be maintained during subsequent mitotic divisions (19) (Figure 1A). This can be an advantage when treating hereditary and chronic disorders, but can also present a number of potential risks, including insertional mutagenesis and toxicity associated with overexpression of the therapeutic gene. However, the major limitation of retroviral vehicles, for example those derived from the Moloney murine leukemia virus (MoMLV), is that the target cells must be proliferating at the time of infection (20). Retroviral vectors are therefore better suited for *ex vivo* gene therapy, in which isolated cells are propagated in culture, transduced and subsequently transplanted into a recipient patient. Moreover, heterologous promoters can be overridden by

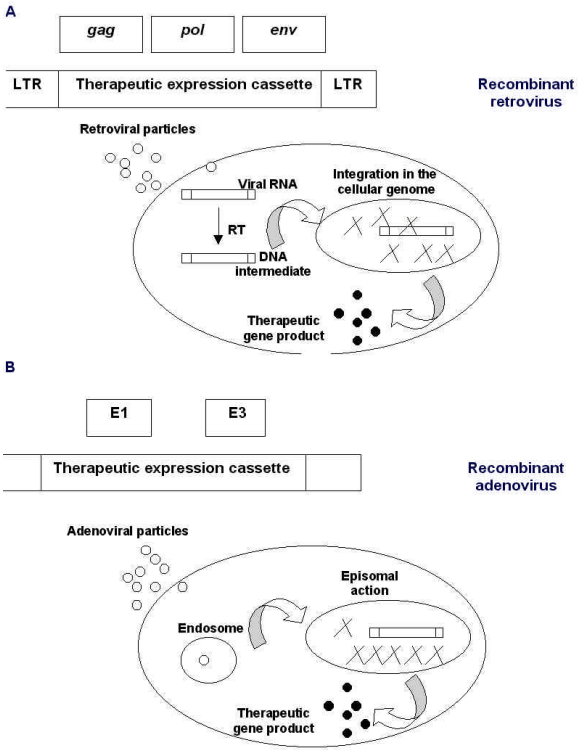

Figure 1. Retro- and adenoviral vectors for gene therapy. A. Retrovirus-based DNA vehicle. The viral genes *gag*, *env* and *pol* can be replaced by the therapeutic expression cassette, flanked by the long terminal repeats (LTRs). Once entered into the target cells, the viral RNA is copied to a DNA intermediate by the reverse transcriptase (RT), carried by the vector. The transgene is randomly integrated into the host genome and the expressed at the target. B. Adenovirus-based DNA vehicle. The early genes *E1* and *E3* can be removed to host the therapeutic expression cassette. After receptor binding and internalization, the vector enters a cytoplasmic endosome. Following endosomal escape, the viral DNA remains episomal to direct the expression of the therapeutic gene product.

the strong constitutive activity of the retroviral LTRs. In clinical trials, safety has been demonstrated, but transfection efficiencies were rather low (21-23). These results may be due to the slower growth rate of spontaneous human malignancies compared to experimental rodent tumors, in which this strategy was initially tested.

Lentiviruses, such as the human immunodeficiency virus type-1 (HIV), are unique among retroviruses because of their ability to infect both replicating and non-replicating cells, mediating efficient delivery and long-term expression. High transduction has been observed in the lympho-hemopoietic and the central nervous systems, without significant inflammatory response, however liver and muscle were characterized by poor transgene expression (24). The development of efficient vector packaging systems and addressing the obvious issue of biosafety will determine the potential of these vectors.

Of the viral delivery systems, *adenoviruses* (Ads) are probably the best characterized and most extensively used. Ads are non-enveloped, double-stranded DNA viruses, with a genome of approximately 36 kbp. Cellular attachment and internalization of adenoviral particles is mediated by the interaction of a 182 kDa fiber protein in the viral coat with the cell surface Coxsackie and Ad receptor (CAR), and of the arginine-glycine-aspartic acid (RGD) motives in the penton base with cellular integrins (25,26). Ads are stable and easy to manipulate, can be grown to high titers (~10^{12} viral particles/ml) and early genes (E1, E3) deleted viruses can accommodate up to 8.5 kbp of foreign DNA (27-28). Also, Ads can infect different cell types independently of their proliferation status (29-30). Because of their non-integrative nature, vector sequences are not inherited in progeny cells, resulting in a transient expression of foreign genes (Figure 1B). This may not be a limitation in cancer gene therapy approaches that aim to directly kill the cancer cells, rather than permanently modify their genome. Mutant Ads have been designed to replicate selectively in tumor cells, for instance the many that lack a functional p53 (ONYX-015; refs. 12,31). ONYX-015 contains no therapeutic gene and its antitumor efficacy relies on the ability of the Ad to kill tumor cells by direct cell lysis. Clinical trials have demonstrated safety and efficacy (17), although some uncertainties remain concerning the p53-dependent specificity of the viral replication (32). Ad-mediated targeted delivery could be achieved by modifying the viral capsid proteins, fiber and penton base to permit the recognition of cell-specific receptors (33,34). The main drawbacks of Ads are a strong B cell- and T cell-mediated inflammatory response that reduces the effectiveness of repeated administration (35), and, in clinical settings, concerns about patient safety (36).

Vectors based on the enveloped, linear double-stranded DNA *Herpes simplex virus type-1* (HSV) are also able to infect non-dividing cells. HSV vectors can be "helper" virus dependent (amplicons) or independent. Amplicons are defective HSV genomes that arise spontaneously by recombination and are amplified by serial passage at high multiplicity of infection (37). Since amplicons are largely deprived of coding sequences, they can accommodate as much as 150 kbp, but proteins necessary for viral structure, replication and exocytosis must be provided by a "helper" virus. Drawbacks include some instability (due to selective advantage for small amplicons), contamination with helper viral DNA and low ratio of amplicon to helper virus (38). "Helper" virus-independent vectors have deletions in some essential viral genes, and therefore need to be grown in appropriate cells. They replicate to high titers and can host up to 40 kbp of foreign DNA. Although HSV-related toxicity has been observed in a number of cell types (39), a clinical trial involving the delivery of a conditionally-replicating HSV vector to brain tumors has shown safety of this approach (40).

Adeno-associated viruses (AAVs) are small, non-enveloped, linear single-stranded DNA parvoviruses, not associated with any pathology in primates. Wild type AAVs can infect non-proliferating populations and integrate their genome at a specific site in chromosome 19,

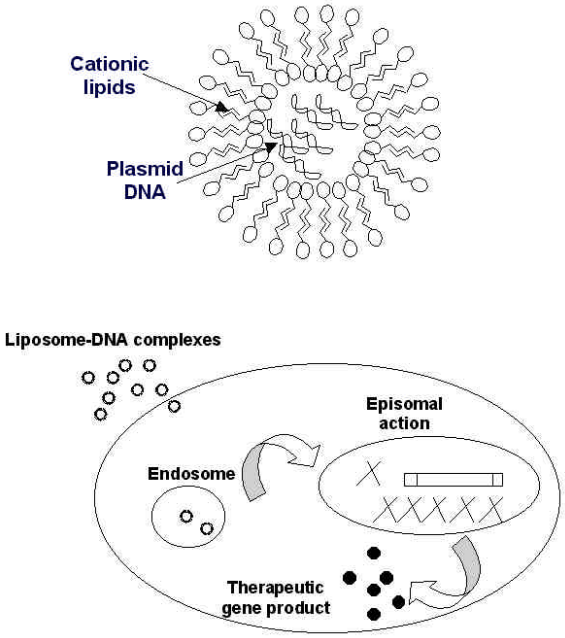

Figure 2. Liposome-mediated DNA delivery. The liposomes used for gene therapy are usually cationic, to electrostatically bind to DNA. The complexes enter the target cells by fusing with the plasma membrane and, once escaped from the endosome, can enter the cell nucleus. It is likely that most of the DNA that enters the nucleus remains episomal.

entering in a latent phase (41). Productive infection is dependent on the presence of a "helper" virus (Ad, HSV or vaccinia). Compared to Ads, AAVs are less immunogenic and present a broad tissue tropism (39). Intramuscular injection of a replication defective AAV vector resulted in transduction of muscle fibers without activating destructive T cell responses (42). Other tissues where AAV gene delivery has been successfully demonstrated are the brain and the retina (43). However, AAVs have limited packaging capacity (only 4-5 kbp of DNA insert) and are difficult to prepare in high titers.

Hybrid viruses may represent an interesting solution by adopting the most advantageous characteristics of the viral vectors described so far. For example, a hybrid between an Ad and an Epstein-Barr virus (EBV) may combine high titers of adenoviral vectors with long-term genome persistence, without integration, as a stable episome, as is seen in natural EBV infections (44).

5. NON-VIRAL DELIVERY SYSTEMS

Compared to viral vectors, non-viral systems (Table 1) are particularly suitable for gene therapy with respect to simplicity of use, lack of immune response, ease of large-scale production and DNA packaging. Moreover, non-viral systems can be controlled and characterized as well as any other pharmaceutical products, in terms of delivery components, complex size, DNA and vector concentration. However, there are some disadvantages, mainly low efficiency of gene transfer. Non-viral systems can be broadly divided into physical (electroporation, sonoporation or gene gun) and chemical (lipids, polymers, proteins).

In *electroporation*, brief high or low voltage electric pulses are applied to cells in culture and tissues *in vivo* to induce transient pores in the cell membranes. This method is applied in clinical settings for delivery of chemotherapeutic drugs, with high antitumor efficiency and negligible side effects (45). Recent studies have shown delivery of plasmid DNA to different tissues *in vivo* (46,47). Electric pulse parameters are important for optimal gene expression, but they appear to differ greatly depending on the target tissue.

The *gene gun* approach consists in the propulsion of gold microspheres coated with plasmid DNA into the target cells, where the DNA is released into the nucleus (48). The transfection efficiency varies among tissues, from 1-5% for muscle cells to 10-20% for skin epidermal cells (49). *In vivo*, major limitations include shallow penetration into the tissue, and short-term, low-level expression of the gene product.

Cationic liposomes (Figure 2) are clinically well tolerated, easy to produce, non-infectious, non-immunogenic and can package large DNA molecules. Even though liposome-DNA complexes can also transfect slowly dividing cells, the overall transfection efficiency *in vivo* remains low. Moreover, the route of administration can affect the physical properties and transfection ability of the lipoplex. For example after intravenous (i.v.) injection, serum can cause lipoplex to aggregate and disintegrate, resulting in DNA release and degradation (50). The introduction of foreign gene products into human cells via DNA complexes consists of a series of discrete steps. First the vector must bind to the cell surface, then enter the cell by an endocytic or phagocytic process. Efficient escape from the resultant vesicle is necessary to evade enzymatic degradation in the acidified environment of the maturing endosome. Ad-based vectors possess mechanisms of endosomal escape into the cytosol mediated by the viral penton base (51). For lipoplex systems, inclusion of fusogenic helper lipids, such as the neutral dioleoyl posphatidylethanolamine (DOPE), can improve transfection and facilitate DNA release through their detergent or buffering properties. Increase in transfection efficiency has been achieved coupling liposome-mediated gene delivery with ionizing radiation (52). A single 2 Gy dose of X-Rays delivered immediately after incubation with Lipofectamine-DNA complexes enhanced the uptake and expression of the transgene luciferase by three-fold (52). Alterations in the membrane and/or nuclear transport functions by the radiation may be involved (53,54).

Receptor-mediated gene delivery may further improve both safety and efficiency, by direct binding to DNA (*polyplex*) or coupled to the delivery systems described above. Ligands to surface receptors have included asialoglycoprotein (55), basic fibroblast growth factor (56), transferring (57) and adhesion molecules (58).

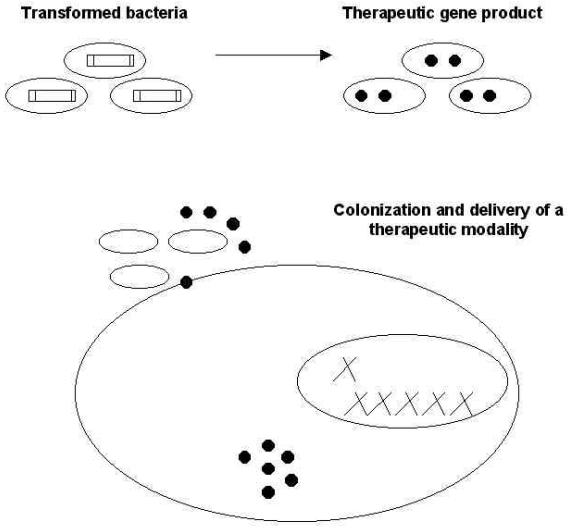

Figure 3. Bacterial delivery of genetic therapeutics. Prokaryotic vectors such as the anaerobic bacteria of the genera *Clostridium*, *Bifidobacterium* and *Salmonella* auxotroph can be transformed at high rate and colonize hypoxic and necrotic tumor areas. While the ability to replicate in tumor tissue provides inherent anti-tumor activity, it is their ability to deliver therapeutic proteins to cancer cells *in vivo* that may confer utility for gene therapy strategies.

Peptides containing an RGD motif with high affinity for integrins (59) and a short polylysine segment for electrostatic binding of DNA could efficiently transfer genetic material to different cell types (60-62). The transfection efficiency was enhanced by 100-fold by incorporating the cationic liposome Lipofectin (L) into the peptides (P)/DNA (D) complex (LPD). Efficient transfection was demonstrated in a number of cell types *in vitro* (63-65) and in bronchial and alveolar cells *in vivo*, with transgene expression sustained for at least three to seven days (66). However, direct intramyocardial (67) or intratumoral (47) injection of LPD complexes resulted in negligible marker gene expression.

6. DELIVERY OF THERAPEUTIC MODALITIES

Besides viruses, other live vehicles have been investigated for cancer gene therapy. Because of their large genome size, bacteria can readily express multiple therapeutic transgenes (Figure 3). Moreover, they are motile and, if necessary, their spread can be controlled with antibiotics. Examples of prokaryotic vectors include anaerobic bacteria of the genera *Clostridium*, *Bifidobacterium* and tumor-invasive *Salmonella* auxotroph. The ability of *Clostridia* to selectively germinate and replicate in necrotic and hypoxic regions of solid tumors (68,69) makes them a promising tumor-selective vehicle for gene therapeutics. Spores of *C. beijerinckii* genetically engineered to produce the *Escherichia coli* (*E. coli*) enzyme nitroreductase (NTR) have been i.v. injected into tumor-bearing mice, and NTR protein was detected in all tumors tested but not in normal tissues (70). *In vitro* conversion of the prodrug CB1954 into a cytotoxic agent by *Clostridia*-produced NTR demonstrated the therapeutic potential of this approach (70,71). *In vivo*, i.v. injection of spores of cytosine deaminase (CD)-transfected *C. sporogens* followed by systemic administration of the prodrug 5-fluorocytosine (5-FC) induced significant antitumor activity (72). Tumor selective spore germination was also observed in rhabdomyosarcoma-bearing rats injected with five different bacterial strains, the most efficient species being *C. acetobutylicum* and *C. oncolyticum* (73).

Attenuated hyperinvasive auxotrophic mutants of *Salmonella typhimurium* can selectively target tumor tissues and amplify in necrotic spaces to levels in excess of 10^9 bacteria per gram of tissue (74). When these auxotrophs were inoculated intraperitoneally into melanoma bearing mice, they suppressed tumor growth and prolonged average survival to twice that of untreated mice. Moreover, when the animals were inoculated with *Salmonella* expressing the HSV thymidine kinase (TK), ganciclovir-mediated, dose-dependent suppression of tumor growth and was observed. *Bifidobacteria* are Gram-positive non-pathogenic anaerobes, found in the gastrointestinal tract. Immediately after i.v. inoculation of *B. longum* to tumor-bearing mice viable bacilli could be detected throughout the animal body, but following 4-7 days the bacteria proliferation was restricted to the tumor mass (75). Compared to *Clostridia* and *Salmonella*, the lack of pathogenesis of *Bifidobacterium*, used in the preparation of fermented milk products, might be advantageous if used in human therapeutic treatment.

Macrophages may be effective vehicles for cancer gene therapy (76). It is known that macrophages infiltrate solid malignancies to form a significant proportion of the tumor solid mass, dominating in areas of hypoxia and necrosis (77). Differentiated macrophages transduced with an adenoviral vector containing the human cytochrome P450 2B6 (CYP 2B6) gene were found to infiltrate human tumor spheroids. Tumor cell death was induced when the spheroids were incubated with the prodrug cyclophosphamide (CP), which was converted by CYP 2B6 into a cytotoxin (76). A hypoxia-responsive promoter (78) conferred an additional level of selectivity to the system. The macrophages themselves did not appear to be affected by the CYP 2B6/CP treatment.

7. PERSPECTIVES

The need for local control of tumor growth in the cure of cancer is a matter of crucial importance. Therapeutic strategies aimed at delivering high and localized concentrations of cytotoxic agents to clinically resistant solid tumor populations may provide a fundamental clinical gain, improving not only the efficacy of standard treatments, without concurrent systemic complications, but overall survival and patient quality of life.

Gene therapy is a promising approach, and 12 years after the approval of the first clinical trial, it is still in

the early stages of development. Some major problems remain to be solved before these new strategies become routinely adopted in the clinic. One of the main challenges is the improvement of gene delivery, and therefore therapeutic efficacy. Given the limitation of the current delivery systems, a crucial feature of a cancer gene therapy approach is the induction of a bystander effect. The bystander phenomenon, initially described by Moolten (79), can be defined as an extension of the killing effects of the active agent to untransfected neighboring cells. This implies that even if only a fraction of the target cells are genetically modified and express the therapeutic gene, tumor eradication may still be achieved. The impact of the bystander effect on the success of gene therapy strategies is so important that a number of studies are currently focused on its enhancement. For instance, the HSV virion protein VP22 once synthesized in infected cells can spread very efficiently via a Golgi-independent pathway to surrounding uninfected cells, where it specifically accumulates in the nucleus (80). Due to these peculiar trafficking properties, delivery of DNA constructs containing the VP22 coding sequence fused to that of the marker green fluorescence protein (GFP) resulted in a significant spread of the VP22-GFP fusion to the nucleus of untransfected cell monolayers (81). The VP22-EGFP spread appeared to be a general phenomenon, common to all cell types tested to date (82). Therapeutic advantage of this "biologically active" bystander effect was demonstrated in suicide (83) and p53-based (84) gene therapy approaches.

The development of the "magic" vector will determine the success of a number of different gene therapy systems. To date, viral vectors are characterized by the highest transfection rates *in vivo*, and major clinical experience has been gained with Ads. Modification of the capsid proteins and fiber may allow increased selectivity via the recognition of cell-specific receptors, but biosafety is at present still disputable. Non-viral systems, although safe and easy to produce, are limited by poor gene transfer. Tumor-specific therapeutic strategies, such as bacteria and macrophages, may represent interesting approaches for cancer gene therapy and the analysis of their pathogenesis and the induced inflammation in humans are the main problems that will need to be answered in future clinical studies. Nevertheless, in the field of gene therapy, the data collected so far are encouraging, and illustrate both feasibility and future promise for cancer treatment. As technology fulfils the requirement for efficient delivery, it can be anticipated that the results observed in the pre-clinical studies will more quickly translate into clinical benefit. Therefore, for cancer gene therapy the program remains mainly one: delivery, delivery, delivery.

8. ACKNOWLEDGEMENTS

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