Part V

Medical and gene therapy

Chronic Critical Limb Ischemia: Medical Treatment

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Chronic critical limb ischemia (CCLI) is a progressive clinical condition of limb artery disease with risk of limb amputation (1,2).
Nosographical criteria have been well defined by the 1991 Consensus Conference and by the 2000 Tasc Force.

The best diagnostic and therapeutic methods for diagnosis and treatment of chronic critical limb ischemia remain controversial.

The first and second European Consensus and Task Force on peripheral occlusive artery disease (POAD) have presented pathophysiological hypotheses as well as recommending therapeutic guidelines. Considering the current social and pharmaceutical economies, it is more cost-effective that all patients with CLI undergo therapy and aim at limb preservation compared with amputation. All this in light of a better quality of life (3,4,5).

Optimal therapy is revascularization whereas in other cases it may be primary amputation. Though limb preservation is persevered thanks to greater availability of pharmacological products and better surgical and revascularization techniques, the incidents of CLI amputation remains very high (6).

The number of CLI patients are 500-1000 per million per year. In diabetic patients these figures are 5-10 times greater compared with sclerotic patients. CLI in patients over 55 yrs. is 1.5%.

Limb amputated patients have a peri-operative mortality of 15-20% when amputation is above the knee and 5-10% mortality when amputation is below the knee.

Many factors influence medical treatment. When ischemia is critical, diagnosis and treatment must be aggressive (7).

Treatment chosen for critical ischemia, be it surgical, revascularization or conservative, depends on a series of factors:

1) Patient's condition:
   a) General and cardiovascular condition
   b) Mobility and neurological disabilities
   c) Previous therapeutic treatment
2) Factors adherent to location and type of arterial lesion:
   a) Location and extent of arterial lesion
   b) Type of arterial lesion
   c) Runoff

Last but not least is the hospitals' capability to foresee a multidisciplinary therapeutic approach, diagnostic possibility, vascular surgery and revascularization.

Conservative medical treatment for CLI is applicable exclusively in cases that are not recommended for surgical and revascularization treatment or when these have failed. Conservative medical treatment is always recommended as supporting treatment.

The principal objective of pharmacological therapy in CLI patients is to:

1) Increase blood flow
2) Treat the presence of infection
3) Reduce pain.

The first point regards the actual pharmacological therapy. The prerequisite is good general circulation and good cardiac activity.

On the bases of pathophysiological criteria and the results of therapeutic treatment on patients with CLI, medicines commonly used are prostanoids. Various prostanoids have been used to treat CLI patients. Chronologically speaking, the first to be used was prostacylin PGI2 (epoprostenol), a very strong vessel dilator and platelet inhibitor. It has a short blood life that is less than one cycle, it is unstable and is rapidly degraded at 6-keto-prostaglandin F1alpha. The following products later became available: Iloprost (PGI2), Alprostadil (PGE1) Taprostene (Iloprost prostacycline analogue stabile) and Sodium Betaprost (oral prostacycline analogue stabile). Alprostadil derives from prostaglandin endoperoxide PGH1. It is a vessel dilator and platelet inhibitor. It has a short blood life of 1min because it is inactivated by the pulmonary cycle. On the contrary, Iloprost (Ilomedin) is a stable carbacycle prostacyclin derivative. It has a blood life of 4min from the moment of distribution and 20-30min to the moment of elimination. The first PGE1 study was conducted by Carlson on 4 patients, without a group control, via artery injection of 1ng/Kg/min. The results showed reduction in rest pain and ulcers. After a short time further controlled studies took place with ATP and Niacinate but showed no difference with PGE1 treated patients and control patients.

Intra-arterial perfusion has been abandoned due to risk of infection, haematoma, etc. and because of the paradoxical increase of TcpO2 in pre-stenotic areas compared to post-stenotic areas. Carlson successfully tested the medicine via intravenous injection on 8 patients at stages III and IV (with control) and the results were satisfactory (8).

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All long term studies that followed have had positive effects where as the effect of short-term studies have been negative (9-14).

The most recent study on Alprostadil is an Italian study by "The ICAI Study Group" (15). It is an open study without a control group but has a randomised control. It stands as today's largest study on 1560 patients followed long-term. The medication administered was 60 micrograms-die every 28 days. Critical ischemia was defined according to clinical criteria: evident ulcers or gangrene and rest pain for more than 15 days.

Ankle pressure was not considered as part of the criterion. Low aspirin doses were used for pain treatment. The treatment's effectiveness was evaluated after 28 days. After 6 months effectiveness was evaluated for persistence of CLI, major amputations and death.

Cases of death not attributable to CLI where considered together with other complicated but not vital vascular cases. The study shows significant short-term advantages and after 6 months there was no difference between the treated group and the control group.

As for the Iloprost therapy, Dormandy results show improvement in 51.9% of patients with CLI compared with 29.1% of those treated with placebo (16). During that same period there was a multi-centre study by 4 European countries (France, UK, Germany and Sweden) on 735 patients. The end point of each study varied such as: analgesic consumption, ulcer deduction, healing, rest pain relief and amputation (17-22). Short-term results are good and statistically significant. ($p<0.005$)

The Tasc Force in its 1985 recommendations considered prostanoids the medication to use in case of impossible revascularization or surgical failure. In reality, no study conducted on CLI patients has given impressive results. Thought should be given to the cost effectiveness of these pharmaceutical products. If they are used in the early stages and in oral form, this could improve the aspect of, less cost better quality of life, in patients with artery disease of the limbs. Furthermore, it is not sufficiently underlined that these pharmacological products are highly vessel dilating and as such can determine blood spills from unhealthy vascularized areas to healthy areas worsening the ischemia especially under the skin.

Together with prostanoids, there are also a series of drugs regularly used with good results in patients with chronic POAD. These same medicines are used in patients with CLI even if few controlled studies exist.

The most commonly used among these are; Haemorelogical products (defibrinogens and destran 40), Propionyl L-carnitine (PLC), anti-aggregants, anti-coagulants, profibrinolytics and fibrinolytics).

This occlusive arterial disease is associated with an increase of blood viscosity and a decrease of the erythrocyte deformity. Defibrinogen medicines and haemodilution react on the blood's overall viscosity whereas other medicines (vessel active) such as pentoxifyllin and buflomedil react more on the erythrocyte deformity. The pharmaceutical role of defibrinogens has yet to be established although various studies have been conducted in the past with Ancrod and Batroxobina a purifying enzyme extracted from viper venom. These studies are now historic (23-27).

The most used haemoreological product is low molecular weight Destran. Destran is a glucose polymer with a molecular weight of 40,000 daltons. It decreases the blood's thickness, interferes on the phenomenon of membrane potential inhibiting aggregation of platelets and erythrocytes. The main effect is haemodilution essentially due to liquids being drawn from the tissues to the blood because of the oncotic-osmotic effects. A 10% concentration in physiological or glucose solution is generally administered by means of IVD. The utmost attention must be given to avoid serious side effects such as: circulation run off, kidney failure, Pseudo-allergic-reaction P.A.R. as those produced by dye exposure ASA, FANS. PAR symptoms are similar to allergy syndromes that go from skin rash to shock but have a differing extra-immunological mechanism. With Destran, the qualitative reactions are abnormal, unpredictable and depend on the dose and the speed at which it is administered. Therefore, before initiating Destran treatment, it is advisable to exercise a tolerance test on the patient to investigate possible reactions for allergies. One or two drops of the medicine in 10cc of physiological solution are administered very slowly by IVD. Ensure that strong cortisone dose, depamin and adrenalin are well at hand. In Italy, this test has been written on the leaflet inserted in the medication pack.

Few studies exist that show the effects of haemodilution in patients with CLI. On the other hand, one cannot deny the fact that an increase in blood pressure, a decrease in HCT and distal perfusion improvement, can only be a benefit for ischemic tissues.

Unfortunately, this therapy can only bring temporary advantages and contemporarily increases the risk of cerebral ischemia and heart failure in patients that have had already polivascular diseases. The therapy must therefore be under strict observation of heart and kidney parameters. Haemodilution can be hypovolemic, normovolemic and hypervolemic in relation to the blood volume actually present after treatment. Hypovolemic haemodilution represents normal bleeding.

Normovolemic and hypervolemic haemodilution was highly used in the past and results were positive but they are now disregarded since the introduction of new pharmacological products to the point that even Tasc Force 2000 barely mentions its existence.

The school of Cologne (Rieger & Coll.) in 1983 published an excellent contribution on the effects of normovolemic haemodilution in POAD patients with CLI. 45 patients were treated according to Reiger's therapy. On the first day the patient gave 500 ml of blood replaced with 300-500 ml of destran 40. This treatment was repeated until HCT was reduced by 30%. After a few days there was also an increase of platelets that lead to the introduction of antiaggregants. The clinical effects were resolution of trophic lesions in 7 patients and improvement of necrosis in 18 patients with POAD. The resolution of the trophic lesions was 100%. Rudofsky and Dormandy also obtained excellent results for CLI patients after using normovolemic dilution. Bartolo` & Coll. used hypervolemic dilution (Rheomacrodex) in 93 patients with CLI. After 20 days of treatment 48.8% of patients had a complete regression of night pain, complete ulcer resolution in 22.8% of patients and an improved healing tendency in 25% of patients. Red blood cells decreased by 67.7% (500,000), platelets by 80.5% (-124,000). The peripheral pletismogramme improved in 77.5% of patients (28).

Vessel active drugs are not easily classified because their mode of action is ambiguous. They have a vasodilation effect that takes place via different mechanisms:

1) global sympatico effect or alpha 1 and alpha 2 receptor blockage
2) specific stimulation of beta 2 receptors
3) myorelaxation effect or papaverin.

Other properties include action by anti-aggregants, anti-serontonics, calcium antagonistic, free anti-radicals, the capability of increasing erythrocyte deformity and to diminish blood viscosity.

Vessel active medicines that are supported by many experiments are pentoxifyllin and Buflomedil.

Pentoxifyllin increases the deformity of the red blood cells through intracellular increase of ATP; it blocks aggregation and production of leukocyte oxidants; it contains a modest platelet and fibrin anti-aggregant that is administered via intravenous injection or orally. Possible side effects are gastro disturbances (nausea, vomiting, flatulence); contra-indications are heart failure and serious haemorrhaging.

There are two multi-centric trials conducted double blind towards placebo in CLI. In these two trials 600 mg pentoxifyllin was used via intravenous injection ever 12 hrs for 21 days. Both the European Study group and the Norwegian Study have shown a reduction in rest pain which is slightly higher in patients treated with pentoxifyllin but this difference is not statistically significant (29,30).

Buflomedil has a calcium antagonistic action with a level that is similar to smooth muscular fibrocells of the pre-capillary sphincter that contrasts with the arterial spasm. It inhibits the platelet aggregation and increments erythrocyte deformity. It is administered in the same way as pentoxifyllin, by way of intravenous injection or taken orally. Buflomedil is preponderant at microcirculation level. In a double blind study by Fagrell, 22 patients with gangrene revealed that this drug administered orally for 12 weeks in doses of 450mg/die significantly improved microcirculation in ischemic areas (31).

In a similar work Sunder-Plassman showed a significant increase of TcpO2 after having administered buflomedil via intravenous injection (32).

Other medicines used when treating CLI are nitro derivatives, calcium antagonists, alpha-blockers and betagonists. These can be used when arteries maintain their elasticity even if their effectiveness in critical ischemia can be opposed by a maximal vessel dilation condition that is a characteristic of this pathological condition.

Propionyl L-carnitine (PLC), a medicine recently used in clinical practice, represents one of the strong analogues of L-carnitine. Administered via mouth or injection it is captured by ischemic muscular cells where it is divided into free carnitine and propionyl-CoA at mitochondria level. Propionyl-CoA is transformed into succinil-CoA and can be used in the Krebs Cycle as an energetic underlay or in the case of reduced availability of acetil-CoA it can be used for low flow ischemia.

Free L-carnitine, increases the availability of Coenzyme A needed to make use of lipids and carbohydrates.

L-propionyl also has a protective action on the endothelium and on the smooth muscle vessels.

From a clinical view, this medicine increases effective autonomy and reduces healing time in patients with trophic lesions of the arteries (33,34,35).

More commonly used platelet anti-aggregants are acetylsalicylic acid (ASA), dypiridamol, tyclopidin, indobuphen and the most recent clopidogrel. Their aim is to fight the progressive disease of atherosclerosis and possible complications of thrombosis. ASA in low doses irreversibly blocks cycloxigen, an enzyme that intervenes in the synthesis of thromboxan A2 and is a strong aggregant. ASA also blocks prostacyclina that is a strong anti-aggregant. The effects on prostacyclin is visible only when doses of ASA are high and the effect is irreversible. Dypiridamol blocks the platelet's phosphodiesterase an enzyme with a predisposition towards the degrading of the AMP cycle. The intraplatelet concentration is increased together with its antiaggregant activity. Tyclopidin produces an irreversible inhibition of platelet aggregability and induces a visible increase of haemorrhage time. Indobufen irreversibly inhibits cycloxigenase platelets and essentially interferes with the synthesis of thromboxan.

Numerous studies show how long-term treatment with aspirin or tyclopidin reduces the progression of

atherosclerosis plaques, while a metanalysis shows a 25% reduction of other vascular events (cerebral ictus, myocardial infarction, vascular death) in all patients treated with antiaggregants.

None of these studies have been performed on patients affected by CLI not even the most recent Caprie study that shows a slight advantage of clopidogrel compared to ASA when treating patients with POAD (36).

Use of anticoagulants (heparin, oral anticoagulants) in critical ischemia has the same aim as use of anti-platelets, and that is to fight arterial thrombosis occlusion and maintain an open passage of the bypass.

A recent open study conducted with the use of low molecular weight heparin has revealed relatively good results on the condition of rest pain and on ulcer improvement.

Amongst profibrinolytics, defibrotide is the activator of plasminogen tissue activators aimed at spontaneously increasing fibrinolysis.

Bacterial infections are the most important factor for extended ulcer lesions and may stop the healing process. On the other hand ischemic tissue has a high risk of bacterial infection especially in diabetic patients. Daily medication is indispensable for ulcerative lesions. Debridement of necrotic tissue is required paying particular attention not to damage healthy tissue and not to spread the infection. The lesion must not be severely dried out or dampened therefore avoiding possible mortificiation of the flesh. If necessary one can practice flat medication that includes Dakin based cleaning or the use of physiological solution in accordance with the seriousness of the infection. General antibiotic treatment is also useful when using an antibiogramme tampon.Proteolysis creams may be use if the wound presents a fibrin layer.

Use of topic antibiotics, growth factors o debriding agents, apart from being most expensive, are not supported by randomised controlled studies that can show effectiveness. These agents can also be the cause of allergy reactions.

The presence of edema doesn't only inhibit wound healing but actually favours its presence.

Patients suffering from critical ischemia gain benefit when standing erect. This position increases the hydrostatic pressure in ischemic areas of the limbs where there is already an alteration in permeability hence forming and increasing the edema.

Edema can be controlled only from a recumbent position that causes a worsening of the pain. Relieving the pain is important. This can be done by using antalgic blockers and spinal cord stimulation (SCS) (37,38) when common oral intra-muscular or intravenous analgesic treatment does not have effect.

According to Allen's pain classification, stages II, III and IV of the peripheral arterial disease in critical ischemia, one can distinguish two types of pain in stages III and IV:

1) graded pain as in Fontaine's classification stage III alleviated by dangling the limbs,
2) continuous pain in the presence of skin ulcers and gangrene (stage IV) to which one adds neuropathy ischemia that is an aspect of critical ischemia.

The limb nerves are made up of sensitive afferent fibres and efferent fibres mainly from the sympathetic nervous system. Some of the afferent fibres are nociceptive. There are many central nervous system routes that conduct the pain such as neurone connections, ascending, descending and segmental connections that all transmit, block and modify the pain stimulation.

In critical ischemia there are two types of pain: ischemic pain and somatic pain.

Ischemic pain occurs during absence of blood flow that follows the sympathetic efferent; somatic pain expresses trophic damage and is felt by nociceptors whose efferents are associated with somatic nerves.

During the phase of critical ischemia the serious reduction of blood flow determines the passage of aerobic and anaerobic glycolisi with an accumulation of lactic acid; mitochondria change and that of the sodium-potassium pump bring about intracellular potassium loss and a fall in cytolitic processes.

Nociceptor substances are let into the extra-cellular liquid together with halogenous chemicals such as H+, K+, p, serotonine, histamine and prostaglandin.

Critical pathophysiological ischemia pain is very complex and has transmission mechanisms at central level, spinal reflexes and hyperreactivity of the sympathy that can determine chronic pain. This complexity permits the use of a vast range of pharmacological and analgesic products such as FANS, spinal cord stimulators (SCS) that strengthen the endorphin systems, opium based medicines that react on both the central and peripheral level and analgesic blockers.

Analgesic treatment must be effective, efficient and from each one must be able to evaluate the hiatrogen risk. There are no guidelines on pain therapy in CLI but only lists of medicines from which to chose on the bases of the situations described above and the response from patients. Pain killing medicines can be classified in three groups:

a) FANS
b) opium based
c) analgesics

The most effective against CLI pain are: morphine and temgesic (buprenorphine)

Morphine and morphine-associated products contrary to FANS, have an analgesic efficiency ceiling with

open-ended side effects. Speaking of opium-based doses in the case of chronic pain IV there are two contrasting schools of thought. On one hand low dosage is encouraged to avoid side effects whereas on the other hand it is said that with low-dosage you will not benefit from pain relief and you will only have side effects.

In cases of CLI opium-based medicines are used through continued infusion with elastomers associated with FANS or via continued peridual associated with anaesthetics.

Peripheral artery disease coexists with a neuropathy component that can have relief with Gabapentin and lamotrigin. These medicines strengthen the effect obtained from opium-based products therefore permitting a decrease in dose. It also has a different receptor site, spinal in the case of the first two and central for opium products.

Antidepressants and anticonvulsives are analgesics.

As in all chronic diseases, patients with CLI need antidepressant therapy and above all psychological support.

Pain therapy is used according to the need and benefit criteria for the patient and is not separated from vascular therapy as is the case with experimental protocol.

There is a synergy between pain killing therapy and pharmacological vascular therapy. What is certain is that neither aspirin nor paracetamol that are normally used in experiments, alleviate the pain in patients with real CLI.

Hope is now placed on the new gene therapy for this vascular disease. It is still at an experimental stage but the first clinical trials are underway (39). For those patients with POAD of the limbs, gene therapy is based on the capacity to induce some cells to synthesise and to secret proteins that are coded in a sequence on nucleic acids know as "plasmides". The application of gene therapy in vascular pathology experimental science is wide spread. The most audacious project is to obtain a "therapeutic angiogenesis".(Hockel 1993) by introducing growth factors (FGF and VEGF165) that have the capacity to stimulate the proliferation and differentiation of endothelial cells to the point that they form new capillary structures. The aim is to promote the development of collateral circulation in ischemic areas (40).

Even though the first results seem encouraging care must be given as was the case with prostanoids.

Checking and eliminating risk factors is also useful even though this is of secondary importance in CLI. The most important risk factor is smoking whether associated with an age factor or associated with the number of cigarettes smoked.

The mechanism with which tobacco acts to determine OPAD consists in the increase of catecolamin release, LDL oxidation and consequent monocyte adhesion, plasmatic fibrinogen increase, increase in platelet adhesion, Von Willebrand factor and the endothelial synthesis reduction of nitric oxide.

90-98% of smokers are affected with artery disease compared to 70% of the general population. Important studies: Framingham, Paris, and Glostrup, the incidence of vascular disease in smokers is on the average 3-4 times higher that non smokers. The most recent study, VAHIT (1988) on 2531 patients has compared smoking to other risk factors in POAD and has revealed a greater prevalence of the disease in smoking patients with diabetes, hypertension. Both affections are compared in patients with the same risk factors but non-smokers (31.4% in diabetic smokers, 8.8% in diabetic non smokers; 14.7% in hypertension smokers, 8.0% in hypertension non smokers; 21.1% in hypertension, diabetic smokers, 13.7% in hypertension, diabetic non smokers).

Another risk factor for all cardiovascular diseases is high blood pressure. Regular pressure checks are essential. A drastic reduction leads to reduced autonomy of movement in chronic artery disease patients, a pain increase and worsening of necrotic lesions in CLI.

Diabetes Mellitus is amongst the most important risk factors for POAD patients but it is certainly determinant in the insurgence CLI in patients. Diabetic neuropathy and the infections that are typical of diabetes Mellitus can only worsen the pain and necrotic lesions.

According to the Tasc Force glycemia should be maintained at approx 120 mg/dL and below 180 mg/dL after meals. The haemoglobin glycosilate must be kept under 7% (recommendation 23).

Another blood parameter to keep under control is hypercholesterolemia. All patients will CLI should keep LDL cholesterol level equal to or less than 100 mg/dL.

The best treatment for critical ischemia is often a combination of various methods of therapy; surgical, revascularization, and conservative all variably combined (41-44). It is obvious that the right decisions depend on the collaboration between vascular surgeon, radiologist and angiologist (45-48).

Concluding, we can say that in order to make the right therapeutic choice for each patient it is of vital importance to have a multidisciplinary team with experience in all methods of treatment. The treatment of artery disease must be seen in the same context as the treatment of the patient's quality of life.

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Gene Therapy for Peripheral Arterial Disease

Michael J. Mann

With the advent of molecular cardiovascular biology has come an opportunity to apply our understanding of the genetic blueprint of disease pathogenesis toward more sophisticated and more powerful interventions aimed at the roots of cardiovascular disorders. Along with this greater understanding of molecular disease targets have evolved a growing armamentarium of tools that can be used to manipulate gene function in intact tissues in vivo. These two phenomena have allowed the birth of Cardiovascular Gene Therapy as a clinical entity that carries the promise of improved care for patients with peripheral arterial disease.

Gene therapy has come to embrace both the introduction of functional genetic material into living cells as well as the sequence specific blockade of certain active genes. These systems have included recombinant viral vectors that allow relatively efficient insertion of genetic information, and oligonucleotides that can be used to alter native gene expression (1,2). This increased breadth of gene manipulation technology has accompanied the identification of genes that are either activated or repressed during disease. Recent discoveries have uncovered therapeutic targets both for the improvement of conventional cardiovascular therapies, such as balloon angioplasty or bypass grafting, and for the development of entirely novel approaches, such as the induction of angiogenesis in ischemic tissues (2, 3). As enthusiasm grows for these new experimental strategies, it is important for clinicians to be aware of their limitations as well as their strengths, and for careful processes of evaluation to pave the possible integration of these therapies into routine practice. This chapter will explore general principles of gene manipulation in the cardiovascular system and will review a number of prominent examples of experimental reduction to practice.

Gene therapy can be defined as any manipulation of gene activity, or gene "expression," that influences disease. This manipulation is generally achieved via the introduction of foreign DNA into cells in a process known as transduction or transfection. Gene therapy can involve either the delivery of whole, active genes (gene transfer), or the blockade of native gene expression by the transfection of cells with short chains of nucleic acids known as oligonucleotides (Figure 1). The gene transfer approach allows for replacement of a missing gene product, or for the "overexpression" of a native or foreign protein that can prevent or reverse a disease process. Vectors for gene transfer can be as simple as a circular plasmid DNA, which is taken up by muscular tissue and expressed without integration into the cells native chromosomal DNA. To enhance the efficiency of gene transfer, researchers have developed strategies for altering viral genomes to yield recombinant viral vectors. These vectors are generally rendered replication deficient by

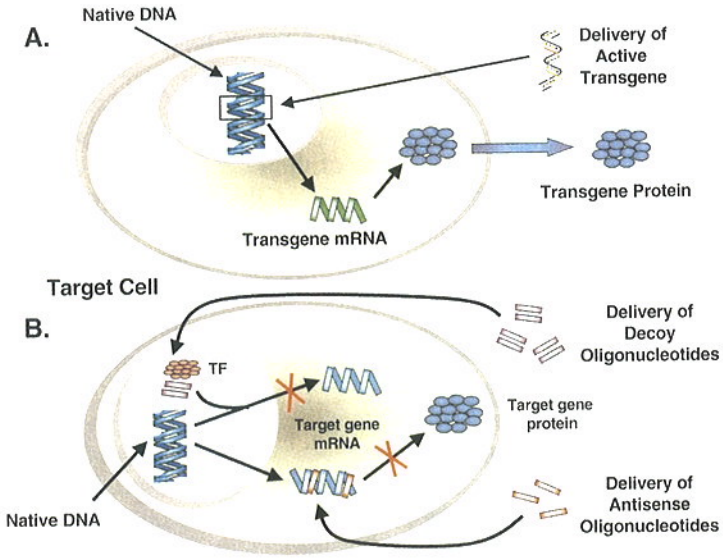

FIGURE 1

Gene therapy strategies. (A) Gene transfer involves delivery of an entire gene, either by viral infection or by non-viral vectors, to the nucleus of a target cell. Expression of the gene via transcription into mRNA and translation into a protein gene product yields a functional protein that either achieves a therapeutic effect within a transduced cell or is secreted to act on other cells. (B) Gene blockade involves the introduction into the cell of short sequences of nucleic acids that block gene expression, such as antisense ODN that bind mRNA in a sequence specific fashion and prevent translation into protein.

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means of deletion of several genes that are crucial to a successful viral life cycle in an infected cell. The first recombinant vectors used for gene transfer were based on retroviruses that required target cell replication for integration into the cell's genome prior to expression of the delivered gene. Since then, adenoviral vectors have proven the most versatile vectors in delivering genes to generally quiescent, non-dividing vascular cells. The immunogenicity of these vectors, however, has been limiting both in terms of tissue toxicity and in terms of reduced efficiency and duration of transgene expression. More recently, recombinant adeno-associated viruses, which are much less pathogenic and immunogenic, have been explored as alternative vectors for vascular gene delivery (4). Although these vectors are generally less efficient than their adenoviral counterparts, they can yield long term expression via integration into the genome of even non-replicating cells. Targeting of transgene expression exclusively to vascular cells may also be accomplished in future via incorporation of gene promoters that allow gene expression only in certain cell types, such as endothelial or vascular smooth muscle cells (5).

Gene blockade can be accomplished by transfection of cells with short chains of DNA known as antisense oligodeoxynucleotides (ODN) that bind to mRNA in a sequence-specific fashion and block protein translation (6). Another form of gene blockade is the use of "ribozymes," segments of RNA that can act like enzymes to destroy only specific sequences of target mRNA (7). A third type of gene inhibition involves the blockade of gene regulatory proteins known as transcription factors. Double-stranded ODN can be designed to mimic the chromosomal binding sites of these transcription factors and act as "decoys," binding up the available transcription factor and preventing the subsequent activation of target genes (8).

Much of vascular pathobiology, including the development of atherosclerosis, revolves around abnormal cell growth, and vascular cell proliferation has logically become a primary target of early molecular strategies aimed at altering the onset and/or progression of occlusive arterial disease (9). Cardiovascular cells, however, are no longer viewed simply as the building blocks of a passive conduit system; they are now known to play dynamic roles both in the maintenance of appropriate blood flow and as an interface between blood borne elements and the tissues. This more sophisticated appreciation of vascular cell phenotype has broadened the range of targets for molecular intervention (10). Nevertheless, translational research has only recently begun to attempt to bridge the distance between the laboratory and clinic, and definitive "proofs of concept" remain an important milestone.

The peripheral arterial tree may provide an important arena for these early investigations, in part because of the tremendous prevalence and impact of occlusive arterial disease both in terms of cost and patient morbidity. Furthermore, the accessibility of peripheral vascular structures, both to manipulation and to physiologic evaluation, may allow a more effective application of early molecular strategies for clinical manipulation.

Prevention of Bypass Vein Graft Atherosclerosis

Saphenous vein remains the conduit of choice for bypass of infrainguinal occlusive disease.

Atherosclerotic disease in bypass vein grafts, which is responsible for failure rates of up to 30-50% in these grafts, provides a particularly attractive target for molecular study as well as intervention. Unlike native vessel atherosclerosis, this disease process has a discrete beginning and instigating event. Furthermore, these initially normal vessels are unusually accessible to effective and safe treatment during their ex vivo passage between harvest and re-implantation. The first stage of vein graft disease involves neointimal hyperplasia as a response to the acute vascular injuries associated with grafting (11). Neointimal thickening of the initially thin vein graft wall does allow a reduction of increased wall stress after exposure to the arterial circulation. The growth of this abnormal neointimal layer, however, involves not only the proliferation and migration of medial vascular smooth muscle cells, but also a phenotypic activation of these cells. The result is the expression of cytokines, adhesion molecules and growth factors that not only amplifies the proliferative process but also promotes a pro-inflammatory environment in the vessel wall that triggers endothelial dysfunction and leukocyte invasion. In fact, neointimal hyperplasia in vein grafts provides a substrate for aggressively accelerated atherosclerosis that yields mature, occlusive plaque within 1-5 years after surgery.

Our research group hypothesized that bypass vein graft biology could be rerouted away from this disease process and onto a more adaptive pathway of vascular remodeling via intervention in the pattern of gene expression associated with neointimal hyperplasia.

Although many redundant pathways are involved in stimulating smooth muscle cell proliferation and activation, a final common pathway involves the orchestrated upregulation of cell cycle regulatory genes. By inhibiting the increased expression of these genes, we postulated that the grafts would instead pursue an alternative avenue of response to the chronic hemodynamic stress of the arterial environment, namely medial hypertrophy. This more stable long-term adaptation would

not render the vessel susceptible to accelerated atherosclerosis as does the process of neointimal hyperplasia, and might therefore yield a conduit that more closely resembled a native artery in structure and function.

In a rabbit model of interposition vein grafting, oligonucleotide inhibition of cell cycle regulatory genes, was found to block significant neointimal hyperplasia (12). In contrast to arterial balloon injury, however, vein grafts are not only subjected to a single injury at the time of operation, but are also exposed to chronic hemodynamic stimuli for remodeling. Despite these chronic stimuli, a single, intra-operative ODN treatment of experimental vein grafts has resulted in a resistance to neointimal hyperplasia that lasted for at least six months. During that time period, the grafts treated with ODN were able to adapt to arterial conditions via hypertrophy of the medial layer in a manner analogous to the medial hypertrophy that has been documented in models of arterial hypertension.

Furthermore, these genetically engineered conduits displayed preserved endothelial function, as demonstrated via increased endothelial cell nitric oxide synthase activity, normalized vascular reactivity, reduced oxidative stress and resistance to increased adhesion molecule expression and monocyte adhesion (13).

Even more importantly, these engineered grafts proved resistant to diet-induced graft atherosclerosis (Figure 2), again for up to six months after a single intra-operative treatment. The need for blockade of multiple cell cycle genes to achieve maximal efficiency of neointimal inhibition led to the application of a decoy oligonucleotide to block the transcription factor E2F (Figure 3). The E2F family of transcription factors is associated with the coordinated upregulation of as many as a dozen genes involved in movement of cells from the initial G1 phase of the cell cycle through the phases of DNA synthesis and cell division. Delivery of the decoy as a single agent to cells in the vein graft wall was found to inhibit target cell cycle gene expression and neointimal hyperplasia, and to prevent accelerated graft atherosclerosis in cholesterol-fed animals (14).

A prospective, randomized double blind trial of human vein graft treatment with E2F decoy was conducted in a cohort of 41 patients undergoing infrainguinal bypass (15). Efficient delivery of the ODN was accomplished within 15 minutes during the operation by placement of the graft after harvest in a device that exposes the vessel to ODN in physiologic solution and creates a non-distending pressurized environment of 300 mm Hg (16). ODN delivery was documented to greater than 80% of graft cells, and effective blockade of target gene expression was observed. Clinical outcome was measured as graft failure within 12 months after operation, and was reduced by 58% among patients treated with E2F decoy compared to untreated controls. This study represented one of the first attempts to definitively determine the feasibility of clinical genetic manipulation in the treatment of a common cardiovascular disorder. It has been followed up by a larger study of 200 patients undergoing coronary bypass grafting, preliminary results of which have also indicated a reduction in graft failure at 12 months. Multicenter testing of both infrainguinal and coronary bypass grafts has recently been initiated.

With the development of viral mediated gene delivery methods, some investigators have begun to explore the possibility of using these systems ex vivo in auto-

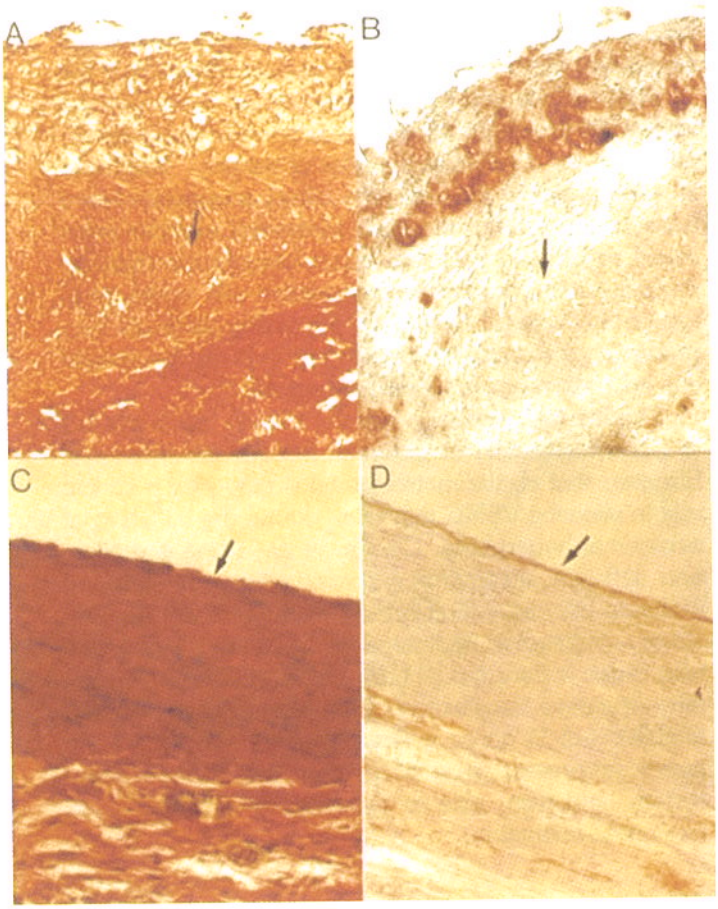

FIGURE 2

Resistance to accelerated graft atherosclerosis in genetically engineered rabbit vein grafts. Foam cell lesions (A) that stain positively for rabbit macrophages (B) and correspond to macroscopic plaque are seen in control ODN-treated grafts in cholesterol fed rabbits six weeks after surgery. In contrast AS ODN-treated grafts remain free of plaque, foam cell lesions (C) or macrophage invasion (D) despite cholesterol feeding.

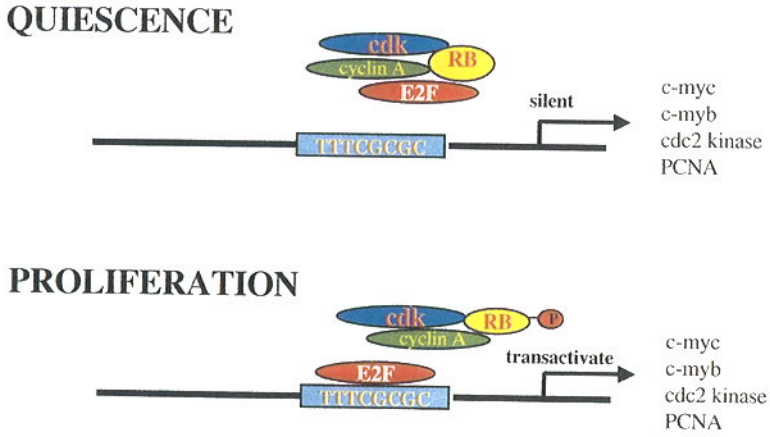

FIGURE 3A

Transcription factor E2F. In quiescent cells, E2F is sequestered in complexes with cyclin A and the retinoblastoma gene product, Rb, and is unable to interact with its binding sites in the chromosomal DNA or to stimulate gene activity. Upon cell cycle stimulation, Rb is phosphorylated, and E2F is released to trigger increased cell cycle regulatory gene expression.

logous vein grafts. Chen et al. (17) demonstrated the expression of the marker gene b-galactosidase along the lumenal surface and in the adventitia of 3-day porcine vein grafts infected with a replication deficient adenoviral vector for 2 hours at the time of surgery. Kupfer et al. (18) explored the use of a novel adenovirus-based transduction system, in which adenoviral particles were linked to plasmid DNA via biotin/streptavidin-transferrin/polylysine complexes. Expression was again greatest on the lumenal surfaces of the grafts, although then presence of occasional transfected cells in the medial and adventitial layers was also reported.

The feasibility of gene transfer in vein grafts has led to the investigation of potential therapeutic strategies. Enzymes that cleave matrix proteins may play a critical role in allowing VSMC migration during the initiation of neointimal hyperplasia. George et al., using a replication deficient adenovirus expressing tissue inhibitor of mettaloproteinasee-2 (TIMP-2), were able to demonstrate a decrease in neointima formation in a saphenous vein organ culture model (19). Bai et al (20) performed intra-operative transfection of the senescent cell-derived inhibitor (sdi-1) gene, a downstream mediator of the tumor suppressor gene p53, using the HVJ-liposome system, and were able to demonstrate a reduction in neointima formation.

Bio-Artificial Graft Conduits

Prosthetic materials, such as PTFE or Dacron, often used as small caliber arterial substitutes or in the construction of arteriovenous grafts have been limited in their long-term use due to their thrombogenic surfaces. A bioengineering, cell-based strategy for decreasing or eliminating this thrombogenicity may therefore yield a prosthetic graft capable of maintaining normal flow. Successful isolation of autologous endothelial cells and their seeding onto prosthetic grafts in animal models has been well characterized (42). Furthermore, it has been hypothesized that one can enhance the function of these endothelial cells via the transfer of genes prior to seeding of the cells on the graft surface. Wilson at al. (43) demonstrated successful endothelialization of a prosthetic vascular graft with autologous endothelial cells transduced with a recombinant retrovirus encoding the beta-galactosidase gene. Successful clinical applications of these concepts, however, have not yet been reported. In an attempt to decrease graft thrombogenicity, Dunn et al. (44) seeded 4 mm Dacron grafts with retrovirally transduced endothelial cells encoding the gene for human tissue plasminogen activator (TPA) and implanted them into the femoral and carotid circulation of sheep. The proteolytic action of TPA resulted in a decrease in seeded endothelial cell adherence, with no improvement in surface thrombogenicity.

Post-Angioplasty Restenosis

Restenosis occurs after approximately 30-40% of coronary angioplasties and in 30-50% of superficial femoral artery lesions within the first year after treatment. Despite the very successful application of intravascular stenting to both coronary and iliac artery disease, stents have not had a similar impact on more distal peripheral disease. A successful inhibition of neointimal disease and post-angioplasty restenosis might provide an opportunity for non-surgical intervention to a large population of patients suffering from peripheral arterial disease. Two basic approaches to the molecular inhibition of arterial neointimal formation have been explored – the cytostatic approach, in which cells are prevented from progressing through the cell cycle to mitosis, and the cytotoxic approach, in which cell death is induced. It has been hypothesized that by blocking expression of

the genes for one or more cell cycle regulatory proteins that one could prevent the progression of VSMC through the cell cycle and inhibit neointimal hyperplasia. Morishita et al. demonstrated near complete inhibition of neointimal hyperplasia after carotid balloon injury via HVJ-liposome-mediated transfection of the vessel wall with a combination of antisense ODN against cell cycle regulatory genes (21). Arrest of the cell cycle via antisense blockade of either of two proto-oncogenes, c-myb or c-myc, has been found to inhibit neointimal hyperplasia in models of arterial balloon injury (22,23), although the specific antisense mechanism of the ODN used in these studies has subsequently been questioned (24).

In addition to transfection of cells with antisense ODN, cell cycle arrest can also be achieved through manipulation of transcription factor activity. Morishita et al. demonstrated the use of a transcription factor decoy, a double stranded ODN bearing the consensus binding sequence recognized by E2F, to block E2F and prevent VSMC proliferation and neointimal hyperplasia after rat carotid balloon injury (25). Alternatively, Chang et al. (26) showed that localized arterial infection with a replication-defective adenovirus encoding a non-phosphorylatable, constitutively active form of Rb at the time of balloon angioplasty significantly reduced smooth muscle cell proliferation and neointima formation in both the rat carotid and porcine femoral artery models of restenosis (Figure 3B). Similar results were also obtained by adenovirus-mediated overexpression a "natural" inhibitor of cell cycle progression, the cyclin dependent kinase inhibitor p21 (27), and more recently of a chimeric molecule p27-p16 (28), that likely prevent hyperphosphorylation of Rb in vivo. In addition to blockade of cell cycle gene expression, interruption of mitogenic signal transduction has been achieved in experimental models as well. For example, Ras proteins are key transducers of mitogenic signals from membrane to nucleus in many cell types. The local delivery of DNA vectors expressing ras transdominant negative mutants, which interfere with ras function, reduced neointimal lesion formation in a rat carotid artery balloon injury model (29).

Restenosis after balloon angioplasty involves not only neointimal hyperplasia, but also a process a remodeling that leads to vessel constriction and lumenal narrowing. The role of TGF-b in this remodeling process has been underscored by the alteration of collagen deposition and a reduction in lumenal narrowing after delivery of a gene for a soluble form of the TGF-b receptor (30). Mechanical prevention of remodeling via stenting of coronary vessels has succeeded in reducing restenosis rates in that circulation; the results of stent placement in the infrainguinal vasculature, however, has not been met with the same success. Recently, unpublished reports have claimed a dramatic reduction in in-stent restenosis after elution of cytostatic drugs, such as the immunosuppressant rapamycin or the anti-cancer drug taxol, from coated stents. It remains to be seen whether a similar application of drug elution will ameliorate the poor outcomes of stent placement in the lower extremity circulation.

Nitric oxide mediates a number of biologic processes that are thought to mitigate neointima formation in the vessel wall, such as inhibition of VSMC proliferation, reduction of platelet adherence, vasorelaxation, promotion of endothelial cell survival and possible reduction of oxidative stress. In vivo transfer of plasmid DNA (31) or an adenoviral vector (32) coding for endothelial cell nitric oxide synthase has shown promise as an investigational paracrine strategy to block neointimal disease. In addition, dietary supplementation with L-arginine,

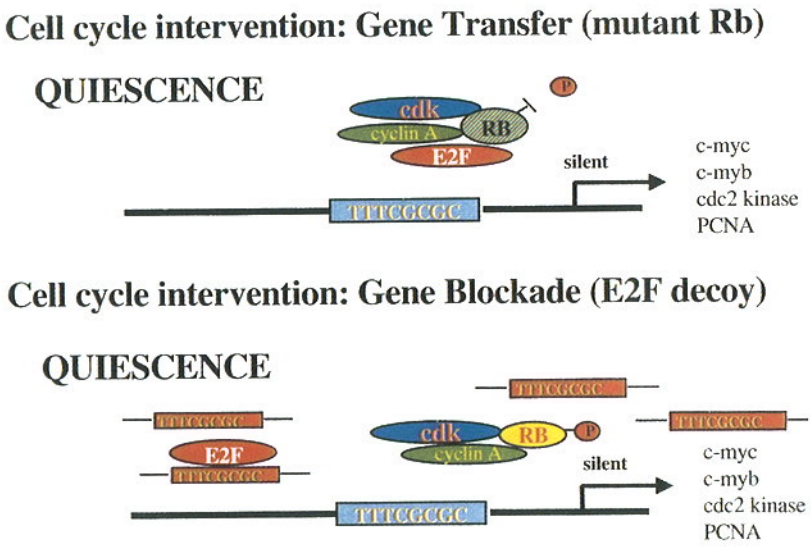

FIGURE 3B

Transcription factor E2F. Transcription factor decoy bearing the consensus binding sequence recognized by E2F, blocks E2F activity and prevents proliferation. Alternatively, a mutant, non-phosphorylatable form of Rb prevents E2F release.

the major substrate for nitric oxide synthase, has been found to induce regression of atherosclerotic plaque, and to reduce neointimal hyperplasia after injury (33,34). Delivery of the gene for guanylate cyclase, the enzyme responsible for the secondary messenger of NO, cyclic GMP, has also been shown to reduce neointimal hyperplasia and improve vascular reactivity after balloon injury (35).

An example of a direct cytotoxic approach to the prevention of neointima formation is the transfer of a 'suicide gene' such as the herpes simplex virus-TK (HSV-TK) gene into VSMC. Using an adenoviral vector, HSV-TK was introduced into the VSMC of porcine arteries rendering the smooth muscle cells sensitive to the nucleoside analog gancyclovir given immediately after balloon injury. Neointimal hyperplasia decreased by about 50% after one course of gancyclovir treatment. (36). Combining the genes for HSV-TK with the native mammalian gene for guanylate kinase, also involved in pro-drug activation, has further improved the efficiency of this cytotoxic strategy (37). Pollman and associates (38) induced endogenous machinery for VSMC "suicide" in a strategy designed to inhibit the growth or achieve regression of neointimal lesions. This strategy involved antisense ODN blockade of a "survival" gene known as Bcl-x, that helps protect cells from activation of programmed cell death, or apoptosis. Similarly, researchers have explored transfer of the gene for Fas-ligand, which triggers apoptosis when presented to vascular cells expressing the Fas receptor, as a means of using programmed cell death to limit neointimal growth in injured vessels (39).

Another potentially relevant biologic target for treatment of restenosis is re-endothelialization, which might be accelerated by local delivery of a pro-angiogenic factor (e.g. vascular endothelial cell growth factor, VEGF) at the angioplasty site. In one early clinical approach, the human VEGF-165 gene was administered as a "naked" circular DNA plasmid directly to the injured arterial wall on the surface of the angioplasty balloon (40). The investigators hypothesize that the low efficiency of this delivery method is balanced by the high biologic potency of this secreted angiogenic cytokine, enabling a significant local biologic effect despite poor gene transfer. Another VEGF isomer, VEGF-121, has also been found to improve endothelial function after intramuscular delivery in experimentally ischemic limbs (41).

Therapeutic Neovascularization

The identification and characterization of "angiogenic" growth factors has created an opportunity for therapeutic "neovascularization" of ischemic tissues. Although it is clear that angiogenic factors can stimulate the growth of capillary networks in vivo, it is less certain that these molecules can induce the development of larger, more complex vessels that would be capable of carrying significantly increased bulk blood flow. Nevertheless, the possibility of improving even the microvascular collateralization as a "biological" approach to the treatment of tissue ischemia has sparked the beginning of human clinical trials in neovascularization therapy.

After the first description of the angiogenic effect of fibroblast growth factors (FGF's) (45), an abundance of "pro-angiogenic" factors were discovered to stimulate either endothelial cell proliferation, enhanced endothelial cell migration, or both. The molecules that have received the most attention as potential therapeutic agents for neovascularization are vascular endothelial growth factor (VEGF) and two members of the FGF family, acidic FGF (FGF-1) and basic FGF (FGF-2). VEGF may be the most selective agent for stimulating endothelial cell proliferation (46). This selectivity has been viewed as an advantage, since the unwanted stimulation of fibroblasts and VSMC in native arteries might exacerbate the growth of neointimal or atherosclerotic lesions. Despite this theoretical selectivity, however, the experimental use of VEGF in animal models has been associated not only with capillary growth, but also the development of more complex vessels involving these other cell types (47). The FGF's are believed to be even more potent stimulators of endothelial cell proliferation, but, as their name implies, are much less selective in their pro-proliferative action (48).

The contribution of gene therapy to the potential development of therapeutic neovascularization is primarily one of drug delivery. The availability of the genetic sequences encoding the paracrine angiogenic factors provides an opportunity for the establishment of local tissue factories for drug production. Both intravascular as well as extravascular modes of gene product delivery are feasible, as gene transfer can be attempted either in the walls of vessels feeding the ischemic tissue or in the target skeletal tissue itself. In fact, muscle tissue is among the most receptive for gene transfer with the simplest of agents, pure plasmid DNA. Adenoviral vectors are also effective at achieving transgene expression in muscle cells.

An increase in capillary density was reported in an ischemic rabbit hind limb model after VEGF administration, and these results did not differ significantly regardless of whether VEGF was delivered as a single intra-arterial bolus of protein, plasmid DNA applied to surface of an upstream arterial wall or direct injection of the plasmid into the ischemic limb (46). Unlike VEGF, FGF-1 and -2 do not possess signal sequences that facilitate secretion of the protein, so that transfer of these genetic sequences is less likely to yield an adequate supply of growth factor to target endothelial cells. To overcome

this limitation, Tabata and associates constructed a plasmid encoding a modified FGF-1 molecule onto which a hydrophobic leader sequence had been added to enhance secretion (49). Delivery of this plasmid to the femoral artery wall, even at very low transfection efficiencies, was found to improve capillary density and reduce vascular resistance in the ischemic rabbit hind limb.

A phase I study of VEGF gene transfer via direct injection of plasmid DNA into patients with inoperable lower extremity ischemia has been reported (50). The modest doses of either protein factors or genetic material delivered in these studies were not associated with any acute toxicities. Concerns remain, however, regarding the safety of potential systemic exposure to molecules known to enhance the growth of possible occult neoplasms, or that can enhance diabetic retinopathy and potentially even occlusive arterial disease itself. Despite early enthusiasm, there is also little experience with the administration of live viral vectors in extremely large numbers to a large number of patients, and it is uncertain whether potential biological hazards of reversion to replication competent states or mutation and recombination will eventually become manifest.

In addition to issues of safety, it is also unclear whether the clinical success of conventional revascularization, which has involved the resumption of lost bulk blood flow through larger conduits, will be reproduced via biological strategies that primarily involve increase microscopic collateral networks. It must also be remembered that neovascularization is itself a naturally occurring process, and that the addition of a single factor may not overcome conditions that have resulted in an inadequate endogenous neovascularization response in patients suffering from myocardial and lower limb ischemia. Despite these limitations, angiogenic gene therapy may provide an alternative not currently available to a significant number of patients suffering from untreatable disease, and may offer an adjunct to traditional therapies that improves their long-term outcomes.

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