# II. TERAPIA GÉNICA

# *Gene therapy in the vasculature and the kidney*

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## INTRODUCTION

Gene therapy refers to the in vivo use of DNA or rarely RNA, in order to replace the function of genes in which null mutations have occurred, to correct point mutations or, most commonly, to inhibit or enhance production of very specific molecules. Targeting of specific molecular pathways in vascular cells by gene transfer techniques is currently in an explosive growth phase, fueled by the revolution in molecular biology, advances in vector technology and gene transfer techniques. Currently close to 200 human gene therapy trials are underway, most of them related to treatment of tumors, HIV, and genetic diseases like cystic fibrosis. A few trials are addressing vascular disease[1,2]. A number obstacles remain to be overcome for gene therapy to become a widely used therapeutic tool. For instance, efficient delivery of the constructs to the desired cell types, and their controlled expression for the desired length of time has not been achieved. Nevertheless, while this new technology is undergoing development, it has already become a highly useful tool to study disease mechanisms in vivo in many experimental models. This section will review several basic concepts in the field of vascular gene therapy. These type of approaches also may become very useful in renal gene therapy.

## CONSTRUCT DESIGN

DNA constructs may be delivered into cells as antisense oligonucleotides, double stranded DNA decoys, chimeric RNA/DNA oligonucleotides and full promoter-cDNA constructs designed to produce specific protein products. Antisense oligonucleotides consist of short complementary DNA strands that hybridize with transcribed RAN, so that RNA processing and/or translation are aborted[3]. Double stranded DNA decoys are copies of promoter sequences designed to compete for transcription factor (TF) binding, thereby preventing TF interaction with, and activation of their usual promoter targets[4]. A multitude of cDNAs constructs encoding specific protein products have also been transferred into cells in vivo and in vitro.

Complementary DNA (cDNA) refers to DNA transcribed from mRNA by reverse transcriptase. Double stranded cDNA constructs complementary to the open reading frame of mRNA therefore can be viewed as artificial «genes» lacking the normal intronic sequences. cDNA constructs often are engineered with artificial promoters that allow for high-level expression. Promoters that can be regulated (i.e. by metal ions or by glucocorticoids) are also used to give some control over transcriptional activity, and promoters that direct cell-specific expression has also bene achieved. Full-length cDNAs, if transferred into cells with an adequate promoter, will produce faithful mRNA copies from which protein is synthesized. The cDNA constructs are transferred into cells as components of plasmid or viral vectors. Vectors contain desired promoter elements, cloning sites, markers and resistance elements to make them useful for transient or stable expression of the desired cDNA construct in cells. For example, viral vectors are engineered to be replication deficient, so that they can serve purely for gene transfer without eliciting viral disease in the host.

## METHODS OF GENE DELIVERY INTO CELLS

*Viral vectors:* Uptake of DNA by cells is highly variable, and depends on the vector, as well as the cell

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type. For in vivo application, viral vectors have been used with considerable success[5]. Among these, retroviruses, adenoviruses and adeno-associated viruses are most commonly used as vectors. They are taken up by specific cell-surface receptors. Hence, failure of any cell type to express the appropriate receptor makes them resistant to transduction by that virus.

Retroviruses are single-stranded RNA viruses, which are converted to double-stranded DNA within cells by reverse transcriptase and random integration of this DNA into the genome. Retroviral infection and DNA integration depends on cell replication. Whereas retroviruses have the advantage of resulting in stable integration of DNA which is then passed on to daughter cells after division, most vascular cells in mature hosts are not actively dividing; retroviral gene transfer therefore is most successful in rapidly dividing cells, such as tumors, but it is not very effective in mature solid organs or in the vasculature.

Adenoviruses are double-stranded DNA viruses, that can infect non-dividing and dividing cells. These viruses are taken up into the lysosomal system, and cause lysosomal rupture and discharge of the DNA into de cytoplasm, allowing viral particles to escape lysosomal enzymes. Adenoviral gene transfer highly efficient in many cells. The viral DNA then enters the nucleus where it exists as an episome. Since it does not integrate into the genome, it is lost during cell division. After systemic infusion of adenoviral constructs, high levels of expression are usually observed in liver, making this a particularly useful vector for transient systemic gene therapy. Adenoviral vectors can also achieve high levels of expression in the intima, media and adventitia of arteries and veins, as long as high titer viral solutions are applied directly to the cells in question. However, adenovirus-specific proteins can elicit a very significant immune response causing infected cells to be destroyed by cytotoxic T-cells within a few days after transduction. Though gene expression after adenoviral gene transfer can be prolonged with immunosuppressive therapy, vascular expression tends to be limited to 2-3 weeks at best.

Adeno-associated viruses (AAV) are single-stranded DNA parvoviruses, capable of integrating specifically into human chromosome 19, though integration of transgenes from AAV vectors does not seem to occur. Nevertreless, gene transfer by AAV into pulmonary epithelial cells, heart and skeletal muscle and kidney[6] has been achieved and subsequent expression is much more prolonged than that achieved with adenovirus. Currently, the greatest limitation of AAV vectors is that they are difficult to produce at high titer.

*Naked plasmid DNA with and without cationic liposomes.* Efficiency of naked plasmid DNA uptake by cells is extremely low. When DNA is complexed to various liposomes, cell uptake can be increased severalfold, though in vivo gene transfer using cationic liposomes is still exceedingly low. Nevertheless, liposome-mediated transfer of plasmid DNA into blood vessels has been achieved. Infusion, in vivo, of antisense oligonucleotides leads to relatively high uptake by renal proximal tubule cells[7], and hepatic Kupffer and endothelial cells, and can therefore be used to inhibit translation in these cells[3].

*Hemagglutinin-virus of Japan (HVJ)-liposomes.* Complexes of DNA, liposomes, nuclear protein HMG-1 and HVJ, result in high efficiency transfer of oligonucleotides and cDNA in a variety of cells[8]. Antisense oligonucleotides and plasmid DNA has been introduced into vascular cells and into the kidney in vivo by HVJ mediated gene transfer. As will be discussed below, some therapeutic success has been achieved in preventing vascular restenosis, and glomerular matriz synthesis.

*Physical force -augmented transfection.* Aside from very efficient hepatic uptake of adenoviruses, efficient uptake of cDNA by renal proximal tubule cells, and excellent gene transfer of AAV when injected directly into tissues, most gene transfer in the vasculature and in the kidney is hampered by the fact that endothelium is generally not efficiently transfected or transduced, and that it acts as a barrier to entry of the virus or DNA particles into tissues. For example, it has been extremely difficult to achieve transduction of renal glomerular cells with many of the agents under investigation. It has become clear recently that gene transfer into vessels can be achieved ex vivo under high hydrostatic pressure[9], and that physical injection of DNA on gold particles delivered under high pressure from a gene gun can be achieved[10].

## SYSTEMIC GENE THERAPY

In the cardiovascular system there are a number of processes which participate in generating intravascular lesions and lead to blood vessel malfunction. For instance, systemic factors, which include, among others, abnormalities in cholesterol metabolism can increase the risk of, and accelerate the rate of progression of atherosclerosis. Systemic gene therapy has beem used in a number of animal models to reduce serum cholesterol concentrations, and formation of atherosclerotic lesions[11]. As noted above, systemic infusion of adenoviral vectors leads to transient, high level expression in liver. Adenovirus-me-

diated transfer of LDL and VLDL receptor constructs reverses hypercholesterolemia in a number of animal models. Similarly, apolipoprotein E reconstitution in apoE deficient mice has been achieved using adenoviral vectors, and overexpression of human apoAI has been shown to raise plasma HDL levels in mice and hamsters. Systemic gene therapy with using adenoviral vector mediated expression of the fibrinolytic tissue type plasminogen activator (t-PA), has also met success in mice[11].

In patients with renal disease, the lack of erythropoietin was a major cause of morbidity, until it was overcome in the last decade this problem has been overcome by recombinant erythropoeitin therapy. Recent trials in animals have shown that long-term expression of erythropoietin can be a achieved with peritoneal adenovirus based gene transfer and with transfer of the erythropoietin cDNA in stably transfected cells[12]. If such constructs could be introduced into venous endothelium under a hypoxia-regulated promoter, it could even be possible to obtain long-term and appropriately regulated expression of this gene in dialysis patients.

## LOCAL GENE THERAPY TO PREVENT REMODELING RESPONSE TO INJURY

There are a multitude of published studies demonstrating the efficacy of various gene therapy approaches in preventing neointimal hyperplasia and vessel stenosis after balloon injury, a model of vessel restenosis after angioplasty. The advantages of local (vs. systemic) gene therapy are at least two fold. First, potential untoward effects at other locations and in other tissues are minimized, and second, as concentration of vector is often limiting, gene transfer in a confined region can be achieved more easily. Since neointimal hyperplasia results from endothelial cell injury, that then causes proliferation and migration of myofibroblasts and/or smooth muscle cells, the most common approaches have been to interrupt local cell proliferation in blood vessels after injury. Another approach has been to overexpress, locally, normal endothelial cell proteins known to inhibit smooth muscle cell proliferation.

Introduction of antisense oligonucleotides which inhibit synthesis of proteins involved in cellcycle regulation in the blood vessel wall has met with considerable success in the rat arterial balloon injury model[11]. Antisense oligonucleotides against cdc2 kinase, c-myc, c-myb and proliferating cell nuclear antigen (PCNA) all demonstrated reduction of neointima formation, when compared to areas in the same vessel not treated with the oligonucleotide[13,14]. Though inhibition of growth factor or growth factor receptor synthesis might also be considered as a potential strategy to inhibition of proliferation, the enormous redundancy and interplay between growth factors and other mitogens makes inhibition of their common downstream mitogenic effector system more attractive as a therapy. Nevertheless, the antisense strategy has been successful in inhibiting TGF-β1 mediated glomerular matrix deposition in the anti thy 1.1 model of mesangial proliferative glomerulonephritis[15].

The transcription factor binding decoy approach, using HJV-liposome mediated gene transfer, has also been used with success in the balloon injury model. Decoys that bind the transcription factor E2F, important in the activation of several cell-cycle control proteins. The presence of the DNA decoy capable of binding E2F leads to inhibition of proliferation in injured arteries[4], and similarly inhibition of mesangial cells proliferation in the anti thy 1.1 model of glomerular proliferation[16].

Viral gene transfer techniques are also being used to alter the vascular response to injury. Studies in which proteins that normally function to inhibit cell proliferation have been introduced. For instance, the retinoblastoma gene product (Rb), a tumor suppresser protein that arrests cell cycle progression, inhibited local cell proliferation and subsequent neointima formation, when transferred into rat carotid artery using a replication deficient adenoviral vector, after balloon injury[17]. Similarly, adenoviral transfer of the cDNA encoding the antiproliferative transcription factor *gax,* inhibited vessel stenosis in the rabbit iliac artery[18].

Exogenous genes that render cells susceptible to toxic drugs have also been used to prevent vascular smooth muscle proliferation. E. and G. Nabel used adenoviral gene transfer to introduce the herpesvirus thymidine kinase cDNA into porcine arteries that had been subjected to balloon injury[19]. Thymidine kinase converts the nucloside analog gancyclovir, through phosphorylation, into active drug which inhibits DNA elongation and therefore kills dividing cells. Local expression of thymidine kinase significantly inhibited cell proliferation at the site of injury and subsequent neointima formation in animals given gancylcovir. It is generally difficult to achieve gene delivery into most cells at the site of injury in a blood vessel. However, local expression of thymidine kinase in some cells profoundly inhibited proliferation of all cells at the site of injury, hence demonstrating a local «bystander» effect. The concept of gene transfer into some cells, which can then have an effect on all cells in the vicinity is an important one, given that gene transfer does not usually achieve 100% efficiency.

The endothelial nitric oxide synthase cDNA has also been transferred into vasculature subjected to injury, using simple plasmid- and adenoviral vectors[20,21]. Both, enhanced blood vessel reactivity to acetylcholine and reduced proliferation of neointimal cells was shown.

A large number of other targets in the vascular response to injury have been subjected to interruption or enhancement using gene transfer techniques. In addition, gene therapy is being applied to vein bypass grafts, which are notorious for rapid development of stenosis, and in the field of transplantation, where accelerated arteriosclerosis of vessels within the transplant can lead to organ failure. Interruption of any mechanism involved in cell migration/proliferation, in regulating thrombosis/fibrinolysis, matrix synthesis/degradation, inflammatory cell recruitment/activation, complement activation, could theoretically be used in vascular gene therapy.

One of the principal obstacles to be overcome in the field of vascular gene therapy is the efficient delivery of the gene of interest to the target cell population. Transfer efficiency can be augmented when the endothelial cell barrier is breached, as is common in the balloon injury model. However, efficient gene transfer in vein grafts, for instance, and in blood vessels of organs to be transplanted, is still problematic. In many cases, effective endothelial cell gene transfer can be achieved, but medial smooth muscle and other underlying cells are transduced only if the endothelium is first disrupted in some way. Since endothelial cell injury itself elicits a remodeling response in veins and large vessels, this approach may be counterproductive. Another problem concerns the efficiency of gene transfer, already discussed above. For adenoviral vectors, uptake of the vector can itself elicit an immune response, which can limit the length of transgene expression, and can stimulate a local inflammatory response that can, itself, lead to blood vessel remodeling. For AAV, gene transfer into some cells is much more efficient than into other cells, owing, presumably, to the lack of expression of the appropriate viral receptor by some cells.

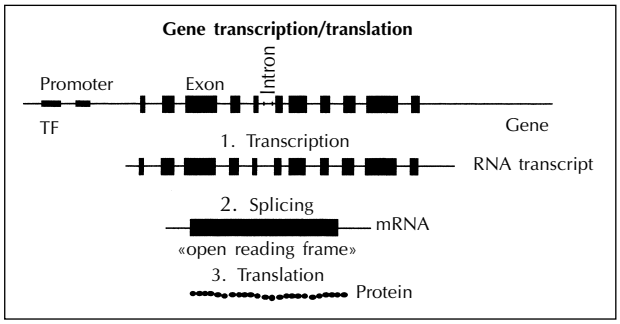


*Fig. 1.—Schematic of gene, RNA and protein structure. Genes consist of translated (exons) and untranslated (introns, promoter and downstream elements) sequences. Transcription factor (TF) bind to specific promoter sequences to activate transcription. Splicing removes intronic sequences from the pre-RNA transcript to produce messenger RNA (mRNA). mRNA contains upstream (5′) and downstream (3′) untranslated sequence. Only the «open reading frame», which corresponds to exons, is translated. For gene therapy, it is possible to introduce double stranded copies of a portion of the promoter region, which then competes for transcription factors. It is possible ot express the cDNA, which represents an artificial gene lacking all introns, in order to replace the function of a gene or to introduce a mutated form. Introduction of antisense oligonucleotides can halt mRNA translation. All of these approaches are temporary forms of gene therapy. It is also possible to fix point mutations using gene conversion approaches, which produces a permanent change in a subset of target cells.*

## LOCAL GENE THERAPY TO STIMULATE COLLATERAL VESSEL FORMATION

There are several instances in which it may be advantageous to stimulate the formation of collateral vessels. These include ischemia and wound healing. In humans with myocardial infarction, it has been shown that local injection of recombinant FGF-2, an angiogenic growth factor stimulated microvessel growth[22]. Were more prolonged expression of angiogenic growth factors, mRNA such collateral formation might be beneficial under circumstances where surgical bypass procedures are fraught with risk or are impossible. Indeed, gene transfer of plasmids containing acidic FGF or VEGF constructs are successful in stimulating collateral blood vessel formation in rat ischemic hindlimb models[23,24]. It has been postulated that constructs under the control of relatively weak promoters containing the hypoxia response element might be useful in producing collateral blood supply, as their expression would be turned off once hypoxia was relieved.

## KIDNEY-SPECIFIC GENE THERAPY

Many of the general approaches outlined above for gene therapy in the vasculature have been applied to studies of kidney disease in animal models[25]. Both adeno- and adeno-associated viral vectors have been used successfully. As noted above, one problem in renal gene therapy is the profound avidity of proximal tubule cells for most plasmid DNA and oligonucleotides. However, significant transgene expression in the renal interstitium has been reported using direct injection of adeno-associated viral vector into the renal parenchyma[6]. Another approach

that may prove fruitful in the kidney is the direct transfer of mesangial cells stably expressing a transgene into glomeruli in vivo[26]. Cultured transgenic mesangial cells are large enough to lodge in glomerular capillaries after intraarterial injection, causing them to set up residence there. If engineered to release a desired gene product, they could potentially alter the glomerular response to injury. Another recent exciting suggests that it is even possible to correct point mutations in renal proximal tubule cells in vivo. Lai and co-workers[27] took advantage of the high efficiency uptake of oligonucleotides by renal proximal tubule cells to correct a point mutation in carbonic anhydrase deficient mice. Chimeric RNA/DNA oligonucleotide designed to correct the known point mutation were infused systemically. Stable correction of the genomic defect and production of functional protein was achieved. Such data suggest that it may even be possible to correct some inherited genetic mutations in mature somatic cells using gene transfer technology.

## SUMMARY

Gene therapy has many potential applications for the treatment of vascular disease. Though a number of tough problems remain to be solved, the potential specificity with which an almost limitless number of mechanisms could be targeted, and the success that has been achieved in animal models in vivo make it likely that we will see further rapid expansion of this technology, and therapeutic use of gene therapy in humans in the future.

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