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Angiogenic gene therapy in the treatment of ischemic cardiovascular diseases

Review Article

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Abbreviations: basic fibroblast growth factor, (β-FGF); complementary DNA, (cDNA); coronary artery bypass grafting, (CABG); coronary artery disease, (CAD); cytomegalovirus, (CMV); electromechanical mapping, (EMM); extracellular matrix, (ECM); fibroblast growth factor-4, (FGF-4); hepatocyte growth factor, (HGF), herpes simplex virus, (HSV); interventional MRI, (iMRI); kilobases, (kb); left ventricular, (LV); magnetic resonance imaging, (MRI); percutaneous transmural coronary angioplasty, (PTCA); peripheral vascular disease, (PAD); human immunodeficiency virus, (HIV); tumor necrosis factor alpha, (TNF-α).

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Summary

Encouraging preliminary data suggest that gene therapy may soon be an option for the treatment of patients with advanced coronary artery disease that is not amenable to conventional treatment. A critical consideration in developing cardiovascular gene transfer as a therapy is the ability to deliver the vector, viral or plasmid, to the desired tissue in a safe fashion. Attempts at developing non-viral direct DNA therapy delivered through the intravenous route are currently underway and with the use of advanced technology the possibility of making gene therapy a simple outpatient procedure does not seem out of the realm of possibility. Several clinical trials are currently underway that should help characterize the risk–benefit profile of various products, the optimal dose that should be administered, and the patient population likely to derive greatest benefit.

I. Introduction

Gene therapy is most often defined as the transfer of nucleic materials to the somatic cells of an individual to elicit a beneficial therapeutic effect. A transferred gene can be targeted to specific tissues, organs or to the entire body. The potential advantage of gene therapy over drug administration is the single administration with long lasting beneficial results and minimal systemic toxicity. There are a couple of techniques that need to be developed for the success of gene therapy namely; the isolation and cloning of the desired therapeutic genes, the vectors which are the vehicle for these genes and finally delivery of gene to target tissues. The proposed mechanisms of action of gene therapy are replacement of non-functional genes with functional counterparts, correction of a defective gene, enhancement of normal gene expression and restriction of the expression of certain genes (Clowes et al, 1997).

The two types of gene delivery for therapy are the ex vivo where the cells to be transfected by the gene are cultured outside the body under a controlled environment and then re-introduced back into the body and the in vivo where the genetic material is directly delivered into the body affecting the desired the cells. Gene therapy is evolving as a new therapeutic alternative for the treatment of patients with advanced coronary artery disease (CAD) not amenable to bypass surgery or catheter based interventions.

II. Development of vectors

The transfer of plain DNA known as “naked” DNA directly into the body has yielded less than satisfactory results owing to the fact that only a small fraction of transferred DNA enters the cell and once inside is subjected to destruction by the cytoplasmic enzymes. Therefore, mechanisms of facilitating DNA entry into cells were developed, namely through the use of vectors, which are vehicles carrying the genetic material to the target tissues or cells. The ideal vector would be the one that delivers genetic material efficiently to target tissue producing the desired level of gene expression with minimal systemic and local adverse effects and for the specified duration of time. To fit all these characteristics in one vector is challenging and has not been completely successful. The vectors used in cardiovascular gene

therapy, as well as gene therapies directed at other diseases, include viral vectors, such as retroviruses, adenoviruses and adeno-associated viruses, and nonviral vectors, such as polymers, cationic liposomes, and liposome-viral conjugates. In order to develop clinical gene therapy strategies, a clear understanding of the advantages and shortcomings of current vector systems is mandatory (Zuckerbraun et al, 2002) (**Figure 1**).

A. Viral vectors

For delivery of the genetic load into cells, viral vectors first must attach to the cell membrane through binding proteins, then fuse with the cell membrane injecting their genetic material into the cytoplasm. The viruses' capability to replicate in the host cell is annulled by removing certain genes and replacing them with the desired genes to be incorporated into the host's genome.

1. Retrovirus

This is a class of viruses that have a lipid envelope containing a single stranded RNA genome. Once the virus transfects a cell and enters the cytoplasm, the viral genome is reverse transcribed into double stranded DNA, which integrates into the host genome [called complementary DNA (cDNA)] and is further expressed as proteins (**Figure 2, 3**).

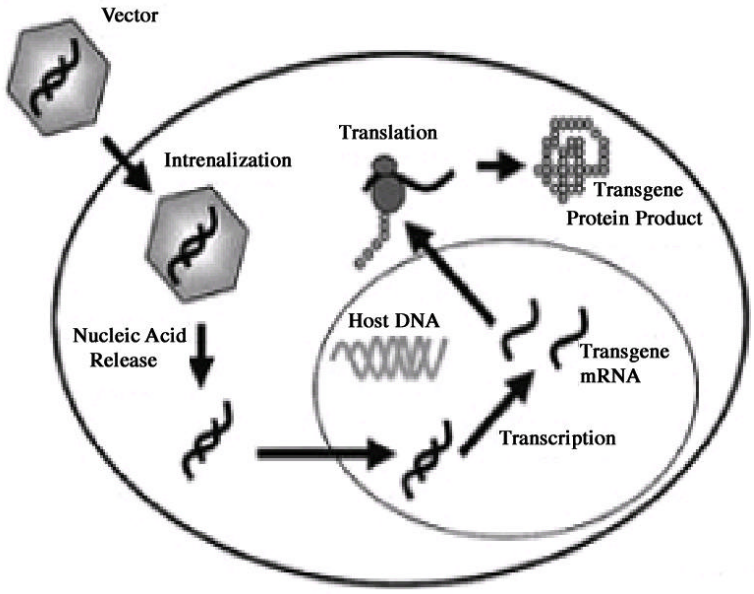

Figure 1. The vector gets internalized into the cell and releases its nucleic acids (containing transgene). The nucleic acids are translocated into the nucleus, where they may remain distinct or become incorporated into the host DNA. Vector (transgene) messenger RNA (mRNA) is transcribed in the nucleus then translated by ribosomal complexes in the cytoplasm to yield the final transgene protein product. It is the over expression of this protein that is intended to be of therapeutic value. Reproduced from Zuckerbraun and Tzeng, 2002 with kind permission from Archives of Surgery.

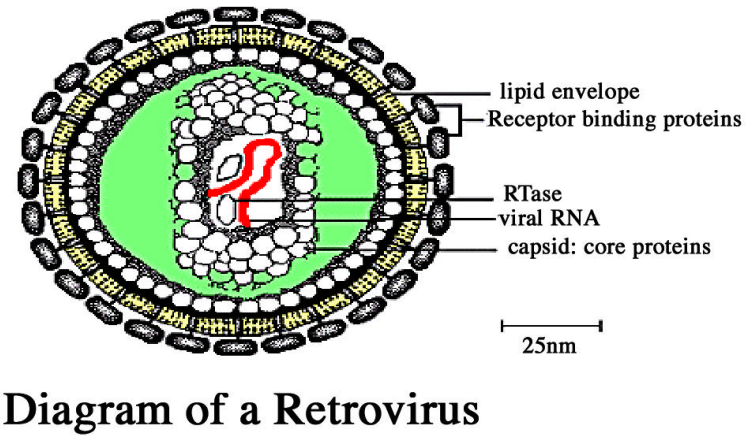

Figure 2. From The Online Biology Book hosted by Estrella Mountain Community College Website, in Sunny Avondale, Arizona: Biological Diversity: Viruses (revised 6/18/01).

The viral genome is approximately 10 kilobases (kb), containing mainly these three genes: gag (coding for core proteins), pol (coding for reverse transcriptase) and env (coding for the viral envelope protein), which are replaced with the transgene of interest (**Figure 3**) (Nabel, 1989, 1990). Retroviruses have the advantage of longer periods of gene expression with relatively minimal stimulation of the immune system and no local inflammatory reactions.

But they attack only proliferating cells with a large variety of cells as a target, which explains why they can't be used in *in-vivo* gene therapy. If the viruses are delivered directly into the body they will be neutralized immediately by the complement system and also the desired target cells are not necessarily in the proliferation phase. The cells desired to undergo the genetic modification are removed from the body and are cultured under controlled conditions then re-transplanted into the body after being transfected by the virus. The retrovirus genome is easily manipulated and replication-deficient retroviruses can hold large transgenes, measuring up to 8 kb (**Figure 3**). Retroviruses theoretically can cause genetic mutations due to the incorporation of an unfamiliar genetic material in the cell's genome. Major limitations to the use of retroviruses are their low titers (number of virus particles proportional to the gene transfer efficiency) but the development of new retroviruses increased the virus titers with more efficient gene transfer (Weiss et al, 1984; Flugelman et al, 1992). Transfecting endothelial cells with retroviruses to be implanted into vascular stents, grafts or injured arteries for a desired therapeutic effect have been studied.

Lentiviruses are a class of retrovirus but unlike retroviruses they can infect non-proliferating, terminally differentiated cells. These advantages of stable gene expression in non-dividing cells with minimal immunogenicity could be promising for gene therapy in the cardiovascular system. The human immunodeficiency virus (HIV) is a member of this family and, as may be expected, there are some concerns about the possible mutation of these recombinant viruses back to a pathogenic phenotype. The use of lentiviruses for gene therapy is on the horizon, and they may be the preferred vectors of the future.

2. Adenoviruses

Adenoviruses are non-enveloped viruses with double-stranded DNA genomes that cause respiratory, intestinal, and eye infections in humans. The virus that causes the common cold is an adenovirus. The virion is spherical and about 70 to 90 nm in size. The genome encodes about thirty proteins and both strands of the DNA encode genes. Some regions of the DNA have to be removed in order to render the virus non-proliferative (**Figure 4**).

Adenoviruses do not incorporate in the host's genome thereby do not cause mutations. This also explains its short duration of action which is usually for 1 or 2 weeks added to the fact that most people in their lifetime have had a natural adenovirus infection thereby evoking an immune response, both at the cellular and humoral levels, against future encounters with the virus. This short duration of action could be seen as a shortcoming in the treatment of chronic diseases and an advantage in the treatment of diseases where a temporary action is required.

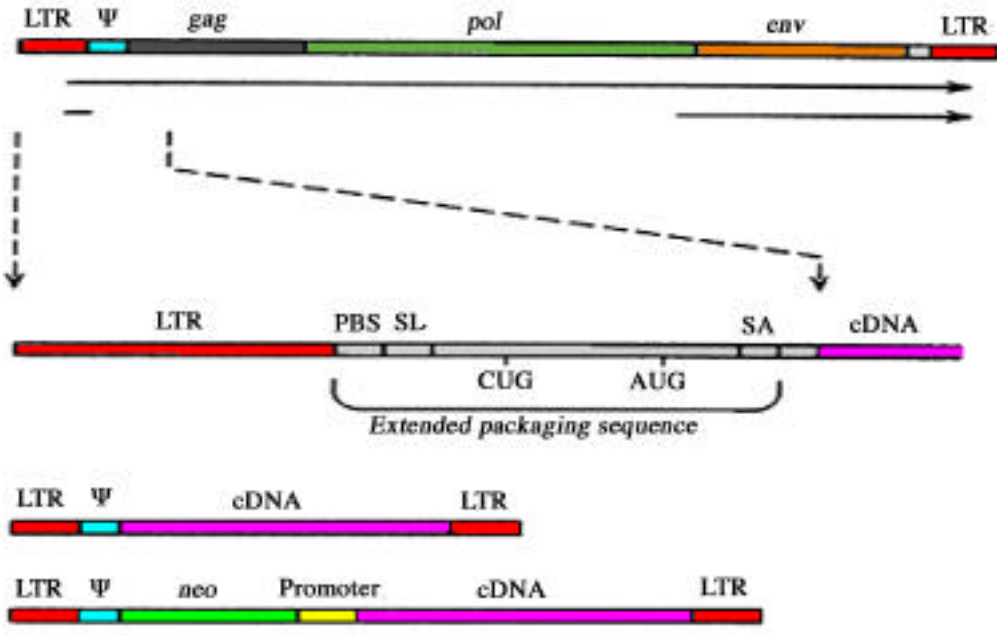

Figure 3. From the Department of Microbiology & Immunology, University of Leicester, UK. MBChB Special Study Module Project Report about Virus Vectors & Gene Therapy Problems, Promises & Prospects by David Peel 1998

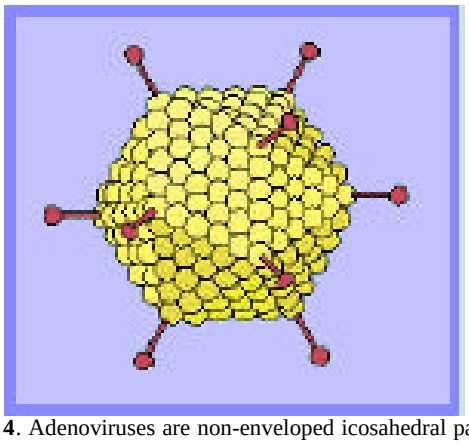

Figure 4. Adenoviruses are non-enveloped icosahedral particles. The capsid is built up from 252 capsomers of which 240 are hexavalent and 12 (situated at the apices) are pentavalent. From the Department of Medical Microbiology Website, University of Cape Town, written by Linda M Stannard, 1995. Virus Ultra Structure

Unlike retroviruses, adenoviruses can be used in *in vivo*, infecting replicating and non-replicating cells equally. They also have high transduction efficiencies with high levels of gene expression (Horwitz, 1990; Clemens et al, 1996). Adenoviruses induce a local inflammatory response and have a large complex genome making it difficult to manipulate (Kochanek et al, 1996; Schiedner et al, 1998).

So several strategies have been developed to improve the use of adenoviruses, and researchers are creating what is called a "gutless" adenovirus that is devoid of all its native genetic material. It has been shown that this new virus causes less stimulation of the immune system with a longer duration of action and the ability to use larger transgenes (Kibbe et al, 2000; Fisher et al, 2001).

3. Adeno-associated viruses (AAVs)

These are small DNA viruses that integrate successfully in one spot of the host's genome (on chromosome 19 in humans). They can't replicate by themselves and therefore require a helper virus, either adenovirus or herpes virus. Also they are non-pathogenic in humans, do not cause mutations and once integrated are stable leading to long term genetic expression which makes AAV an attractive tool for the management of chronic diseases from single gene mutation as well as acquired disorders, such as atherosclerosis (Summerford and Samulski, 1998). Other advantages of AAVs are that proliferating cells are not a requirement for transfection, it is relatively non-immunogenic, and the genome is small and easy to manipulate. A disadvantage of the small AAV genome is that the transferred genetic material is limited in size to a maximum of 4.9kb. It is challenging to produce this vector in large amounts without delivering an equally large amount of the contaminating helper virus. These problems with AAV production will soon be overcome, and it is becoming a very attractive vector for human gene therapy (Cheung et al, 1980; Jolly, 1994) (**Figure 5**).

4. Others

Several others viruses have been used experimentally for gene transfer namely; Herpes Simplex Virus (HSV), Pertussis Virus, Cytomegalovirus (CMV).

B. Non viral vectors

A plasmid is an autonomous, circular, self-replicating and an extra-chromosomal DNA molecule that carries only a few genes and has a single origin of replication. Some plasmids can be inserted into a bacterial chromosome, where they become a permanent part of the bacterial genome. The number of plasmids in a cell generally remains constant from generation to generation. It is here that they provide great functionality in molecular science.

Plasmids are easy to manipulate and isolate using bacteria. They can be integrated into mammalian genomes, thereby conferring to mammalian cells whatever genetic functionality they carry. Thus, we can have the ability to introduce genes into a given organism by using bacteria to amplify the hybrid genes that are created in vitro. This tiny but mighty plasmid molecule is the basis of recombinant DNA technology.

They were originally discovered by their ability to transfer antibiotic-resistance genes between bacteria, so to make plasmids useful these regions of antibiotic resistance had to be removed and replaced with recombinant genes (Feldman and Steg, 1997). Methods to deliver gene-carrying plasmids to mammalian cells for gene therapy include direct microinjection, liposomes, calcium phosphate, electroporation, or DNA-coated particle bombardment.

Liposomes are microscopic artificial vesicles, spherical in shape that can be produced from natural nontoxic phospholipids and cholesterol. When mixed in water under low shear conditions, the phospholipids arrange themselves in sheets, the molecules aligning side by side in like orientation, "heads" up and "tails" down (**Figure 6**). These sheets then join tails-to-tails to form a bilayer membrane enclosing some of the water in that phospholipid sphere. The vesicles can be loaded with a great variety of molecules, such as small drug molecules, proteins, nucleotides and even plasmids.

The simplicity of the liposome preparation and lack of disease transmission associated with viral vectors combined with the ease of plasmid construction make liposomes the most common form of non-viral gene transfer. The genetic material transferred by the liposome will enter the nucleus but will not incorporate into the cell's genome except for a very small amount. However some of its shortcomings are its use only in *in vitro* due to the instability of this complex (liposome-plasmid DNA) in the circulation, gene expression is for a short duration and the efficiency of gene transfer is low (Morishita et al, 1994). Transfection efficiencies vary with DNA/liposome ratio, cell type, and the proliferation status of cells. (Dzau et al, 1996; Armeanu et al, 2000). The non-selectivity of these liposomes has been partially overcome by the insertion of surface markers that attach to specific cell surface receptors (Von der Leyen et al, 1995).

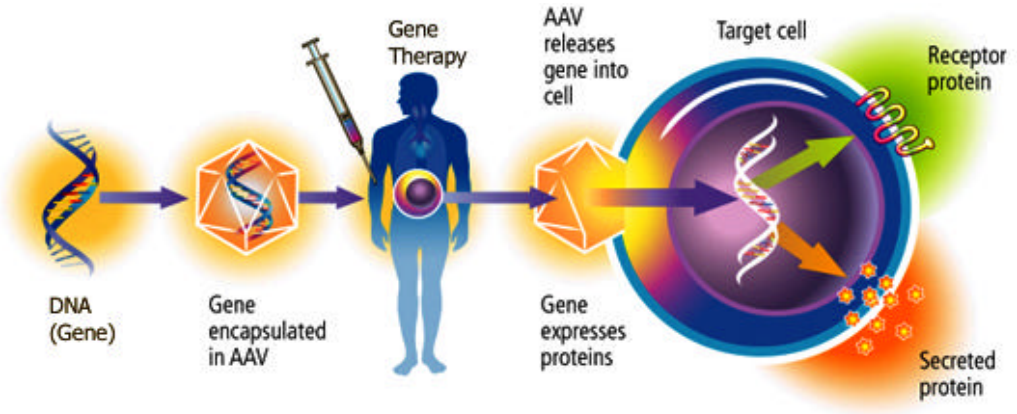

Figure 5. From the Avigen Company Website. 2001. DNA should be single stranded.

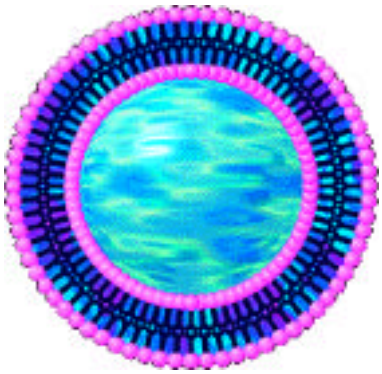

Figure 6. A liposome with showing the lipid bilayer with water inside. From the Collaborative Laboratories Website. Liposomes, controlled delivery systems. Updated April 22nd 2004.

III. New techniques for administering gene-based therapy and assessment of heart function

One of the important considerations in developing cardiovascular gene transfer as a therapy is the ability to deliver the vector, viral or plasmid, to the desired tissue in a safe fashion. This is not a problem in peripheral vessels but proves to be quite a challenge in the coronary arteries (Nabel EG and Nabel GJ, 1999). In the peripheral vessels, adequate exposure at the time of surgery makes gene transfer feasible and also these vessels tolerate long periods of ischemia without serious consequences. In contrast, in the coronary bed, we must be able to access the lesion and occlude the vessel for an adequate amount of time to allow vector attachment and uptake without significantly compromising myocardial perfusion (Bailey, 1996) (**Figure 7**).

In angiogenesis direct intramuscular injection of the desired vector into ischemic tissues, such as skeletal muscle or myocardium, allows local angiogenic factor expression to stimulate collateral blood vessel development (Baumgartner et al, 1998; Mack et al, 1998; Rosengart et al, 1999a). Researchers have modified this by injecting microspheres coupled to plasmids or growth factors that in turn can allow for slow release of the recombinant material into the surrounding tissue. (Arras et al, 1998).

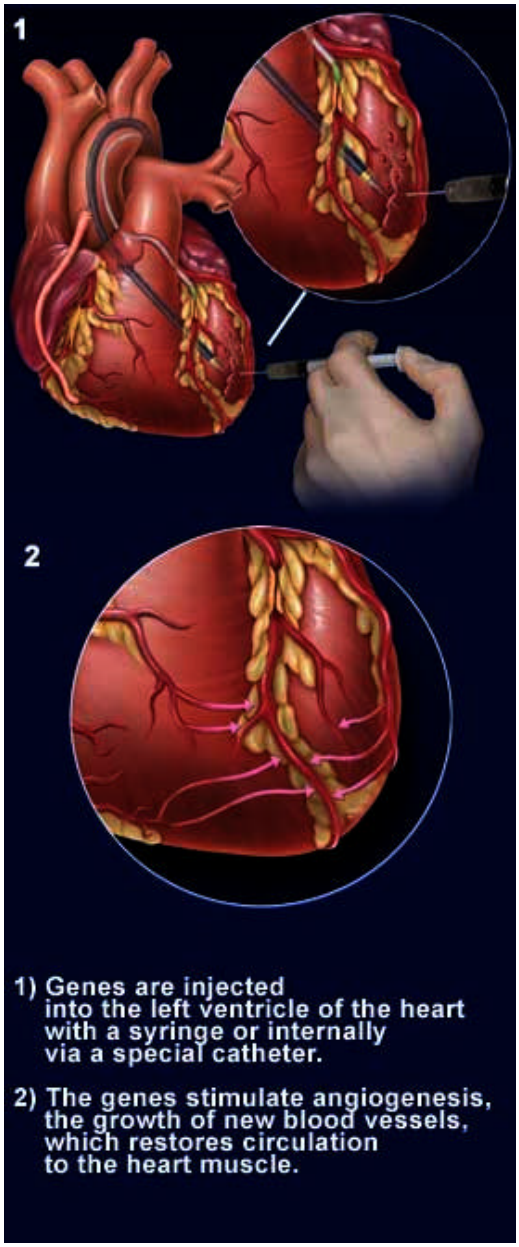

Figure 7. From the Arizona Heart Institute Research Website. 2000-2001

A. Magnetic resonance imaging (MRI)

MRI has evolved as a new non-invasive tool of accurately measuring and quantifying myocardial function and perfusion. The distinct advantages of MRI over current conventional nuclear-based cardiac imaging techniques, such as PET or myocardial scintigraphy, include its spatial resolution and lack of exposure of the patient to ionizing radiation. Also, quantification of cardiac morphology and function by MRI is more accurate and image quality is more reproducible than echocardiography, independent of the operator's skills and experience or each patient's individual anatomy (Lederman et al, 2002).

The new interventional MRI (iMRI) provides a real-time guidance for gene and cell delivery into the heart in addition to being a reliable tool in assessing the ventricular remodeling after myocardial infarction (Barbash et al, 2004).

B. Electro-mechanical mapping

Left ventricular (LV) electromechanical mapping (EMM) can be used to distinguish among infarcted, ischemic, and normal myocardium. This system uses electromagnetic field sensors to combine and integrate real-time information from percutaneous intracardiac electrograms acquired at multiple endocardial locations. The resulting interrogations can be used to distinguish between infarcted and normal myocardium (Gepstein et al, 1998) and thus permit online assessment of myocardial function and viability (Kornowski et al, 1998). This could be used as a tool for assessing the effects of gene delivery in restoring the myocardial function after an infarct.

IV. Angiogenesis and gene therapy

For gene therapy to be successful in angiogenesis, the gene selected should code for a protein with a proven angiogenic activity, the vector used should provide high gene-transfer efficiency, the delivery technique should target the desired ischemic tissues and the procedure should be safe both in the long term and short term.

A. Process of new blood vessel formation

A couple of trials have been done using gene therapy in angiogenesis with some promising results. Three different processes (vasculogenesis, arteriogenesis and angiogenesis) contribute to the growth of blood vessels. *Vasculogenesis* is the primary process responsible for growth of new vasculature during embryonic development and it is characterized by the differentiation of pluripotent endothelial cell precursors into endothelial cells that subsequently form primitive blood vessels (Bussolino et al, 1997). *Arteriogenesis* is the growth of collateral arteries that possess a fully developed tunica media or the enlargement of existing blood vessels that is seen in adult vessels. Recruited monocytes transform into macrophages, which produce numerous cytokines and growth factors (including tumor necrosis factor alpha (TNF-α), and basic fibroblast growth factor (b-FGF) involved in arteriogenesis (Wolf et al, 1998). These proteins stimulate remodeling and dilatation of arterioles leading to the development of functional collaterals. *Angiogenesis* is a process that also occurs in adult tissues whereby new capillaries develop from preexisting vasculature. It is a dynamic, multi-step process and requires interaction of a variety of cells which involves retraction of pericytes from the surface of the capillary, release of proteases from the activated endothelial cells by VEGF family proteins, degradation of the extracellular matrix (ECM) surrounding the pre-existing vessels, endothelial migration towards an angiogenic stimulus and their proliferation, formation of tube-like structures, fusion of the formed vessels and initiation of blood flow. Matrix degradation and endothelial and smooth muscle cell/pericyte migration are modulated by interplay of numerous factors, including plasminogen activators, matrix metalloproteinases and their inhibitors. There are multiple additional regulators of endothelial and smooth muscle cell proliferation that are also important components of the angiogenic process (Ruel and Sellke, 2003). Initial trials with gene therapy using adenovirus have used a replication-deficient virus, serotype 5 (Ad5) in which the E1A and E1B genes have been removed and replaced with fibroblast growth factor-4 (FGF-4) may be promising.

B. Regulation of angiogenesis

Angiogenesis is held delicately in a balance, well orchestrated by the interplay of many cells and controlled by both positive and negative regulators. In the body, angiogenesis is controlled through a series of "on" and "off" switches. The "on" switches are angiogenesis-stimulating factors, and the "off" switches are angiogenesis-inhibiting factors. There are more than 20 known angiogenic growth factors, and 30 known angiogenic inhibitors. Under normal physiological conditions, angiogenesis is "turned off" because there is more production of inhibitors than stimulators. But, this balance is a double-edged sword. Improper regulation of stimulators and inhibitors contributes to more than 70 pathological conditions such as tumor growth, rheumatoid arthritis, psoriasis, and diabetes mellitus (Sellke and Ruel, 2003). VEGF is the most widely studied and used factor for therapeutic angiogenesis. Several studies have been done where VEGF was directly delivered to a patient's leg with known peripheral vascular disease (PAD) in the area surrounding a diseased artery. Within a few days, stimulation of the growth of new blood vessels around the blockage in the ailing blood vessel was found and this obviated the need for an amputation. Improved myocardial perfusion and function after the administration of angiogenic growth factors has been demonstrated in animal models of chronic myocardial ischemia. A recent clinical study reported beneficial long-term effects of therapeutic angiogenesis using FGF-2 protein in terms of freedom from angina and improved myocardial perfusion on nuclear imaging (Ruel and Sellke, 2003). For successful angiogenesis in ischemic heart disease and PAD, a sustained but transient expression of growth factors is required, which makes gene therapy a particular attractive therapeutic option.

V. Gene therapy trials

A. FGF trials

Initial pre-clinical trials using animal models of chronic myocardial ischemia have shown that adenovirus-5 with a gene coding for fibroblast growth factor-4 (Ad5FGF-4) delivery into coronary vessel reverses myocardial dysfunction and increases blood flow with a sustained response of approximately 2-3 months. This ultimately led to the initiation of the multi-center clinical trials known as the AGENT trials.

1. AGENT and AGENT 2 trials

This was the first multi-center US clinical, randomized, double-blinded, placebo-controlled trial using Ad5-FGF4 for the treatment through the stimulation of angiogenesis of myocardial ischemia.

The main focus of this trial was safety of intra-coronary route for gene delivery. Patients with chronic stable angina were given incremental doses of ad5fgf-4 to know the optimum dose for use in future trials. It was not powered to evaluate the dose response or the efficacy. Both the treatment and placebo groups were well matched in terms of disease characteristics. Results showed that administration of ad5fgf-4 by intra-coronary route is safe and well tolerated and patients had a significant increase in their exercise tolerance when compared to placebo suggesting an improvement in myocardial dysfunction (Grines et al, 2002).

AGENT 2 was designed to evaluate the potential of Ad5FGF-4 in promoting new blood formation thus reversing the ischemic insult and to reassess its safety (Grines et al, 2003).

Seventy-nine were included in the first and 52 patients in the second trial. Patients who received Ad5FGF-4 experienced complete resolution of symptoms (30% vs. 13%) and less usage of medications to relieve their angina (43% vs. 17%) when compared to patients who received placebo. In addition, the incidence of worsening/unstable angina and revascularization by coronary artery bypass grafting or angioplasty was considerably lower in the Ad5FGF-4 group (6% and 6%, respectively) compared with those in the placebo group (24% and 16%, respectively). But some of these results did not reach a statistical significance (Data on file, Berlex Laboratories, 1998 Report No. A02854, 2000 Report No. A02856).

2. AGENT 3 and AGENT 4 trials

The results from the first two AGENT trials have provided preliminary encouraging data about the safety and anti-ischemic effects of Ad5FGF-4. Larger, long-term trials that could evaluate better the potential risks, benefits and complications looking into the short- and long-term safety and efficacy parameters were needed.

AGENT 3 and AGENT 4 are 2 ongoing double-blind, placebo-controlled trials with AGENT 3 is being conducted exclusively in the United States, whereas AGENT 4 is a multinational study (Europe, Canada, United States, and Latin America). Each trial will recruit 450 patients (150 patients each on low-dose Ad5FGF-4, high-dose Ad5FGF-4, and placebo) from centers with expertise in multiple vessel percutaneous revascularization procedures (Data on file, Berlex Laboratories, 2000 Report No. A02858) and patients will be followed clinically for up to 5 years and tracked for a further 10 years (Grines et al, 2003).

Other potential areas for investigation include the use of Ad5FGF-4 as an adjunct to angioplasty, as well as the value of repeated administration of Ad5FGF-4 (Grines et al, 2003).

B. VEGF trials

In one of the first human clinical trials, patients with ischemic heart disease were injected with naked plasmid encoding for VEGF directly into diseased myocardium and results showed marked improvement in blood flow and with reduction of symptoms related to ischemia (Losordo et al, 1998; Vale et al, 2000).

In a more recent trial, patients (n=13) with symptomatic disease in spite of being treated with conventional modalities of therapy [medications, percutaneous transmural coronary angioplasty (PTCA) and /or coronary artery bypass grafting (CABG)] demonstrated significant reduction in infarct size after direct myocardial injection of phVEGF165 measured by serial single-photon emission CT-sestamibi imaging (Lathi et al, 2001).

Also patients with advanced CAD (class 3 or 4 angina) receiving naked DNA-encoding VEGF165 through direct myocardial injection reported to experience reduced angina and sublingual nitroglycerin consumption and this improvement was maintained throughout a whole year of follow-up measured at different time points (Lathi et al, 2001; Fortuin et al, 2003). Following this success, a phase I study using intramuscular injection of adenoviral vector of VEGF121 gene demonstrated clinical safety with no evidence of systemic or cardiac related adverse effects related to the vector (Rosengart et al, 1999a; Hedman et al, 2003).

Using the intra-coronary route for gene delivery encoding for VEGF165 produced promising results with significant increase in myocardial perfusion although no differences in clinical restenosis rate or minimal lumen diameter were present after the 6-month follow-up (Aoki et al, 2000).

C. HGF trials

Another angiogenic factor that looks promising is hepatocyte growth factor (HGF), which was reported to promote angiogenesis in animal models of myocardial infarction (Ueda et al, 1999).

HGF has been found to inhibit collagen synthesis and through different mechanisms stimulate its degradation and this interesting function can be used as a tool in the treatment of post myocardial infarction fibrotic cardiomyopathy (Taniyama et al, 2002).

VI. Conclusion

Encouraging preliminary data suggest the possible use of gene therapy in the treatment of advanced coronary

artery disease that is not amenable to conventional treatment options (Dzau et al, 2003; Sleight, 2003).

Indeed, larger-scale, clinical trials are currently underway at centers throughout the world. These trials will characterize further the risk–benefit profile of various products, the optimal dose that should be administered, and the patient population likely to derive greatest benefit (Dzau, 2003).

Attempts at developing non-viral direct DNA therapy delivered through the intravenous route are currently underway and with the use of advanced technology the possibility of making gene therapy a simple outpatient procedure does not seem remote.

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