Current therapies for the treatment of atherosclerotic vascular disease are aimed at either disrupting or bypassing flow-limiting lesions. Preventative strategies are necessary to decrease the burden of disease but are limited by genetic predispositions to certain diseases and the body’s innate response to injury. Gene therapy, defined as the purposeful therapeutic overexpression or attenuation of a gene product, has enormous potential benefits in vascular disease prevention and treatment strategies. This article reviews the scientific considerations involved in the development of gene therapy strategies and outlines some of the gene products that are currently being used. These interventional genetic approaches will be reviewed in the context of specific vascular disease processes, including atherosclerosis, restenosis, and thrombosis. Gene therapy will serve an enhancing and adjuvant role to evolving surgical therapies.

Atherosclerotic cardiovascular disease is the leading cause of morbidity and mortality in the United States and Europe, and gene therapy has become a potentially important therapeutic tool in the treatment of cardiovascular disease. Gene therapy is most often defined as the transfer of nucleic acids to the somatic cells of an individual to elicit a beneficial therapeutic effect. These nucleic acids are in the form of genes that either replace a specific genomic deficit, as with single gene mutations, or encode proteins that may have therapeutic applications. Gene therapy also includes the transfer of nucleic acids to alter patterns of gene expression. Instead of full coding sequences, DNA molecules called oligodeoxynucleotides, which encode sequences that are complementary to those of specific messenger RNA, can be used to block the expression of certain genes (Figure). A transferred gene can be targeted to specific tissues, organs, or the entire body. The delivery of a gene can be likened to the administration of a drug in classic pharmacology. However, a theoretical advantage of gene transfer is that a single administration of a gene can result in highly specific, long-lasting therapeutic effects with reduced systemic toxicity.

Several hurdles must be overcome in the development of cardiovascular gene transfer strategies. First, there must be a disease process that is amenable to treatment with a gene product. Such targets for cardiovascular gene therapy may include postangioplasty restenosis, angiogenesis, atherogenesis, thrombogenesis, and transplantation allograft vasculopathy. Second, the therapeutic gene of interest must be identified and cloned. Third, gene therapy uses vectors that serve as vehicles to facilitate the efficient transfer of genetic material into targeted cells. Finally, gene therapy strategies must consider the accessibility of the targeted tissue to vector delivery. The advent of catheter-based delivery systems and other technologies contributed to the feasibility of cardiovascular gene therapy as a clinical application. Ultimately, the advancement of cardiovascular gene therapy will rely on our ability to improve on current vectors and delivery systems.

HISTORY

Although the phrase “genetic engineering” was first coined in the 1930s in reference to the application of genetic principles to animal and plant breeding, the concept of selective breeding predates this by centuries. Our current definition
of genetic engineering evolved during the 1950s and 1960s when genes were first transferred to bacterial and mammalian cell lines. As gene transfer technology developed over the ensuing decades, efforts were originally focused on its use for the correction of inherited disorders stemming from single gene mutations. However, as our application of gene transfer broadened to include the concept of a genetic means of drug delivery (ie, the use of genes expressing therapeutic proteins), acquired diseases, such as cancer and atherosclerosis, also became candidates for gene therapy. The first report of gene therapy in the cardiovascular system was by Nabel et al in 1989, in which retrovirally transduced endothelial cells were seeded onto porcine iliac arteries. Although these engineered cells were detected on the vessel wall, this was clearly an inefficient method of in vivo gene delivery and a far reach from clinical application. Since this report, many technological advances have been made that enable the use of gene therapy in the care of patients with cardiovascular disease.

VECTORS

When “naked” DNA comes into contact with a cell membrane, only a minute amount of it will pass into the cell, and, once in the cell, the DNA is rapidly degraded by cytoplasmic nucleases. Naked DNA gene transfer is a highly inefficient process and certainly one that would be prohibitively limiting for human gene therapy. Therefore, mechanisms of facilitating DNA entry into cells were developed, namely through the use of vectors. An ideal vector should enable efficient delivery of genetic material to a targeted tissue with minimal local or systemic toxicity while allowing a desired level of gene expression for a specific duration of time. Thus far, no single vector possesses all these characteristics, and vector design is an active area of research. The vectors used in vascular gene therapy, as well as gene therapies directed at other diseases, include viral vectors, such as retroviruses and adenoviruses, and nonviral vectors, such as polymers, cationic liposomes, and liposome-viral conjugates. An understanding of the advantages and shortcomings of current vector systems is critical to the design of any clinical gene therapy strategy.

Viral Vectors

Viruses are commonly used as vectors in cardiovascular gene therapy research and clinical trials. Their popularity centers on the natural life cycle of viruses, which involves the infection of mammalian cells to propagate viral genomic material and produce infectious viral offspring. Often, however, these life cycles result in lytic death of the host cell. Recombinant DNA technology has allowed the reengineering of these viral genomes such that the majority of the viral coding sequences necessary for replication are deleted and foreign therapeutic DNA sequences are inserted. Disrupting the normal reproductive pathways of these viruses allows the safe infection of mammalian cells. Propagation of these modified viruses requires the use of specially engineered “packaging” cells that carry the deleted viral genes.

Gene delivery for overexpression of therapeutic proteins vs oligonucleotides to inhibit expression of deleterious host proteins. In gene delivery by a generic vector (A), 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 overexpression of this protein that is intended to be of therapeutic value. Antisense oligonucleotides are single-stranded nucleic acids (B), antisense oligonucleotides can prevent protein synthesis at several different levels in the gene expression pathway. They can hybridize to host DNA to form a triple helix or to host mRNA and thus prevent mRNA transcription or splicing and modification, respectively. Additionally, they can prevent mRNA export to the cytoplasm and ribosomal translation into protein. Decoy oligonucleotides are double-stranded nucleic acids (C). These oligonucleotides contain consensus sequences for transcription factor–binding sites. When transfected into cells, transcription factors will bind to these consensus sequences on the decoy oligonucleotides in the cytoplasm and will be prevented from translocating into the nucleus. This will prevent binding to host DNA and activation of host transcription.

One of the original viral vectors was the retrovirus. Retroviruses are RNA viruses that infect cells by a specific receptor–ligand interaction. Once inside a host cell, the viral RNA is reverse-transcribed to DNA, which then undergoes stable integration into the host genome. Theoretically, retrovirally transferred genes can be expressed for the lifetime of the organism treated. Nabel et al have shown expression of a marker transgene in arteries as long as 5 months after gene delivery. However, the integra-
tion of the reverse-transcribed DNA requires active cellular proliferation. In their natural environment, both endothelial and vascular smooth muscle cells have low proliferative activity and are very poor targets for retroviral infection. Transfection efficiencies may be as low as 0.1% to 1.0% in vivo in these cells. Additionally, because retroviral integration occurs randomly, there is the theoretical potential for insertional mutagenesis with malignant transformation, although this has never been reported to date. The retrovirus genome is easily manipulated and replication-deficient retroviruses can hold large transgenes, measuring up to 8 kilobases (kb). Retroviruses have been used more effectively for ex vivo cell-mediated vascular gene transfer. These strategies use harvested endothelial cells that can be proliferated and transduced in vitro. Such techniques have been used in cardiovascular gene therapy for seeding stents, grafts, or injured arteries.

Lentiviruses are a class of retrovirus that can infect quiescent, terminally differentiated cells. The ability to generate stable gene expression in nondividing cells with minimal immunogenicity is promising for gene therapy in the cardiovascular system. The human immunodeficiency virus is a member of this family and, as may be expected, there are a number of safety concerns about contamination or 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.

Adenoviruses are DNA viruses that enter the cell via specific receptor interactions and are typically associated with the transmission of the common cold. After infection, adenoviral DNA enters the nucleus, where it remains episomal. The lack of genomic integration enables adenoviruses to efficiently infect proliferating and nonproliferating cells. In addition, there is no risk of insertional mutagenesis. Compared with recombinant retroviruses, adenoviral vectors have high gene transfer efficiencies in susceptible cells. Also in contrast to retroviruses, adenoviruses have a very complex genome and are more difficult to manipulate using recombinant technology. Significant limitations of our current “first-generation” adenoviral vectors are the resultant immunologic and cytotoxic responses that are generated against adenoviral proteins within the infecting virus and the low-level viral protein expression in the infected cell. Most people have been exposed to natural adenovirus infection, and the production of viral proteins by recombinant adenovirus can stimulate a cellular immune response that eliminates transfected cells. The immune response and the lack of gene integration limit the duration of transgene expression, which usually lasts 1 to 2 weeks.

Several strategies are being explored to improve adenoviral vectors for gene therapy. Many researchers are creating “gutless” adenoviruses by removing nearly all the native adenoviral genes. This reduces the immune response, creates room for larger transgenes, and has been shown to improve the duration of expression in vivo. Others are altering the viral capsid to improve virus-receptor interaction or using polymer-coated adenoviruses to shield them from antibodies and permit more selective targeting.

Adeno-associated viruses (AAVs) are small DNA viruses that gain entry into cells by binding to heparan sulfated proteoglycan receptors. Viral DNA then localizes to the nucleus where it is integrated into the host genome. This occurs preferentially at chromosome 19q. Because AAV is not capable of replication on its own, it is dependent on coinfection with a helper virus, either an adenovirus or a herpesvirus. Additionally, AAV is not associated with any known human disease or insertional mutagenesis. Integration of the recombinant viral genes leads to stable expression within the host cell and, therefore, long-term recombinant gene expression. This is a very important property that makes AAV particularly attractive for the treatment of chronic disorders resulting from single gene mutations as well as acquired disorders, such as atherosclerosis. Other advantages of AAVs are that they can infect proliferating and nonproliferating cells, it is relatively nonimmunogenic, and the genome is small and easy to manipulate. A disadvantage of the small AAV genome is that it also limits the size of the transgene to a maximum of only 4.9 kb. To date, there

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<th>Vector</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<td>Retroviruses</td>
<td>Integrates into host genome; months of gene expression</td>
<td>Potential for insertional mutagenesis</td>
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<td>Relatively nonimmunogenic</td>
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<td>Easily manipulated</td>
<td>Infects proliferating cells only</td>
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<td>Low transfection efficiency of endothelial and smooth muscle cells</td>
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<td>Limited by host immune response</td>
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<td>Adenoviruses</td>
<td>High transduction efficiencies</td>
<td>Large genome; difficult to manipulate</td>
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<td>Infects replicating and nonreplicating cells</td>
<td>Lack of integration and immune response limit duration to &lt;2 wk</td>
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<td>High level of gene expression</td>
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<td>Adeno-associated viruses</td>
<td>Infected replicating and nonreplicating cells</td>
<td>Only small transgenes</td>
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<td>Nonpathogenic in humans</td>
<td>Difficult to produce in large quantities</td>
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<td>Easily manipulated</td>
<td>Potential for insertional mutagenesis (not documented)</td>
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<td>Stable integration</td>
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<td>Nonimmunogenic</td>
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<td>Catonic liposomes</td>
<td>Easy to manipulate</td>
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<td>Nonpathogenic</td>
<td>Days of gene expression, in general</td>
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<td>No size limit</td>
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<td>Transfects all cell types</td>
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is no accepted packaging cell line for recombinant AAV propagation, and it is difficult to produce viral stocks in high concentrations with adequately low levels of contaminating helper virus. These problems with AAV production will soon be overcome, and it is becoming a very attractive vector for human gene therapy.

**Nonviral Vectors**

Plasmids are circular DNA molecules that were originally found to transfer antibiotic-resistance genes between bacteria. The regions carrying the antibiotic resistance were deleted and replaced with recombinant genes, and these plasmids have been employed in the bulk preparation of a variety of proteins for pharmacologic use. 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 the most common nonviral form of gene transfer. This method takes advantage of cationic lipid bilayers that incorporate the nucleic acids and facilitate cell entry. Liposome-coated DNA will enter the nucleus and remain extrachromosomal, although a very small amount will undergo spontaneous genomic integration. Transfection efficiencies vary with DNA/liposome ratio, cell type, and the proliferation status of cells. The advantages of liposome-mediated gene transfer include the ease of plasmid construction, the lack of the risks associated with viral vectors, and the simplicity of liposome preparation for clinical use. However, limitations of this method of gene transfer, such as low gene transfer efficiencies and transient durations of gene expression, are significant. Advances are being made to improve transfection efficiency and enhance the specificity of DNA delivery. An example of such a modification is the combination of liposomes with proteins of the hemagglutinating virus of Japan. These proteins mediate cell attachment and membrane fusion and can dramatically improve gene transfer efficiency. Hemagglutinating virus of Japan–liposome constructs carrying antisense oligonucleotides or therapeutic genes have been used in vascular gene therapeutics to prevent intimal hyperplasia (IH).

**LOCAL GENE DELIVERY**

One of the important considerations in developing cardiovascular gene transfer as a therapy is our 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. In peripheral circulation, vessels can be transduced at the time of surgical exposure for intervention, and extremities also tolerate quite well the periods of ischemia necessary to perform the gene transfer. 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. With the evolving technology in angioplasty and endovascular devices, these hurdles are surmountable. The various types of catheters include double-balloon, channel-balloon, microporous, hydrogel-coated, and infiltrating. Catheters can be subdivided based on the mechanism of delivery of the genetic material, either by passive diffusion, pressure facilitation, or mechanical facilitation. The purpose of all these devices is to deliver the vector to an isolated segment of the artery. Still in the developmental phase are stents coated with a hydrogel containing viral or plasmid vectors that can be used for gene delivery, but these devices are limited by production concerns and the ability to sterilize the stents without destroying the vectors. Bypass grafts, such as saphenous veins, can be easily transduced ex vivo prior to implantation into the arterial circulation. Prosthetic grafts can also be modified with autogenous vascular smooth muscle or endothelial cells that have been engineered in vitro to express recombinant genes prior to implantation. In the clinical setting, the barrier of atherosclerotic plaques can significantly lower gene transfer efficiency, and devices have been designed that allow vectors to be injected directly into the arterial wall, penetrating these plaques.

An alternative to intraluminal gene delivery is transduction of the outer wall, or adventitia, of blood vessels. Numerous studies have demonstrated that adventitial gene delivery results in equivalent levels of biological effect in reducing IH in injury models of restenosis. Certainly, this method of vascular gene transfer obviates the inherent problems of transient interruption in blood flow through a targeted blood vessel and access to the intravascular space. Biodegradable polymers mixed with vectors and placed on the outside of vessels have been used to deliver antisense oligonucleotides, plasmid DNA, and adenovirus. Other investigators have used biodegradable cuffs that isolate vector solutions to the adventitial space or direct adventitial injection. Adventitial gene delivery is a particularly attractive route of gene transfer for the surgeon who has direct access to blood vessels during operative exposure.

In angiogenesis, intravascular gene delivery is not needed. Instead, 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. Researchers have modified this by injecting microspheres coupled to plasmids or growth factors. The microspheres can allow for slow release of the recombinant material into the surrounding tissue.

**VASCULAR GENE THERAPIES**

**Prevention of IH**

Common interventional therapies for atherosclerotic vascular disease focus on physically disrupting or bypassing hemodynamically limiting lesions. These treatments produce perturbations in normal flow dynamics and induce injury, both of which provide stimuli for cellular proliferation and IH. Clinically significant in-stent stenosis occurs in 20% to 40% of patients within 6 months of balloon dilation and stenting of coronary vessels. The proliferative process is more protracted in vein grafts, and significant stenosis typically occurs in 50% of grafts by 5 years. Pharmacologic agents, such as antiplatelet and
anticoagulant drugs, they have generally been unsuccessful at preventing neointima formation. Instead, the use of gene therapy technology that locally modifies the vascular “injury” response and inhibits cellular proliferation has become an attractive option. Gene therapy strategies for the prevention of IH have targeted several different processes, including cell cycle progression, DNA synthesis, cell viability, and cytoprotection.

Many gene therapy studies have focused on the modulation of cellular proliferation through regulation of the cell cycle. Cell cycle progression is controlled by the phosphorylation of cyclin/cyclin-dependent kinase (CDK) complexes. Activated cyclin/CDK complexes will then phosphorylate and inactivate the retinoblastoma protein that results in the release of transcription factors E2F and ERF. Inhibitors of CDK (CDKIs) are endogenous inhibitors of the cyclin/CDK complexes and can arrest cell cycle progression. Gene therapy strategies have included manipulation of the expression of a number of these cell cycle regulatory proteins. An example is the overexpression of the CDKI p21Cip1/Waf1. In 1995, Chang et al infused rat carotid arteries following balloon injury with an adenovector carrying the p21 CDNA and demonstrated histologically that p21-expressing carotid arteries had a 46% reduction in the intima-media ratio at 20 days postinjury as compared with control treated arteries. This has been reproduced in both rat and pig models.37,38 Chang et al also used gene transfer of a constitutively active retinoblastoma protein to dramati-}

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control arteries at 14 days. These results have been reproduced by other investigators using eNOS gene transfer by either liposomal or adenoviral gene delivery methods; eNOS was a natural candidate for these studies because it is believed that vascular injury results in the loss of eNOS function and, therefore, nitric oxide. This enzyme produces nitric oxide in low levels in response to mechanical and agonist stimulation. In contrast, the inducible NOS (iNOS) is expressed following cellular stress and is capable of producing significantly greater quantities of nitric oxide. A theoretical advantage of iNOS for gene therapy is its ability to produce adequate local concentrations of nitric oxide in the vasculature with lower gene transfer efficiencies. This is an important consideration in the coronary circulatory system, where even short periods of blood flow disruption can result in myocardial injury. In 1996, Tzeng et al performed ex vivo delivery of retroviral human iNOS to balloon-injured pig arterial segments and essentially abolished myointimal thickening, despite a very low (1%) gene transfer efficiency. Subsequently, adenoviral delivery of iNOS to balloon-injured rat carotid arteries using low titers of virus resulted in a 97% inhibition of IH compared with controls at 2 weeks. This therapy was similarly effective in a porcine model of in vitro injury where iNOS gene transfer reduced IH by more than 50%. Finally, iNOS gene transfer into jugular vein grafts implanted into the pig carotid circulation also protected against graft IH by 35%. These studies suggest the great potential utility of NOS gene therapy for the prevention of IH and have fueled the approval by the National Institutes of Health (Bethesda, Md) and the Food and Drug Administration (Rockville, Md) of iNOS gene transfer in a clinical trial to treat in-stent stenoses following coronary angioplasty or stenting.

These studies demonstrate that a number of different approaches can be effective in preventing IH by targeting smooth muscle cell proliferation. This discussion is by no means complete and many other studies have been performed using genes directed against extracellular matrix deposition, cytokines, and other antiproliferative agents. Further investigations into vascular biology and delineation of molecular mechanisms will help to identify additional targets for gene therapy as well as improve on our current strategies.

Angiogenesis

The treatment of ischemic disease with the goal of increasing the number of small vessels within ischemic tissue is termed therapeutic angiogenesis. Studies from tumor neovascularization and cardiovascular development have helped to identify vascular endothelial growth factors (VEGF) and fibroblast growth factors (FGF) as potent mediators of angiogenesis. The VEGF family is large but VEGF-A is the best-characterized form in the study of angiogenesis. Hypoxia and several cytokines induce VEGF expression, which then signals through tyrosine kinase receptors to mediate downstream effects. A mitogen for endothelial cells, VEGF also promotes cell migration and is a potent hyperpermeability factor. It has been shown to improve collateral vessel development in animal models of hind limb ischemia and myocardial ischemia. Earlier studies with FGF demonstrated similar results. In the clinical setting, Baumgartner et al treated 9 patients with limb-threatening lower-extremity ischemia with intramuscular injections of plasmid DNA containing the VEGF complementary DNA (cDNA). This treatment improved blood flow to the ischemic limbs as evidenced by angiographic evaluation, improved hemodynamic indices, relieved rest pain, and improved ulcers and limb salvage when evaluated at an average of 6 months posttreatment. Other clinical trials, however, failed to show such definitive benefit, and additional trials are ongoing using claudication as the treatment criterion as opposed to limb-threatening ischemia. Trials are also being carried out using VEGF administration, either liposome-mediated or adenoviral-mediated, to stimulate angiogenesis in ischemic myocardium. Patients are still being evaluated for these trials.

Despite these studies, many concerns have been raised regarding these therapies. Although gene therapy with FGF, VEGF, and other growth factors has led to angiogenesis, additional studies have not shown the formation of functional collateral vessels that persist after the withdrawal of the growth factor. There are many unanswered questions and concerns. The biological effects of VEGF are remarkably dose-dependent. The potential risks of therapeutic angiogenesis include hemangiomatous formation, formation of nonfunctional leaky vessels, and the acceleration of incidental tumor growth. Accelerated tumor growth was observed in a patient with an occult lung tumor receiving VEGF therapy and resulted in the halting of that trial by the Food and Drug Administration. This event brought to light the need to be extremely cautious about the clinical application of these gene therapies and the need to be rigorous about the screening of the patients we subject to such experimental therapies.

Prevention of Atherosclerosis

The origin of atherosclerotic lesions is multifactorial and involves many genes. Before we can treat this process in the vasculature, there has to be a better understanding of the precise roles of the gene products involved in this progression. For this reason, vascular-directed gene therapy approaches to treating atherosclerosis are still early in the development phase. On the other hand, certain inherited defects that lead to accelerated atherosclerosis may potentially be treated with systemic therapies. For example, low-density lipoprotein–receptor deficiency may be treated by low-density lipoprotein–receptor gene transfer to the liver to increase low-density lipoprotein uptake and clearance. Similarly, patients with specific defects in enzymes of lipoprotein metabolism, such as lipoprotein lipase or hepatic lipase, could be treated with gene therapies to express functional forms of these defective enzymes in the liver or even the skeletal muscle.

Prevention of Thrombosis

A thrombus forms in the vasculature when there is a local defect in the normal antithrombotic function of the
vessel. This typically occurs at sites of vascular injury, either from disease states or secondary to therapeutic maneuvers. Gene therapy approaches have been developed to prevent thrombus formation. Examples of such genes include tissue plasminogen activator (t-PA), which activates plasminogen to plasmin that can then mediate fibrinolysis, tissue factor pathway inhibitor, because tissue factor is the primary stimulator of the coagulation pathway, and hirudin. These may be very useful in preventing early thrombosis following bypass surgical or angioplasty procedures.

**FUTURE DIRECTIONS AND SURGERY**

Research in the field of human genetics will continue to affect our understanding of the molecular mechanisms involved in disease. Technological advances, such as DNA microarrays and proteomics, continue to help us study the molecular responses of tissue to vascular injury and all disease processes. The Human Genome Project will help link specific genes to disease, identify new genes, and discover single nucleotide variations (polymorphisms) within a gene that alter phenotype and, thus, physiological behavior. These advances will enable physicians to better predict and diagnose disease. For the surgeon, this may translate into optimal timing for some surgical therapies and identifying patients at higher risk for complications both before and after surgical intervention. Gene-based strategies will improve late complications that have limited the surgical outcomes for patients, such as neo-intima formation, graft failure, and transplant arteriosclerosis. More important, gene therapy and molecular medicine have tremendous potential to affect our understanding of the molecular mechanisms involved in disease states or secondary to therapeutic maneuvers.Gene therapy approaches have been developed to prevent thrombus formation. Examples of such genes include tissue plasminogen activator (t-PA), which activates plasminogen to plasmin that can then mediate fibrinolysis, tissue factor pathway inhibitor, because tissue factor is the primary stimulator of the coagulation pathway, and hirudin. These may be very useful in preventing early thrombosis following bypass surgical or angioplasty procedures.

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