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The future of human gene therapy

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Abstract

Human gene therapy (HGT) is defined as the transfer of nucleic acids (DNA) to somatic cells of a patient which results in a therapeutic effect, by either correcting genetic defects or by overexpressing proteins that are therapeutically useful.

In the past, both the professional and the lay community had high (sometimes unreasonably high) expectations from HGT because of the early promise of treating or preventing diseases effectively and safely by this new technology. Although the theoretical advantages of HGT are undisputable, so far HGT has not delivered the promised results: convincing clinical efficacy could not be demonstrated yet in most of the trials conducted so far, while safety concerns were raised recently as the consequence of the "Gelsinger Case" in Philadelphia. This situation resulted from the by now well-recognized disparity between theory and practice. In other words, the existing technologies could not meet the practical needs of clinically successful HGT so far. However, over the past years, significant progress was made in various enabling technologies, in the molecular understanding of diseases and the manufacturing of vectors. HGT is a complex process, involving multiple steps in the human body (delivery to organs, tissue targeting, cellular trafficking, regulation of gene expression level and duration, biological activity of therapeutic protein, safety of the vector and gene product, to name just a few) most of which are not completely understood.

The prerequisite of successful HGT include therapeutically suitable genes (with a proven role in pathophysiology of the disease), appropriate gene delivery systems (e.g., viral and non-viral vectors), proof of principle of efficacy and safety in appropriate preclinical models and suitable manufacturing and analytical processes to provide well-defined HGT products for clinical investigations.

The most promising areas for gene therapy today are hemophilias, for monogenic diseases, and cardiovascular diseases (more specifically, therapeutic angiogenesis for myocardial ischemia and peripheral vascular disease, restenosis, stent stenosis and bypass graft failure) among multigenic diseases. This is based on the relative ease of access of blood vessels for HGT, and also because existing gene delivery technologies may be sufficient to achieve effective and safe therapeutic benefits for some of these indications (transient gene expression in

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some but not all affected cells is required to achieve a therapeutic effect at relatively low [safe] dose of vectors).

For other diseases (including cancer) further developments in gene delivery vectors and gene expression systems will be required. It is important to note, that there will not be a "universal vector" and each clinical indication may require a specific set of technical hurdles to overcome. These will include modification of viral vectors (to reduce immunogenicity, change tropism and increase cloning capacity), engineering of non-viral vectors by mimicking the beneficial properties of viruses, cell-based gene delivery technologies, and development of innovative gene expression regulation systems. The technical advances together with the ever increasing knowledge and experience in the field will undoubtedly lead to the realization of the full potential of HGT in the future.

1. The early promises and subsequent disappointments during the first decade of human gene therapy

The application of recombinant DNA technology and gene cloning (which started in the 1980s) and the resulting increase in genomics data during the 1990s have contributed to define some disease-causing genetic factors and to explore the potential of new therapies based on engineered genes and cells (Watson, 1990; Anderson, 1998; Gage, 1998).

Human gene therapy (HGT) is one of the new therapeutic approaches emerging from this molecular biology and biotechnology revolution (Anderson, 1998). Gene therapy is aimed at correcting genetic defects or to express gene products that are therapeutically useful. In most applications, gene therapy can be defined as the transfer of nucleic acids to the somatic cells of an individual with a resulting therapeutic effect. It represents a new, innovative drug delivery system making use of the technical and scientific advances of, among others, microbiology, virology, organic chemistry, molecular biology, biochemistry, cell biology, genetics, and genetic engineering. It is more than "gene transfer", which is only a part of the complex, multiphase process of identification, manufacturing, preclinical testing and clinical development of gene therapy products (Carter, 2000).

The principle of gene therapy has indisputable therapeutic advantages over existing therapeutic modalities (such as small molecules or biologics). These include (i) correction of the genetic cause of a disease, (ii) selective treatment of affected (diseased) cells and tissues (the cells and tissues themselves produce their own "remedy") and (iii) long-term treatment after single application. Based on these theoretical principles, at the time of its first introduction a decade ago, gene therapy promised to be an effective and safe treatment modality, which will soon cure diseases and replace classical therapies.

The first clinical test of gene therapy was started 10 years ago with the transfer of the missing *adenosine deaminase* (*ADA*) gene into isolated lymphocytes (using ex vivo gene-transfer technology) of patients with severe combined immune-deficiency

(SCID) syndrome. It is ironic, that until today, *ADA* gene transfer could not be accomplished in a clinically successful way. However, the recent report of successful gene therapy of SCID-XI patients (Cavazzana-Calvo et al., 2000), and the success in hemophilia B patients receiving factor IX gene therapy delivered by adeno-associated viral vectors (Kay et al., 2000) raised renewed optimism about the future clinical success of this new treatment modality.

It is fare to state that till today, gene therapy has not been able to live up to its original promise. Despite the early, enthusiastic prophecy of achievable unparalleled efficacy and safety, the existing clinical experience indicate insufficient therapeutic efficacy coupled with increasing safety concerns and ethical issues as well (Verma and Somia, 1997). This chapter gives a brief overview of the success factors which are essential for clinical efficacy and safety, the specific technical hurdles each of these factors faces today, and potential technical solutions of these obstacles, which will undoubtedly make gene therapy a successful therapeutic approach in the future.

2. Success factors for gene therapy

Gene therapy consists of multiple complex biological processes in the body, the molecular nature of which is in most cases still unknown (Fig. 1). Gene therapy

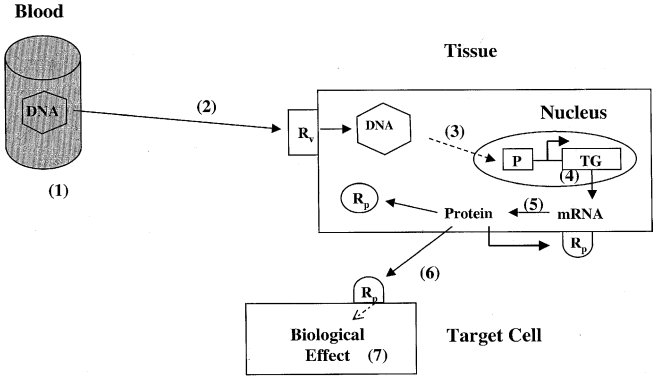

Fig. 1. *Multiple biological processes of in vivo gene transfer.* The following main steps are required for successful gene transfer in the human body: (1) vector (viral, non-viral, cell-based) delivery (localized tissue delivery or systemic delivery via blood circulation); (2) vector "recognition" by specific receptors (Rv) on cells in target tissue; (3) uptake of the vector by cells, trafficking to the nucleus and delivery of vector DNA in to the nucleus; (4) transcription (expression) of therapeutic (trans) gene in the nucleus; (5) translation of mRNA into therapeutic protein in the cytoplasm; (6) interaction of therapeutic protein with its receptors (Rp) within the "producing" cell (intracrine mechanism), on the surface of "producing" cell (autocrine mechanism) or on neighboring "target" cell (paracrine mechanism). For some applications, the therapeutic protein enters the circulation and acts distant from the target tissue (endocrine mechanism) (e.g., erythropoietin, coagulation factors VIII and IX, growth hormone, etc.); (7) after interaction with its receptor, the protein induces a biological effect which results in therapeutic benefits.

starts with the introduction of an appropriate vector (viral, non-viral or cell based) into the body either locally (direct tissue injection) or into the blood stream (systemic delivery). The vector needs to "find" its target tissue, it needs to enter the target cells and traffic through the cytoplasm to reach and enter the nucleus. Once there, the therapeutic (trans)gene needs to be transcribed and the formed mRNA needs to be appropriately translated into the therapeutic protein. The protein then acts on its receptor(s) either on the cell which produced it (intracrine or autocrine mechanism), on neighboring cells (paracrine mechanism) or at distant sites after entering the blood circulation (endocrine mechanism, e.g., erythropoietin, coagulation factors, growth hormone, etc.). Finally, after interacting with its receptor, the protein needs to induce an appropriate biological effect which results in therapeutic benefits.

The generic factors needed for successful gene therapy are not different from any new therapeutic modality: they include technical (gene delivery and expression), clinical (therapeutic efficacy and safety) and socioeconomic factors.

However, the specific technical success factors are unique for gene therapy approaches. They include the choice of appropriate therapeutic gene(s) (with proven role in the pathomechanism of the disease, specifically targeted and of sufficient potency) gene delivery systems (of sufficient targeting ability, transfection efficiency, and safety) and gene expression regulation systems to control the level and timing of therapeutic protein expression.

The therapeutic and socioeconomic success of gene therapy products include the requirement that the benefits of gene therapy should outweigh the risks and should offer advantages over conventional (usually less expensive) treatments, before this new approach will become accepted in the general medical practice.

3. Technical hurdles to be overcome in the future

3.1. Gene delivery vectors

The ultimate goal for gene therapy is the replacement, in a site-specific manner, of a disease-causing gene with its "healthy" counterpart (Dyer and Herrling, 2000). The long-term goal aims at a corrected gene surrounded by appropriate regulating sequences and expressing its product in a physiologically relevant manner. More realistically, shorter-term goal for development of gene therapy vectors is to deliver and express genes at the appropriate site and at therapeutically meaningful levels in a controlled manner (Anderson, 1998). The first-generation gene therapy vectors have used the ability of viral systems to deliver genetic information to human cells. Attempts are also made to develop non-viral synthetic vectors (Li and Huang, 2000) and hybrid synthetic-viral systems (Kaneda, 1999) that are safer alternatives for gene delivery. A third approach uses human stem cells as a means to introduce the therapeutic genes into specific human cell populations where the therapeutic product is required (Gage, 1998).

3.1.1. Viral vectors

Viruses "acquired" numerous biological properties over millions of years of evolution which allow them to effectively recognize and enter cells, traffic within the cytosol to the nucleus, translocate into the nucleus and express their genes in the host cell (Fig. 2). These properties made them among the first choices for gene delivery vectors in early gene-transfer studies.

The most frequently used viral vectors in clinical trials so far are retroviruses and adenoviruses (Table 1). Several other viral vectors are in preclinical development or are under early clinical evaluation, including adeno-associated virus (AAV) (Monahan and Samulski, 2000), lentivirus (Amado and Chen, 1999), herpes simplex virus (HSV), pox virus, etc. Retroviruses can lead to a stable integration of the transfected gene into the host genome and produce long-lasting gene transfer. Replication-deficient retroviruses are produced in vitro in specific packaging cells transfected previously with retroviral genes (*G*, *P*, *E*) that have been deleted from the genome of the therapeutic retroviruses. Major limitations of the retroviruses are their low titres, their inability to infect non-dividing cells, and the potential risk of insertional mutagenesis (Nabel and Nabel, 1994). The development of new pseudotyped retroviruses has increased virus titres that will permit more efficient gene transfer (Yee et al., 1994).

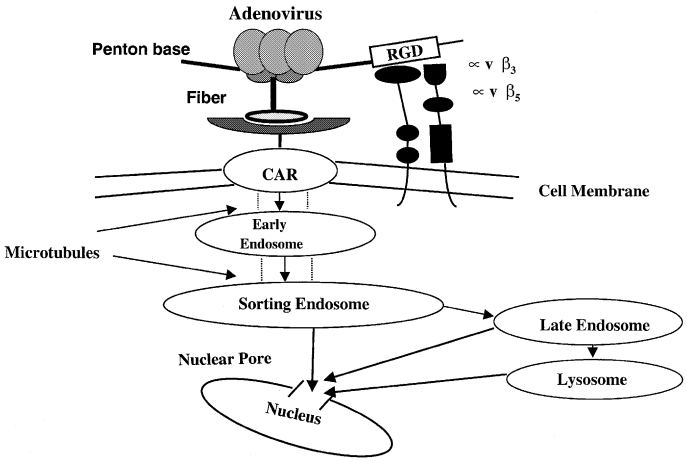

Fig. 2. *Cellular recognition, uptake and trafficking of adenoviral gene delivery vectors.* Receptor-mediated entry of adenovirus into cells depends on interaction of two if its coat proteins with two different cell surface receptors. The viral fiber knob protein mediates attachment to the cell via the CAR. Following attachment an RGD tripeptide motif in the penton base protein binds to integrins (αvβ3 or αvβ5) which mediates internalization. Knowledge of these domains and processes helped to design novel strategies to change the natural tropism of adenoviral vector ("re-targeting"; see text for details). Once inside the cell, the adenovirus effectively and quickly (within less than 60 min) reaches the nucleus (via the endosome, microtubules and the lysosome) and translocate into the nucleus via nuclear pores with the help of nuclear localization signal peptide in the viral coat protein.

Table 1
Gene delivery systems (vectors) used in gene therapy clinical trials (1992–Present) (Wiley clinical trials database)

Delivery system	Number of trials
Retrovirus	67
Liposome	59
Adenovirus	54
Cell-based	20
Poxvirus	26
Other methods[a]	16
Naked DNA	8

[a] Electroporation, gene gun, HSV, etc.

Adenoviruses can be produced in high titre. They do not lead to stable integration of the transgene into the host genome (only at very low frequency in cell culture) (Harui et al., 1999), and they usually remain extrachromosomal and cause only a transient transgene expression. Replication-deficient adenoviruses are produced in vitro in specific packaging cells that complement gene products (e.g., *E1*, *E3*) deleted from the genome of the therapeutic adenoviruses. They give an effective transient gene expression in proliferating and non-proliferating cells, but first-generation adenoviruses have the disadvantage of producing immunological and inflammatory reactions by themselves (Newman et al., 1995) or via the proteins encoded in the transgene (Tripathy et al., 1996). These complications should be lessened with second-generation adenoviral vectors (Wilson, 1996) (see below). AAVs have been used for effective gene transfer to muscle (Svensan et al., 1999). Further development of viral vectors is clearly needed since, although many may give useful results, none is optimal yet for achieving the full potential of HGT in patients.

The *E1*/*E3*-deleted, non-replicating recombinant adenovirus of serotype 5 (Ad5) expresses most of the genes of its genome in the host cell driven primarily by the *E4* gene. These ''foreign'' proteins (when expressed on the surface of the host cell) elicit local immune/inflammatory responses which can lead to elimination (by cytotoxic T-cells) of the infected host cells curtailing the duration of therapeutic gene expression. Second-generation (*E1*, *E2*, *E3* and *E4* gene deletions) adenoviral vectors or ''gutted'' adenoviruses (where most of the viral genome is deleted) have all shown to be devoid of such immune reaction (allowing longer-term transgene expression) and also reduced inflammatory effect in the liver after systemic or intrahepatic application (Wilson, 1996). However, the level of transgene expression is reduced in these second-generation adenoviral vectors (compared to *E1*-deleted first-generation adenoviral vectors), which can be restored by ''adding'' back one or more of the several open reading frames (e.g., ORF3 and 4) of the *E4* gene (Christ et al., 2000).

Adenoviruses are human pathogens, and most patients have already been exposed to them during their lifetime resulting in the presence of various levels of circulating neutralizing anti-viral antibodies. This may hinder the effectiveness of their systemic

application. Use of viral vectors which are not human pathogens (e.g., various serotypes of adenoviruses, AAV or non-human adenoviruses) can avoid this problem. Immune response evoked by the first application of the viral vector (human or non-human) may interfere with their repeated application (although the immune response may depend on the route of delivery and target tissue involved). First- and second-generation adenoviral vectors have limited cloning size (<10 kb), which prevents the use of large therapeutic genes, multiple genes or complex gene regulatory elements. The use of "gutless" adenovirus avoids this problem allowing cloning capacity of up to ~30 kb, but filling the vector with "useless" DNA is a challenge, and so is the proper manufacturing of these modified vectors, which require the presence of helper viruses. Change in viral coat proteins (fibers, pentons, etc.) will be needed to redirect the tropism of the virus. Finally, future viral vectors will consist of specific gene regulatory systems (gene-switches, tissue-specific promoters, etc.) to allow proper timing, duration, extent and localization of therapeutic gene expression.

3.1.2. Non-viral vectors

The existing synthetic vectors (naked DNA, cationic liposomes, etc.) are far from being perfect delivery systems. Although they are less pathogenic and may have reduced toxicity compared to some of the existing viral vectors, depending on the dose injected, liposomes may aggregate in the blood and can cause severe toxic reactions (Li and Huang, 2000).

Plasmid and liposome complexes are easy to produce and are safe, but they have low gene-transfer efficiency. However, novel lipid formulations and synthetic cationic polymer carriers have clearly improved the effectiveness of plasmid-mediated gene transfer (Stephan et al., 1996; Plank et al., 1996; Turunun et al., 1999).

In the future, the "perfect" gene delivery vector may be synthetic, incorporating many of the advantages of viruses (which over millions of years of evolution acquired the perfection of gene delivery to host organisms, such as dense DNA "packaging", cell recognition, cellular uptake, cytosolic trafficking, efficient nuclear uptake and gene expression in the host cell nucleus) (Fig. 3), but avoiding the unwanted properties of viruses (e.g., pathogenicity, cell toxicity, immune and inflammatory reactions, etc.).

Dense DNA packaging (a prerequisite for efficient gene transfer) can be achieved by using, for example, protamine sulfate (a "trick" borrowed from sperm cells, which similar to viruses, package DNA efficiently). The particle should be "shielded" from binding to plasma proteins, blood cells or to each other to allow longer circulating plasma half-life and more efficient uptake in target tissues (e.g., at the site of leaky vessels in tumors) ("passive" targeting). They are also formulated to resist breakdown of packaged DNA by nucleases (e.g., PINC™ system developed by Valentis Corporation). They will be engineered to contain cell recognition ligands (e.g., transferrin for proper "active" targeting of cancer cells), cell membrane fusion proteins and nuclear localization signals (all "borrowed" from viruses) for efficient cellular trafficking. Chromosomal localization and insertion, for long-term expres-

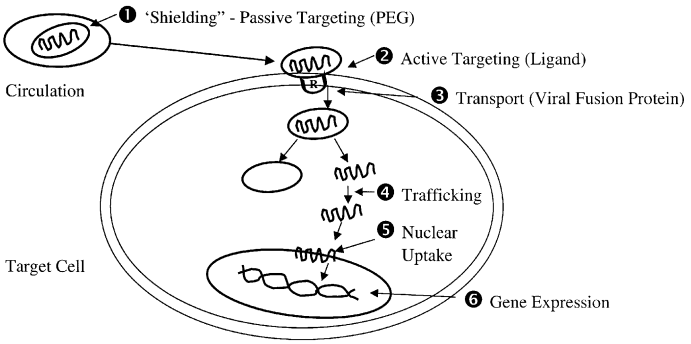

Fig. 3. *The "ideal" synthetic (non-viral) gene delivery vector.* After dense DNA packaging is accomplished (e.g., by protamine sulfate) the surface of synthetic particles (which is usually positively changed) needs to be "shielded" (e.g., by polyethylene-glycol [PEG]) so that they do not attach to blood elements or to each other, and therefore have an extended circulating plasma half-life (1) (passive targeting to "leaky" vessels). The surface of the particles will contain specific ligands for active targeting to selected cells/tissues (2). By engineering viral fusion proteins to the particle coat, cell-entry is facilitated (3). Cellular trafficking will be enhanced and intracellular degradation of DNA prevented (4). Nuclear uptake will be facilitated by viral nuclear localization signal (NLS) peptides (5). Chromosomal localization will be augmented (e.g., by the *rep* gene which allows targeting to chromosome 19) and gene expression regulated by specific transcriptional control elements (6).

sion of the therapeutic gene will be achieved by adding, for example, the *rep* gene, which allows targeting and insertion of the DNA to chromosome 19 (Young et al., 2000). These are but a few of the examples, already being tested today. However, even more sophisticated systems can be expected in the future, along with appropriate manufacturing and analytical processes, which will allow their introduction to human subjects.

3.1.3. Cell-based delivery of therapeutic genes

Although cell therapies have been used in medicine for several decades (e.g., blood transfusion), the use of cells manipulated ex vivo with therapeutic genes and then reintroduced into patients offers a new strategy by which to deliver therapeutic genes. Of particular interest are human stem cells, which give rise to various cell lineages in particular organ systems (Asahara et al., 2000).

Human hematopoietic stem cells, mesenchymal stem cells, neuronal stem cells, and embryonic stem cells are the focus of present research efforts (Gage, 1998; Asahara et al., 2000). The panel of stem cells available for gene therapy purposes will increase as isolation and culturing procedures improve and appropriate factors are identified which can be used to drive their differentiation along distinct cell lineage pathways. Issues currently being addressed include the development of vectors for efficient stem cell gene transduction, expression and regulation of therapeutic genes during lineage progression from stem cells to differentiated cells, control of stem cell growth, expansion ex vivo and engraftment and differentiation in vivo. Genetically engineered stem cells or less pluripotent progenitor cells are currently being tested for

therapeutic angiogenesis (endothelial cell progenitors), Parkinson's disease (neuronal stem cells), bone marrow transplantation (hematopoietic stem cells) and AIDS (e.g., hematopoietic stem cells transfected with the *RevM10* gene) (Gage, 1998; Asahara et al., 2000; Su et al., 1997).

3.1.4. "Customized" gene delivery vectors

The "perfect" or "ideal" vector will meet all of the requirements of the successful treatment of a specific disease target. It is important to emphasize that there will not be a "universal" vector, optimally useful for all indications. On the contrary, each disease target will have a specific set of technical requirements, and the "perfect" vector for a specific disease should be optimized according to these specific criteria. For example, some diseases will require local delivery (e.g., ischemia, restenosis, retinitis pigmentosa, Parkinson's disease, etc.), while others necessitate systemic delivery (e.g., cancer, atherosclerosis). For certain diseases, the gene of a secreted protein (e.g., coagulation factors VIII and IX for hemophilia A and B, respectively, growth hormone, erythropoietin, etc.) can be expressed in almost any tissue of the body. Sometimes only transient, short-lived gene expression will be needed (e.g., therapeutic angiogenesis, cancer) while in other cases long-term (sometime life-long) gene expression duration will be necessary (e.g., most monogenic diseases, such as familial hypercholesterolemia, hemophilia, SCID, etc.) For certain disease targets most if not all target cells need to be transfected (cancer), while in other cases this will not be necessary (e.g., with most secreted therapeutic proteins).

In certain diseases tight control of gene expression will not be important (e.g., coagulation factors VIII and IX for hemophilias), while in others very tight regulation of the gene expression will be essential (e.g., insulin for diabetes). Some diseases will require specific targeting of the vector for efficient and safe delivery after systemic application (e.g., cancer). Other disease targets will require tissue or disease-specific promoter elements (e.g., arteriosclerosis, cancer). In some instances conditionally inducible gene expression regulation (gene-switch) will allow precise dosing and timing of gene expression.

Although they will be different, most of the vectors optimized for a certain disease target will consist of multiple "parts", each fulfilling some necessary technical need. Since the different elements will probably be perfected (and patented) by different companies, the introduction of the optimal multicomponent vector may be difficult because of intellectual property rights and commercial obstacles.

3.2. Gene delivery targeting

The effectiveness of gene therapy is determined by a combination of the effects of gene delivery into the target tissue, the entry of the new genetic material into cells, and the expression of the transfected gene in the target tissue (Fig. 1). When specific physical or biological targeting methods are available, they generally improve the expression of the transfected gene in the target organ.

3.2.1. Physical targeting

A variety of physical gene delivery methods have been introduced to achieve better local tissue targeting of vectors. An example of the effective physical targeting is catheter-mediated gene transfer to various regions of the circulation (e.g., feed arteries of organs, such as leg muscles, heart and liver, or retrograde injection via veins) (Takeshita et al., 1994; Boekstegers et al., 2000; Giordano et al., 1996). Intramuscular injection of plasmid DNA or viral vectors encoding angiogenic growth factors has been used in ischaemic myocardium (Mack et al., 1998) and peripheral vascular disease (Tsurumi et al., 1996; Shyu et al., 1998). Another approach to local delivery to small arterioles and capillaries is injection of biodegradable microspheres coated with recombinant growth factors or plasmid DNA (Banai et al., 1994). Ultrasonography (Lawrie et al., 1999), alone or in combination with microbubbles (Villanueva et al., 1998), can also potentially be used to improve the efficiency of gene transfer. For facilitation of intramuscular, intratumoral or intradermal delivery of naked, plasmid or cationic liposome-carried DNA, gene gun technology (Yang and Sun, 1995) and electroporation (Mir et al., 1999) can be used. Although these delivery methods offer certain advantages in specific disease targets, they will be replaced by more specific biological targeting methods in the future. Most of these physical targeting methods may become obsolete in a few years, and we will look back at them as "desperate" approaches of the pioneering era of gene therapy.

3.2.2. Biological targeting

In contrast to physical targeting, biological vector targeting uses modification of viral coat proteins (for viral vectors) or surface properties of synthetic vectors (e.g., liposomes). Passive targeting makes use of alteration of the pharmacokinetics of liposome vectors by "shielding" them from binding to plasma proteins, unwanted tissues or to each other, allowing them to circulate for longer periods of time in the blood and accumulate in specific tissues with "leaky" blood vessels (such as tumors) (Wu et al., 1993).

The use of targeted viral vectors to localize gene transfer to specific cell types holds many advantages over conventional, non-targeted vectors currently used in gene therapy. The resulting improvements in gene localization from targeted adenovirus vectors are likely to reduce immunogenicity and toxicity, increase safety, and enable the systemic administration of these vectors for multiple indications including cancer, cardiovascular diseases, and inflammatory diseases. Recent advances in the biological understanding of adenovirus structure and adenovirus receptor interactions have lead to the development of targeted adenovirus vectors. Receptor-mediated entry of adenovirus into cells has been found to depend on two of its coat proteins. The fiber knob protein mediates primary attachment to the cell via the coxsackie-adenovirus receptor (CAR) protein (Bergelson et al., 1997). Following cell attachment via fiber–CAR interaction, an RGD tripeptide motif in the penton base protein binds to integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) which mediate cellular internalization (Wickham et al., 1993; Nemerow and Stewart, 1999) (Fig. 2).

Two basic requirements are necessary to create a targeted adenovirus vector: interaction of adenovirus with its native receptors (e.g., CAR) must be first removed and novel, tissue-specific ligands must be added to the virus (Douglas et al., 1996; Wickham et al., 1996, 1997). Two general approaches have been used to achieve these basic requirements. In the "two-component" approach, a bi-specific molecule is complexed with the adenovirus (Douglas et al., 1996; Krasnykh et al., 2000). The bi-specific component simultaneously blocks native receptor (CAR) binding and redirects virus binding to a tissue-specific receptor (e.g., to integrins using the RGD motif). In the "one-step" approach the adenovirus is genetically modified in the fiber protein domains to remove native receptor interactions and a novel ligand is genetically incorporated into one of the adenovirus coat proteins (Wickham et al., 1997; Krasnykh et al., 2000; Wickham, 2000). For example, adenoviral vectors, which contain polylysine motifs or RGD genetically incorporated into the fiber, have been shown to enhance the transduction of a variety of cells which lack adenovirus (CAR) receptors (Wickham, 2000). Further studies using genetically modified, tropism-expanded vectors have shown that they increase the in vivo transduction of both vascular smooth muscle and certain types of tumors (Shinoura et al., 1999). High affinity peptide ligands have been inserted into the HI loop or on to the C-terminus of fiber proteins or into the RGD loop of the penton base proteins.

Engineering the surface of synthetic vectors with specific ligands (e.g., transferrin, EGF, etc.) enabled them to target cancer cells (Yanagihara et al., 2000).

3.2.3. Transcriptional targeting

Receptor targeting technology can be combined with "transcriptional targeting" approaches (e.g., tissue- or disease-specific promoters) to create vectors which deliver genes selectively, safely, and with little immune response.

Tissue or disease specificity of a gene therapy product can be achieved by the incorporation of a tissue- or disease-specific promoter into the vector, which allows therapeutic gene expression only in cells which express transcription factor proteins binding to these specific promoter sites. Some of the tissue- or disease-specific promoters, identified and tested in animal models to date include the promoter of the *prostate-specific antigen* (*PSA*) gene (Pang et al., 1997), *osteocalcin* gene (Ko et al., 1996), and hypoxia response element (HRE) activated by hypoxia inducible factors (HIF) in hypoxic/ischemic tissues (e.g., tumors) (Shibata et al., 2000). The ever growing genomic database and the development of novel technologies to analyze these databases will lead to the identification of other tissue- and pathology-specific promoter elements to be used in gene delivery vectors and gene expression control systems in the future.

3.3. Gene expression control systems

Regulating therapeutic gene expression will be necessary for both the clinical efficacy and safety of most HGT applications. Gene therapy offers the promise of

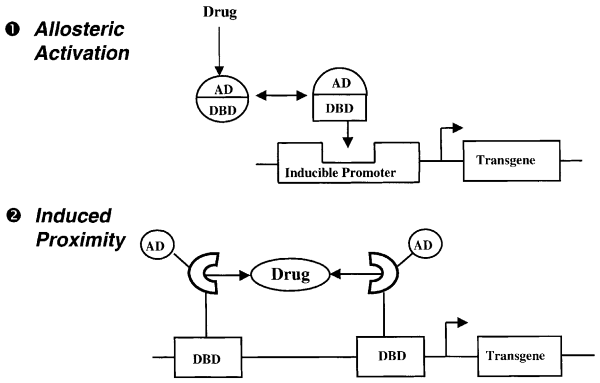

Fig. 4. *Gene expression control systems.* In order to regulate the timing and level of therapeutic protein production within the target cell, several gene expression control systems have been designed. Most of these systems make use of chimeric transcription factor proteins (consisting of an activation domain [AD] and a DNA binding domain [DBD]) which after "activation" by small molecules in a dose-dependent manner, interact with DNA elements incorporated into the vector construct and regulate the expression of a therapeutic transgene. The figure illustrates two different types of such control mechanism, the allosteric activation (top) and induced proximity (bottom) systems.

replacing frequent injections of an expensive protein with an infrequent or even one-time administration of a gene delivery vector, which would then provide continuous therapeutic protein production at the desired site. An appropriate system of gene expression will control and allow titration of protein levels, dosing to be adjusted as the disease evolves, and therapy to be initiated repeatedly or terminated at will. Regulation can be achieved by a physiological/pathological signal (e.g., glucose, hypoxia) (Varley and Munford, 1998; Thule et al., 2000) or via dose-dependent ligand binding and activation of chimeric transcription factor proteins, which then interact with DNA elements incorporated into the vector construct and regulate the therapeutic gene (Clackson, 2000) (Fig. 4). Some of these systems are suitable for the use of orally active low molecular weight drugs to regulate the level and timing of therapeutic gene expression.

Some applications of gene therapy require no precise gene expression regulation, because they involve proteins with large therapeutic windows (such as adenosine deaminase, CFTR and coagulation factors VIII and IX). These applications, however, represent only a small part of the clinical potential of gene therapy. Most therapeutic proteins have limited therapeutic window, both in terms of their level and their duration of action. For gene therapy to achieve its full potential as a widely applicable technology for safe and effective protein delivery, control over the level and duration of gene expression will be essential.

3.3.1. Small molecule regulated transcription systems

Much progress has been made on control systems, where gene expression is regulated pharmacologically by a small molecule drug. Four major systems are

known to date which have already been tested in animals: those regulated by the antibiotic tetracycline (Tet) (Gossen and Bujard, 1992), the insect steroid ecdysone or its analogs (No et al., 1996), the anti-progestin mifepristone (RU486) (Wang et al., 1994), and chemical dimerizers such as the immunosuppressant rapamycin and its analogs (Rivera et al., 1996; Ho et al., 1996; Magari et al., 1997). They all involve the small molecule-dependent recruitment of a transcriptional activation domain to a basal promoter driving the gene of interest, but differ in the mechanism of recruitment (Clackson, 1997) (Fig. 4). Such a pharmacological gene expression regulation system should meet the following criteria. Basal expression should be very low and inducible to high levels over a wide dose range. Induction should be a positive effect (adding rather than removing a drug), and use of an orally active small molecule that has no pleiotropic effects in mammalian cells. The regulatory protein(s) should have no effects on endogenous gene expression, and should be of human origin to minimize immunogenicity.

4. Disease targets for gene therapy

Ever since the first clinical attempt of *ADA* gene transfer 10 years ago, numerous monogenic (Table 2) and complex (multigenic) diseases (Table 3) were targeted with a large variety of gene therapy strategies, including the use of numerous therapeutic genes (Tables 2 and 3) and gene delivery vectors (Table 1), in an exponentially growing number of clinical trials. According to recent counting there were 368 gene therapy clinical protocols submitted or trials in progress world wide (332 in the USA and 36 outside the USA) (Human Gene Marker/Therapy Clinical Protocols, 2000).

Table 2
Disease targets for gene therapy: monogenic diseases

Disease	Gene(s)	Number of clinical protocols/trials (1990–1999)[a]
Cystic fibrosis	*CFTR*, *α-1-anti-trypsin*	24
Severe combined immuno deficiency (SCID)	*ADA*	3
Gaucher disease	*Glucocerebrosidase*	3
Canavan disease	*Aspartoacylase*	2
Hemophilia A	*Factor VIII*	2
Hemophilia B	*Factor IX*	2
Familial hypercholesterolemia	*LDL-R*	1
Hunter disease	*Idurinate-2-sulfatase*	1
Muscular dystrophy	*Sarcoglycan*, *dystrophin*, *utrophin*	1
Fanconi anemia	*Group A* gene	1
Purine nucleoside phosphorylase deficiency	*PNP*	1
Ornithin transcarbamylase deficiency	*OTC*	1

[a] From "Human Gene Marker/Therapy Clinical Protocols" (2000).

Table 3
Disease targets for gene therapy: multigenic diseases

Disease	Gene(s)	Number of clinical protocols/trials (1990–1999)[a]
Cancer	multiple (see text)	280
Cardiovascular disease (coronary and peripheral artery disease, restenosis, vein-graft failure)	*VEGF-A*, *VEGF-C*, *bFGF*, *FGF-4*, *HIF-1α/VP16*, *E2F-Decoy*	20
AIDS	*Rev*, antibodies, antisense, ribozymes, etc.	19
Rheumatoid arthritis	Cytokines, *IL-1RA*	2
Chronic granulomatous disease	*gp91 phox*	2
Multiple sclerosis	Immune-modulation	1
ALS	*CNTF*	1
Carpal tunnel syndrome	*IGF-1*	1
Leukocyte adherence deficiency	*CD 18*	1

[a] From "Human Gene Marker/Therapy Clinical Protocols" (2000).

A majority of these clinical trials are directed at diseases that are life threatening and for which currently available therapies are not highly effective, such as HIV infection (AIDS) and certain cancers. Gene therapy trials are also ongoing for monogenic disorders with low incidence in the population (e.g., cystic fibrosis). These are being addressed through our knowledge of the defective genes in these rare inherited diseases. Clinical trials which do not address immediate therapeutic benefits to the individuals, such as gene-marking studies, are performed in order to test specific hypotheses concerning in vivo vector functioning and targeting or efficiency and fate of gene-modified cells in humans. The most "popular" disease target is cancer (with more than 280 clinical trials in the past decade). Although the number of ongoing clinical trials is lower (~20), one of the most "promising" disease targets to date appear to be among cardiovascular diseases (e.g., therapeutic angiogenesis, restenosis, stent- and bypass graft failure), because of the relative ease of access of the vascular system, and the matching of some of the clinical needs to achieve therapeutic benefits with existing gene therapy technology. Due to space limitations, all of the major diseases targeted by gene therapy today can not be reviewed. However, brief evaluation of two areas (cancer and therapeutic angiogenesis) will help to illustrate the importance of identifying and overcoming specific technical hurdles for clinical success of gene therapy.

4.1. Cancer gene therapy

The therapeutic goal in any treatment modality for cancer is effective killing of most (if not all) cancer cells without serious damage to normal cells and tissues. Therefore, gene therapy should also aim at effective and selective killing of cancer cells. So far, there has been a disappointing inability to reach cancer cells with

Table 4
Cancer gene therapy clinical trials in Phase II/III (Adenoviral vectors)

Product (sponsor)	Indication	Application	Phase
Adeno-p53 (Schering/Canji)	Ovarian carcinoma	i.p. + chemotherapy[a]	II/III
Adeno-p53 (Aventis/Introgen)	Various incl. Head & neck, NSCLC	i.t.[b]	II
CN706 (Calydon)	Prostatic carcinoma	i.t.	I/II
		Hepatic artery	I/II
Onyx 015 (Onyx Pharmaceuticals)	Head & neck tumors	i.t. + chemo therapy	II
	Pancreatic carcinoma	i.t.	I/II
	Hepatocellular carcinoma	i.t.	I/II

[a] i.p., intraperitoneal.
[b] i.t., intratumoral.

sufficient efficacy to generate high enough levels of direct killing. At least in the foreseeable future, clinical advance will come from cooperation with other, more established, therapeutic modalities – such as chemotherapy, radiotherapy and immunotherapy.

The field of gene therapy for cancer now has the experience of numerous clinical trial data to assess the efficacy of both the genes and the vectors which have been used so far. However, the available technology (e.g., potency of therapeutic genes and efficacy of gene delivery vectors) are far from optimal to achieve the desired clinical benefits in the field of solid tumors and their metastasis. The available data suggest that the problems with cancer gene therapy involve primarily the gene delivery technology rather than the choice of the therapeutic gene(s).

Although there are numerous gene therapy clinical trials in the cancer field reaching the Phase II and Phase III stages with both viral (Table 4) and non-viral vectors (Table 5), they invariably use local, intratumoral delivery routes, and not the more optimal systemic delivery route yet, which would allow treatment of not only the primary tumor but also its metastases. The main reason for this is the fact that none of the existing vectors allow effective and selective gene delivery and gene expression in the tumor tissue. In order to accomplish this goal, significant technical hurdles (e.g., bystander effect, conditionally replicating viruses, vector targeting, etc.) need to be overcome in the future.

4.1.1. Therapeutic strategies for cancer

There are at least five major classes of approaches presently developed for cancer gene therapy: (1) inhibition of tumor angiogenesis, (2) immunotherapy, (3) induction of apoptosis, (4) conditionally replicating (oncolytic) viruses, and (5) suicide genes (Palu et al., 1999; Heise et al., 1999; Moolten, 1994; Bischoff et al., 1996; Marcelli et al., 1999; Bouvet et al., 1998; Liu et al., 1999; Frank et al., 1998; Russell, 1994; Vile et al., 1998; Taniguchi et al., 1998; Li et al., 1998; Dranoff et al., 1993; Cavallo et al., 1999; Melcher et al., 1997; Pietersen et al., 1999).

Table 5
Cancer gene therapy clinical trials in Phase II/III (non-viral, immune stimulation)

Product (Sponsor)	Indication	Application	Phase
IL2 (Vical)	Kidney cancer	i.t.[a]	II
	Prostate cancer	i.t.	II
IL2 (Valentis)	Head & neck	i.t.	IIb
		i.t. + chemotherapy	IIb
IL12 (NIH)	Head & neck	i.t.	I/II
IL12 (Valentis)	Head & neck	i.t.	I/II
HLA-B7 (Vical)	Melanoma	i.t.	II
	Melanoma	i.t. + IL2	I/II
	Melanoma	i.t. + dacarbazine	III
IFN-α (Valentis)	Head & neck	i.t.	I/II
	Malignant mesothelioma	i.t.	IIa
E1A (Targeted Genetics)	Head & neck, ovarian	i.t.	II
		i.p. + chemotherapy[b]	I

[a] i.t., intratumoral.
[b] i.p., intraperitoneal.

An expanding blood supply through the process of angiogenesis is the absolute requirement for tumor growth. There is a long list of targets at the tumor vasculature that could be exploited by anti-angiogenic approaches using gene therapy. Most likely candidates to be used first in gene therapy approaches include angiostatin, endostatin, thrombospondin-1 and uPA-fragment (Bouvet et al., 1998; Liu et al., 1999; Taniguchi et al., 1998; Li et al., 1998; Rubanyi, 2000; Folkman, 1995).

The immune system provides two mechanisms, what cancer gene therapy should mimic: (i) amplification of the therapeutic potential following relatively low level of gene delivery, and (ii) high level specificity of body-wide targeted cell killing. This is the reason why the majority of cancer gene therapy protocols have been aimed at immune stimulation to fight metastatic cancer (Plautz et al., 1993). Cytokine gene transfer (e.g., using IL-2, IL-12, IFNα, IFNβ and IFNγ, alone or in combination) was successfully used in animal models of human tumors and the immune response mediated by them also plays a role in suicide-gene-induced tumor cell killing (Marcelli et al., 1999; Colombo et al., 1992). Clinical trials have shown encouraging signs that cytokine-modified vaccines can generate significant immune responses in patients with minimal toxicity (Soiffer et al., 1998). Promising results have been obtained with co-stimulatory molecules (e.g., HLA-B7), T-cell receptor chimeras, ex vivo cell therapy and immunoconjugates. The two most significant areas in which immunogene therapy is likely to progress in the future are the identification of tumor-associated antigens and exploitation of the significance of the dendritic cell in generating anti-tumor immune responses (Banchereau and Steinman, 1998; Huang et al., 1994).

Selective induction of apoptosis (programmed cell death) in tumor cells is also a "popular" approach. Some of the pro-apoptotic genes tested so far include, *p53*,

E1A, *p16/p27*, *FasL*, *caspases*, *Bax*, *Bak* and *apoptin* (Marcelli et al., 1999; Frank et al., 1998; Pietersen et al., 1999).

Oncolytic viruses (e.g., adenovirus, HSV, reovirus, New Castle Disease virus, etc.), with or without conditional replication capabilities, are being developed (Heise et al., 1999; Bischoff et al., 1996; Russell, 1994).

Cancer-cell-specific suicide genes (e.g., *HSV-TK* and *CDA*), activated by small molecule drugs (e.g., ganciclovir and 5-fluorouracil, respectively) are being used in many cancer gene therapy approaches (Palu et al., 1999; Moolten, 1994).

Thus, there are plenty of appropriate therapeutic genes available for cancer gene therapy approaches. However, as already mentioned, the ultimate clinical success of gene therapy for cancer will have to await the development of effective and site-specific gene delivery systems, which will meet the specific technical requirements of these indications.

4.2. Cardiovascular gene therapy

Cardiovascular diseases are relative "newcomers" as targets for HGT, compared to other diseases, such as cancer or monogenic disorders. None the less, cardiovascular diseases are expected to become one of the most promising targets for gene therapy in the short term, because blood vessels are among the easiest to access for gene transfer and, in most disorders, only a temporary expression of the transfected gene in some but not all target cells will be required to achieve therapeutic benefits (Nabel and Nabel, 1994; Yla-Herttuala and Martin, 2000; Finkel and Epstein, 1995; Gibbons and Dzau, 1996; Yla-Herttuala, 1997).

Current targets for cardiovascular gene therapy include therapeutic angiogenesis for coronary artery disease (CAD) and peripheral arterial occlusive disease (PAOD) (Rubanyi, 2000; Dormandy et al., 1999), prevention of postangioplasty and stent restenosis (Sanghong and March, 1998; De Young and Dichek, 1998) and bypass vein-graft failure (Bai et al., 1998; Von der Leyen et al., 1995).

Gene transfer of vascular endothelial growth factors (VEGFs) and fibroblast growth factors (FGFs) have improved blood flow and collateral development in various animal models, including mouse (Couffinhal et al., 1998), rat (Yang and Terjung, 1993) and rabbit (Takeshita et al., 1994; Shyu et al., 1998; Yang and Terjung, 1993) for PAOD, and dog (Banai et al., 1994; Arras et al., 1998) and pig (Giordano et al., 1996) for CAD. Promising therapeutic effects have also been obtained in animal models of restenosis (Sanghong and March, 1998; De Young and Dichek, 1998) or vein-graft stenosis (Bai et al., 1998; Von der Leyen et al., 1995) with the transfer of nitric-oxide synthase (NOS), thymidine kinase (TK), retinoblastoma (Rb), cyclin or cyclin-dependent kinase inhibitors, fas ligand and antisense oligonucleotides against transcription factors or cell-cycle regulatory proteins (Simons et al., 1992; Morishita et al., 1993, 1995, 1997; Indolfi et al., 1995; Pollman et al., 1998; Chang et al., 1995a,b; Yang et al., 1996; Tanner et al., 1998; Yonemitsu et al., 1998; Ohno et al., 1994; Harrel et al., 1997; Sata et al., 1998). First clinical experience of *VEGF* and *FGF* gene transfer for therapeutic

angiogenesis (Laitinen et al., 2000; Isner et al., 1996; Baumgartner et al., 1998; Losordo et al., 1998; Makinen et al., 1999; Rosengart et al., 1999; Selke et al., 1998; Laham et al., 1999a,b; Schumacher et al., 1998; Symes et al., 1999) and decoy oligonucleotides for vein-graft stenosis in human beings have been reported (Mann et al., 1999) (Table 6). However, further developments in vectors, gene delivery techniques and identification of novel genes will be required before

Table 6
Cardiovascular gene therapy clinical trials

Disease	Investigator/company	Gene	Vector	Delivery route
Familial hypercholesterolaemia	Wilson et al.	*LDL receptor*	Retrovirus	Ex vivo hepatocytes
Peripheral-artery disease	Baumgartner et al.	*VEGF-A*	Naked DNA	Intramuscular injection
	Isner et al.	*VEGF-A*	Naked DNA	Intramuscular injection
	Warner–Lambert/GeneVec	*VEGF-A*	Adenovirus	Intramuscular injection
	Yla-Herttuala et al.	*VEGF-A*	Liposome/adenovirus	Infusion–perfusion catheter after angioplasty
	RPR Gencell	*FGF-1*	Plasmid (pCOR)	Intramuscular injection
	Isner et al./Vascular Genetics Inc.	*VEGF-C*	Naked DNA	Intramuscular injection
	Schering AG/Berlex	*FGF-4*	Adenovirus	Intramuscular injection
Coronary artery disease	Losordo et al.	*VEGF-A*	Naked DNA	Intramyocardial injection via thoracotomy
	Yla-Herttuala et al.	*VEGF-A*	Liposome/adenovirus	Infusion–perfusion
	Sylven et al.	*VEGF-A*	Naked DNA	Intramyocardial injection via thoracotomy catheter after angioplasty
	Rosengart et al./GeneVec	*VEGF-A*	Adenovirus	Intramyocardial injection during bypass operation or minithoracotomy
	Berlex Laboratories Inc./Schering AG	*FGF-4*	Adenovirus	Intracoronary injection
	Isner et al./Vascular Genetics Inc.	*VEGF-C*	Naked DNA	Intramyocardial injection
	Isner et al./Vascular Genetics Inc.	*VEGF-C*	Naked DNA	Catheter-based myocardial injection
Restenosis/vein-graft stenosis	Isner et al.	*VEGF-A*	Naked DNA	Hydrogel-coated balloon catheter after angioplasty
	Eurogene Ltd.	*VEGF-A*	Plasmid/liposome	Adventitial delivery with biodegradable reservoir
	Sylven et al.	*VEGF-A*	Naked DNA	Intramyocardial injection via thoracotomy catheter after angioplasty
	Mann et al.	*E2F Decoy*	Oligonucleotide	Pressure ex vivo delivery

the full clinical potential of gene therapy in cardiovascular diseases can be achieved.

4.2.1. Therapeutic angiogenesis

Therapeutic angiogenesis is an innovative approach aimed at increasing the number of collateral vessels delivering blood and oxygen to ischemic tissue (Rubanyi, 2000; Dormandy et al., 1999; Folkman, 1995). None of the existing medical therapies (e.g., β-blockers, Ca^{2+}-antagonists and nitrates for CAD, and PDE-inhibitors and prostanoids for PAOD) promote the growth of collateral vessels. Surgical revascularization procedures restore blood flow in large conduit arteries, without promoting arteriogenesis or angiogenesis. VEGFs and FGFs have been the most widely used agents for therapeutic angiogenesis. FGFs affect various types of cells in vivo, which differentiates them from the VEGF family, whose receptors are present mostly on endothelial cells and monocytes or macrophages (Neufeld et al., 1999). Angiopoietin-1 and angiopoietin-2 have been identified as factors that may modify the response of VEGF gene therapy by affecting maturation and stability of the new vessels (Shyu et al., 1998; Holash et al., 1999). Recent findings in mice suggest a synergistic interaction between VEGF and placental growth factors (PIGF) in angiogenesis (Carmeliet, personal communication). Stimulation of endothelial stem cells by VEGF or other means could be a new approach to achieve therapeutic angiogenesis in ischemic tissues (Takahashi et al., 1999).

Gene therapy might be superior to infusion of recombinant growth factor protein (Isner, 1997) to produce therapeutic angiogenesis, since sustained expression of growth factors in the ischemic tissue is required for the formation of new blood vessels. In addition to higher costs and frequency of administration, infusion of growth factor proteins also has the disadvantage over gene therapy, that the angiogenic factor is exposed from the "wrong" (luminal) side of the blood vessels. Physiologically, these factors are produced by hypoxic parenchymal cells (myocytes) or infiltrating monocytes. Indeed, two clinical trials, one with VEGF (Henry et al., 1999) and another with FGF protein infusion (Laham et al., 2000) have failed to achieve therapeutic efficacy in patients with CAD.

Potential risks of therapeutic angiogenesis could be the production of hemangiomas, stimulation of angiogenesis in tumors (Folkman, 1995; Springer et al., 1998) and in the eye (retinopathy), and neovascularization in atherosclerotic lesions which might lead to plaque rupture (Moulton et al., 1999). These complications may be overcome by an increase in tissue specificity of vectors and gene expression regulation systems.

4.2.2. Success factors and their fulfillment in therapeutic angiogenesis using gene therapy

As it was stated already, each disease indication has its specific technical hurdles to overcome before gene therapy can become successful in the clinic. To illustrate this point, we chose a specific disease target (CAD) and therapeutic approach:

therapeutic angiogenesis with angiogenic gene therapy (AGT). Some of the criteria for successful AGT of ischemic (coronary) heart disease include: choice of (i) appropriate therapeutic gene(s) (e.g., *VEGF* and *FGF*, with proven role in pathogenesis of disease target), (ii) gene delivery vector (e.g., replication incompetent adenovirus 5), (iii) route of vector delivery (e.g., catheter-mediated intracoronary injection). Further success criteria are (iv) sufficient transfection efficiency in target organ (i.e., heart), (v) transgene expression duration appropriate for the indication (transient, to initiate angiogenesis but does not lead to development of hemangiomas), (vi) lack of local tissue reaction to vector/transgene (immune/inflammatory reaction), (vii) lack of widespread vector distribution and transgene expression in the body, (viii) no angiogenesis at distant sites (e.g., eye), (ix) no toxic effect (e.g., liver inflammation, etc.), (x) clinically relevant therapeutic effect in an appropriate animal model of disease, (xi) GLP and GMP process development and adequate analytical tools for quality control for the manufacturing of viral vectors for GLP toxicology studies and GMP clinical trials, (xii) appropriate safety in human patients (phase I clinical trial) and (xiii) significant therapeutic efficacy in patients (Phase II and III clinical trials). With the exception of demonstrating statistically significant clinical efficacy in a large scale, multicenter, double-blind and placebo controlled clinical trial, all of the above criteria (success factors) have already been met in the AGT program, sponsored by Schering AG/Berlex Laboratories (in collaboration with Collateral Therapeutics Inc.), using intracoronary injection of Ad5FGF-4 to patients with CAD (Table 6). The reasons for this success are based on the specific technical and therapeutic requirements of this disease target, which may be satisfied with gene therapy technology available today (see below).

During episodes of exercise or stress-induced myocardial ischemia (stable or exertional angina), endogenous angiogenesis/arteriogenesis occurs, leading to the development of some collateral vessels. Thus, most (if not all) of the factors needed for angio/arteriogenesis (i.e., expression of angiogenic growth factors and modulator proteins and their receptors) are most probably present in the hypoxic/ischemic myocardium of these patients. The problem is that endogenous collateral formation is insufficient to relieve the heart completely from ischemia during episodes of stress-induced angina. Although the exact cause(s) of this lack of sufficient collateralization in the human heart is unknown, it is proposed that either the pro-angiogenic factor(s) are suboptimal and/or that co-existence of anti-angiogenic mechanisms curtail their effect. In any case, delivery and expression of angiogenic growth factor genes (e.g., *FGF* or *VEGF*) in the myocardium, will "tip the balance" for allowing sufficient collateral formation (i.e., there is probably no need to deliver an optimal mixture of all necessary angiogenic factors).

Therapeutic benefits can be achieved by transfection/transduction of some (but not all) target cells because these cells (cardiac myocytes) will serve as "factories" for production of the secreted growth factor protein. Transient gene expression maybe sufficient because the growth factor proteins are secreted and retained in the extracellular space, probably long after gene expression has already been ceased and also because the growth factor is needed for a relatively short period of time to initiate the

angiogenic program, which will result in mature vessel formation. Long-term local expression of VEGF protein may lead to excessive angiogenesis and the formation of hemangiomas (Springer et al., 1998). Catheter-mediated intravascular delivery or intramuscular injection allow local intramyocardial targeted delivery of the gene-transfer vector. Cardiac myocytes express the CAR receptor, a prerequisite for efficient uptake of adenovirus vectors of the serotype 5, allowing effective gene transfer. The endothelium of the coronary microcirculation takes up adenovirus very effectively from the blood, allowing efficient and selective delivery of the viral vector to the heart even after single intracoronary injection (high "first" pass effect). These facts allowed the successful use of first-generation (*E1*/*E3*-deleted, replication incompetent) adenovirus 5 (Ad5) vector for this indication (NB, for most other indications this vector may be inappropriate).

Intracoronary injection of recombinant, replication incompetent adenovirus 5 carrying a potent and secreted angiogenic growth factor gene (*FGF-5*) driven by the constitutive CMV promoter into coronary arteries of pigs instrumented with an ameroid constrictor around the left circumflex coronary artery resulted in efficient vector uptake (25–30% of myocardial cells), transgene expression, stimulation of the growth of new collateral vessels and complete normalization of stress-induced coronary blood flow and myocardial function deficit in the ischemic zone at relatively low (safe) levels of the viral vector (Giordano et al., 1996). Since the coronary circulation of the pig heart shows a lot of similarities to that of the human heart, this animal model is adequate to test preclinical efficacy and safety before introduction of the gene therapy product to human patients. Toxicology and biodistribution studies in pigs using the Ad5FGF-4 vector manufactured under GLP conditions showed no product related untoward effects up to 100 times higher doses (10^{12} vp/animal) than the effective dose (10^{10} vp/animal). Establishment of appropriate GMP manufacturing processes and development of novel analytical assays (Lehmberg et al., 1999) allowed us to initiate a placebo controlled, double-bind, multicenter, dose escalation (3×10^8–3×10^{10} vp) Phase I/II clinical trial using viral vectors which met all regulatory requirements. The clinical experience on 79 patients with stress-induced myocardial ischemia, studied so far in the Phase I/II trial, proved that intracoronary injection of the Ad5FGF-4 is safe up to a dose of 3×10^{10} vp/patient (Grines et al., presentation at the Am. Col. Cardiol. Meeting in March, 2001). Thus, by virtue of coincidence of several biologic and gene-transfer technology factors, therapeutic angiogenesis, using existing gene delivery technologies, may prove to be clinically effective and safe, thereby fulfilling the early promise of gene therapy.

4.2.3. Future improvements for therapeutic angiogenesis

The encouraging results so far, however, do not mean that further optimization of the product for therapeutic angiogenesis will not be necessary. For example using multiple genes ("gene-cocktails") which exert synergistic effect on collateral vessel formation (e.g., VEGF, FGF, NOS, angiopoietins, etc.) could lead to further reduction of the effective vector dose necessary to achieve therapeutic efficacy thereby further improving the safety of the gene therapy product. The use of new vectors

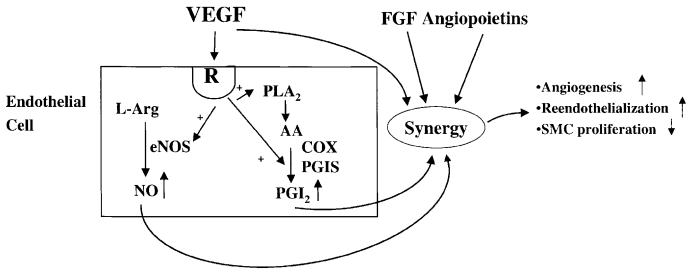

Fig. 5. *Synergy between various angiogenic factors to facilitate therapeutic angiogenesis (collateral formation and vessel maturation).* After new blood vessel growth is initiated by VEGF and FGF, the maturation process of the new blood vessel (i.e., recruitment of pericytes and smooth muscle cells, reduced permeability, etc.) requires the presence of angiopoietins. VEGF (and FGF in some instances) stimulates the release of nitric oxide (NO) and prostacycline (PGI_2) from endothelial cells, which mediate some of the angiogenic effects of VEGF and FGF. Thus, combination therapy with the genes of some of these factors may improve the clinical benefits of angiogenic gene therapy.

(e.g., to reduce further the risk of immune responses) and gene expression regulation systems could also contribute to improved clinical safety and efficacy.

VEGF, FGF, and angiopoietins may exert synergistic effects on the formation and maturation of new collateral vessels (Fig. 5) (Holash et al., 1999). VEGF stimulates the release of nitric oxide (NO) and prostaglandin I_2 (prostacyclin, PGI_2) from the endothelium (Van der Zee et al., 1997; Wheeler-Jones et al., 1997) (Fig. 5). Nitric oxide (NO) appears to mediate some of the angiogenic effects of VEGF and FGF, since in the absence of endothelial constitutive NOS (Murohara et al., 1998) or after its blockade (Terjung et al., 2000), these growth factors loose their angiogenic effect in animal models of limb ischemia. Since in most patients with ischemic heart disease (Rubanyi, 1999) or with peripheral vascular disease (Rubanyi and Dzau, 1997) endothelial NO production is severely impaired, therapeutic angiogenesis with a combination of *VEGF* (or *FGF*) and *NOS* genes could improve the clinical benefits.

More (novel) angiogenic factors will probably be identified through the human genome project and genomics technology. So far, only single growth factors or their genes have been used to induce angiogenesis. More information is needed about the maturation of new vessels, since only under certain (still poorly defined) conditions does neovascularization lead to the formation of persistent functional collateral vessels. Prevention of regression of the newly formed collateral vessels also needs to be addressed in the future.

5. Safety, regulatory and ethical aspects – lessons learned from recent gene therapy fatalities

Recent patient fatalities have been reported in gene therapy clinical studies, including the death of an 18-yr-old patient receiving high dose adenoviral gene therapy

for an inherited deficiency in ornithine transcarbamylase (OTC) (an enzyme involved in the urea cycle and ammonia metabolism) (Lehrman, 1999; Hollon, 2000; Brenner, 2000), and a patient with severe CAD shortly after intramyocardial injection of *VEGF* gene carried by a viral vector (Isner, 1999). Such fatalities underscore the risks involved in pioneering, new approaches in medicine. They also highlight the requirement for comprehensive regulatory guidelines and stringent review procedures for clinical protocols. With regard to the need to monitor and document safety aspects of gene therapy the FDA has published guidelines for academic and industrial researchers, and regulatory agencies in other countries require similar safety testing.

In December 1999, the Recombinant DNA Advisory Committee (RAC) addressed the events at the University of Pennsylvania (''Gelsinger Case'') and the general use of adenovirus vectors. In this meeting, a working group of the RAC listed a number of points to consider for adenovirus gene therapy trials. These points included standards to quantitate the vector, end-point measurements of vector activity, vector quality control, preclinical evaluation, routes of administration and biodistribution, patient evaluation and monitoring, and the need for control arms in clinical studies. All of these points represent basic principles for all drug development of which gene therapy is a subset.

Current gene therapy protocols have addressed gene modifications in somatic cells and have not attempted to modify germ-line cells. In fact, regulatory guidelines require stringent testing of patients receiving gene therapy in order to confirm that germ-line cells have not been modified inadvertently. Gene modification of non-target tissues will remain an important area for testing as in vivo vector technology improves and gene-transfer efficiencies increase.

6. Summary and conclusions

The progress of gene therapy in the past decade has been slower than was expected, which is due to several factors. Gene therapy is a pioneering new therapeutic modality based on complex biological systems occurring at the leading edge of biomedical knowledge. Incomplete knowledge of the genes involved in the pathomechanism of diseases constitutes a limit to generate clinically effective gene therapies, especially in complex diseases with multiple interacting genetic and environmental factors. Stringent and time-consuming safety studies are needed and the establishment of new regulatory frameworks essential to control the applications of gene therapy and ensure safety to the patient and the population at large. High costs are involved in R&D of gene therapy and complex issues of intellectual property and commercial rights need to be resolved.

Even if successful, gene therapy will be first introduced in most instances (e.g., cancer) as part of a combination therapy with other, existing therapeutic modalities. It is expected that existing therapies may find new ''meaning'' in conjunction with gene therapy (for example the stable prostacyclin analogue Iloprost®, may be useful to protect and maintain the new collateral vessels generated by AGT).

Despite the early high exceptions and the subsequent set-backs, one has to recognize that this new therapeutic modality is still in its infancy. It will neither deliver medical "miracles" (as its early prophets predicted) nor will it "disappear" because of a few disappointing cases (as some of its recent antagonists predict). As with all new technologies, gene therapy has to run its course in its present "development phase" before it reaches "maturation", when its full potential will be exploited. This in turn will offer significant opportunities to effectively target the causative factors for several disabling diseases afflicting mankind.

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