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Cardiovascular gene delivery: The good road is awaiting*

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Abstract

Atherosclerotic cardiovascular disease is a leading cause of death worldwide. Despite recent improvements in medical, operative, and endovascular treatments, the number of interventions performed annually continues to increase. Unfortunately, the durability of these interventions is limited acutely by thrombotic complications and later by myointimal hyperplasia followed by progression of atherosclerotic disease over time. Despite improving medical management of patients with atherosclerotic disease, these complications appear to be persisting. Cardiovascular gene therapy has the potential to make significant clinical inroads to limit these complications. This article will review the technical aspects of cardiovascular gene therapy; its application for promoting a functional endothelium, smooth muscle cell growth inhibition, therapeutic angiogenesis, tissue engineered vascular conduits, and discuss the current status of various applicable clinical trials.

Keywords

Thrombosis; Myointimal hyperplasia (IH); Gene delivery; Endothelialization; Endothelial cell (EC); Vascular smooth muscle cell (SMC); Extracellular matrix (ECM)

1. Introduction

Cardiovascular disease is the leading cause of mortality worldwide [1], and the most recent data from the world health organization show this trend continuing [2]. In the United States alone there were over 6.8 million interventions (including both operations and endovascular procedures) performed for patients with cardiovascular disease last year [3]. Unfortunately, many of these interventions, like angioplasty or endarterectomy, eventually fail because the interventions themselves injure or disrupt the vessel intima. The limited ability of the endothelium to regenerate itself in humans directly leads to both the thrombotic and myointimal hyperplastic (Fig. 1) complications that follow vascular interventions, and they frequently occur along an established time frame moving from the former to the latter failure mode (Fig. 2). Acutely, denuded intima may lead to thrombosis via platelet deposition and

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activation of the coagulation cascade, and over time it promotes smooth muscle cell (SMC) migration, proliferation, and extracellular matrix (ECM) deposition, leading to myointimal hyperplasia (IH). IH in turn narrows the vessel lumen (re-stenosis) decreasing blood flow to the point that it may promote local thrombosis or lead to symptomatic ischemia in the relevant distal end organs like the brain (cardiovascular accidents), heart (myocardial infarctions), and extremities (critical limb ischemia) [4].

IH continues to be the most common long-term complication following all types of vascular interventions with 4- and 5-year re-stenosis rates after carotid endarterectomy ranging from approximately 5–25% [5,6]. These are similar to the re-stenosis rates after angioplasty and stenting in the carotid and coronary arteries [6,7]. Further, synthetic grafts, distal lower extremity interventions, and interventions in certain patient populations, such as diabetics and patients with renal disease, continue to be associated with worsened outcomes [8–11]. Results with newer drug-eluting stents (DES) have yielded exciting early outcomes (6 months–1 year after intervention) [12,13], but these stents cannot be used in every lesion or in all patients. In addition, many DES slowly release broadly immunosuppressive agents that are not equivalent in all patient populations [14]. Further, DES are indiscriminant in their suppression of not only SMC proliferation but also that of ECs, limiting endothelial healing. This approach may retard rather than prevent the development of IH [15]. Finally, irradiation of narrowed or occluded vessels is possible, but it too is indiscriminant to cell type, and the benefits of this modality may also be limited temporally as well as longitudinally along the treated vessel [16,17]. Thus, despite voluminous work by scientists and clinicians alike in recent years, we are still in the early stages of understanding and treating the complications of vascular interventions.

Cardiovascular gene therapy offers the benefit of sustained and/or controlled expression of desired proteins in chosen tissue beds or cell types, and these characteristics are why gene therapy may be more beneficial in providing durable clinical benefits than other therapies such as the direct delivery of proteins, peptides, or other drugs; also, gene therapy provides a more specific treatment modality than vascular cells themselves, which would provide a variety of additional and unintended local functional effects. In this article, we will review the technical aspects and clinical application of cardiovascular gene therapy as it applies to the blood vessels themselves; systemic diseases, such as dyslipidemia and diabetes, preventative gene therapies aimed at pre-clinical vascular lesions, and the myocardium itself have been excluded due to space constraints, but the latter is discussed generally as a tissue bed for therapeutic angiogenesis. Since the endothelium mediates both thrombotic and IH complications of vascular intervention, we will focus much of this review on the role of gene therapy, and to a lesser extent protein therapy, in promoting a functional endothelium or limiting aberrant vascular SMC activity after injury. Next, we will review gene therapy's application in the application of gene therapy towards therapeutic angiogenesis and the development of tissue engineered vascular conduits. Finally, the current status of clinical trials will be discussed along with the current limitations of gene therapy and the direction of future work.

2. Technical aspects of cardiovascular gene therapy

2.1. Composition of the vessel wall

For arteries, the intima (*tunica intima*) is composed of a relatively quiescent endothelial cell (EC) monolayer and its surrounding basement membrane proteins (e.g., type IV collagen, perlecan) (Fig. 3) [18]. Together with the internal elastic lamina, they maintain SMCs in their contractile state and inhibit pathologic SMC activity [19]. Deep to the intima and separated by the internal elastic lamina is the medial layer (*tunica media*). It is the thickest arterial layer and composed of SMCs and many ECM proteins (e.g., fibrillar collagens,

particularly type I collagen). The progression of both arteriosclerosis and atherosclerosis is associated with changes in the relative contributions of these ECM proteins, particularly elastin and collagen [19–23], and these changes can be visualized and localized non-invasively through ultrasound screening [24].

Medial SMCs here respond to intimal cues to dilate or contract the vessel. The medial layer in veins is difficult to define because it lacks the internal elastic lamina, but after exposure to arterial flow via vein graft bypass, they develop a defined media that has similarities to that of native artery [25,26]. The adventitial layer (tunica adventitia) is external to the media, and it is composed of loosely arranged connective tissue and fibroblasts. It may play an important role in the progression of restenosis and late interventional failure after angioplasty due to vessel remodeling since adventitial delivery of therapeutic treatments has reduced these complications [27–29].

Genes may be delivered differentially to the various vessel layers in vivo or to specific cell types ex vivo with subsequent cell seeding. In vivo arterial gene therapy to the intimal and adventitial layers is relatively straightforward via the vessel lumen or perivascular space. However, the vessel media is relatively difficult to access efficiently in the presence of an intact or partially intact intima [30–33]; initially this limitation does not exist in vein grafts and may be one of the potential benefits of studying either intimal or adventitial gene delivery in this model system.

2.2. Natural history of interventional failure and the challenges the vasculature presents for effective gene therapy

2.2.1. Natural history—For arteries and vein grafts, vascular interventions such as angioplasty or endarterectomy disrupt the endothelium and its underlying media. Immediately after injury, there is a period of time where the vessel is at an increased risk of thrombosis proportional to the length of disturbed endothelium. Concomitantly overlapping waves of response are initiated within the vessel wall; these waves have been best defined in the rat model of angioplasty injury, and some of the better understood growth factors involved in this process are summarized in Fig. 1B [34].

There are multiple other growth factors involved in IH besides the ones shown in Fig. 1B, and not all ECM proteins act alike. Nevertheless, this table is useful to demonstrate some of the mediators of the vessel's response to injury in the first 2 weeks. Schwartz et al. have extensive reviews of this process and point out the utility and human exceptions to this model system [35,36]. In fact, the role of ECM proteins and growth factors are no longer considered to act in isolation, and the importance of integrins in regulating this cooperation is now evident [37]. As the interaction between growth factors and the ECM is becoming more complex, it is being better understood. Recently, Clowes et al. demonstrated that platelet-derived growth factor (PDGF)-BB-induced SMC proliferation was dependent upon fibroblast growth factor (FGF)-2 release and fibroblast growth factor receptor (FGFR)-1 activation [38]. Further the synthesis of certain ECM proteins like fibronectin becomes up-regulated after injury, and their fibrillar construction provides tracking for subsequent SMC migration, while another ECM protein, perlecan, is up-regulated after the initial waves of response [39], and it is thought to downregulate the SMC response to injury [40].

Obviously, the injured vessel's response in the first 2 weeks after injury is critical to the future responses of the vessel, but IH may have secondary cues that spur a later initiation or progression at subsequent time points. These cues may not be prevented by treatments that are aimed at limiting the vessel's initial response.

2.2.2. Timing and mode of gene therapy—Gene therapy is an appealing therapeutic approach for the treatment of cardiovascular disease because it has the potential to provide sustained expression of growth factors over a defined and predictable duration in order to promote angiogenesis or arteriogenesis in ischemic tissue beds as well as the potential to alter EC recruitment and SMC activities following interventions. Since there is a 12–24-h lag time between gene activation and protein production and the cellular response to injury is instantaneous, there may be inherent efficacy limitations to gene therapy when delivered immediately after injury; this has led to much speculation about the timing of gene delivery in order to achieve maximal effectiveness [41]. Also, subsequent delivery modalities at chosen time points using appropriate vectors may allow constitutively elevated protein levels in the desired vascular segments as the vessel responds to injury in real time.

Catheter-based systems can provide intravascular gene delivery to the intima and media, and they are simple to use. However, the need for total vascular occlusion, the problem of dilutional washout, and inactivation of viral vectors limits this delivery method [42,43]. Alternatively operative approaches can deliver genes intramuscularly and both intra- or extra-vascularly. The latter may be potentially useful in limiting the adventitial response to vascular remodeling. Again, this has been evident with direct application of IH suppressive agents to the adventitia [44].

Finally, direct cellular implantation may have profound effects in promoting cellular regeneration in sites of ischemic injury. Bone marrow mononuclear cells have been shown to convey a global benefit to cardiac function in both animal and clinical reports [45,46]. A better understanding of the mechanisms and molecular agents of these benefits may provide unique genetic insights into future therapies.

2.3. Gene delivery vectors

Genes are double-stranded DNA molecules that are large relative to their corresponding protein's nucleotide sequence. They may be delivered to the desired cells in or ex vivo by a variety of vectors, each with certain benefits and limitations, and none of them being ideal [47]. Broadly, these vectors can be divided into viral or non-viral vectors. More complete reviews of different vectors can be found in other sections of this theme issue or in these reviews [48,49].

2.3.1. Viral vectors—The first viral vectors to be used for cardiovascular gene infection were the retroviral vectors [50]. Retroviral gene delivery establishes prolonged gene expression because it integrates the gene into the host genome. However, efficient gene delivery requires an actively dividing target cell population. Since both EC and SMCs are normally quiescent in the blood vessel wall [51,52], it is not ideal for prophylactic delivery or delivery at the time of injury [53,54], but may be useful in limiting SMCs' proliferative waves of response. Further, the use of retroviral gene infection may be utilized in various models to better define primary and alternative signaling pathways relevant to vascular biology [55].

Lentiviruses are members of the retroviral family (Retroviridae), and lentiviral gene delivery may be the most promising method of gene infection [56]. They provide an efficient method of delivering large genes and the potential for prolonged gene expression. Currently, use of this vector is not well defined in cardiovascular gene therapy, but there is growing utility of this approach in the understanding of gene signaling mechanisms [57], as well as therapeutic initiatives with this approach [58,59].

Adenoviral gene delivery can infect non-dividing cells efficiently, but gene expression is often limited to approximately 2 weeks because the gene is kept episomal. Also, the

efficiency of repeated gene delivery is negated by the immune response generated against the adenovirus. Yet its efficiency and ability to produce high transgene levels makes it an attractive vector for cardiovascular applications [60]. Adeno-associated vectors provide for sustained gene expression and do not elicit an immune response, but they have a lag phase for gene expression that is at least 2 weeks and poor infection efficiency [61]. Finally, cytotoxic viral approaches may either deliver a cytotoxic gene (i.e., interferon) [62] or be used to infect cells with the herpes virus, which then makes them susceptible to antiviral destruction at a chosen time by acyclovir or other anti-herpetic treatments [63–66].

2.3.2. Non-viral vectors—Potentially, the use of non-viral vectors offers several advantages over the use of viral vectors including ease and thrift of production, limited toxicity, lessened immunogenicity, and a lower risk of transfecting untargeted tissue beds. Examples of non-viral vectors are lipoplexes, polyplexes, lipopolyplexes, and naked DNA with various iterative devices such as a gene gun or microspheres [67,68]. The simplest of the non-viral delivery systems is direct DNA injection into the tissue bed of choice or incorporation of the gene into implantable scaffolds (i.e., gene-activated matrices). At high pressures, this injection can yield high levels of expression comparable to that of viral vectors, but in skeletal muscle (a frequent target for neovascularization), it has relatively low and variable transfection efficiency. The efficiency of uptake can be increased 10-fold by electroporation or ultrasound, but these approaches have the untoward effect of cellular injury [69].

Gene-activated matrices incorporate DNA into implantable matrices; here cells are transfected with the desired genes as they colonize the matrix. Alternatively, these genes can be delivered extrinsically through gels or collar-devices [70,71]. This was first shown to be feasible and effective in tissue regeneration by Bonadio et al. [72,73]. Recent work has shown that this approach can be used effectively in the vasculature using matrix scaffolds for delivery [74–78]. Comprehensive reviews of this approach are available [79,80] and generalizable approaches to improve transfection efficiency by non-viral vectors have been discussed in detail elsewhere [81].

Inhibiting gene translation through RNA interference via cell transfection with specific antisense oligonucleotides offers a novel approach in gene therapy. Transfection of these oligonucleotides selectively downregulates the translation of certain proteins by binding to the messenger RNA (mRNA) and targeting it for destruction. Antisense oligonucleotides target mRNA in the cytoplasm, which provides for its rapid effect by abrogating the need for nuclear localization. Currently, this approach requires clinically incompatible delivery pressures and volumes, but nevertheless, it has been useful in refining our understanding of the mechanistic pathways of cell signaling and function. Although its durability is not well defined currently, with refinement it offers great promise. Finally, inhibitors of nuclear cell transcription factors itself have demonstrated efficacy in regulating gene expression [70,82–90]. This approach can be used in vivo at physiologically acceptable pressures [91], but obviously requires nuclear localization for functioning, which may decrease its efficiency [92]. As above, these methods of gene transfer are also susceptible to dilutional washout when exposed to the circulation.

3. Promoting a functional endothelium

Underlying SMC proliferation is directly related to the completeness of the overlying endothelium [93] (Fig. 4). Endothelial regeneration after injury can be promoted by the local delivery of potent growth factor genes or by the growth factors themselves. The importance of the endothelium is supported clinically by the improvement Zilla et al. showed in prosthetic graft patency and function after EC seeding compared to unseeded grafts [94,95].

This is probably the most obvious way to promote a confluent endothelium, and it can be used at sites of intimal disruption [96] or on prosthetic grafts as reviewed by Parikh and Edelman in 2000 [97]. We have been able to promote endothelialization of prosthetic grafts exceeding 30 cm in length without cell seeding through the localized delivery of the potent angiogen FGF-1 in a fibrin glue delivery vehicle to prosthetic vascular grafts [98] (Fig. 5). However, the excitement of these early successes has been tempered by the concomitant induction of IH in these vessels and grafts [99]. This has led us to develop site-directed mutants and bioactive chimeric proteins based on the FGF-1 angiogen that promote favorable characteristics (such as prolonged bioactivity, EC specificity, or increased potency) while removing unfavorable characteristics (heparin-dependent activity, susceptibility to thrombin proteolysis) [100–103]. Although not currently applied as gene therapy, these constructs (and others like them) could easily be delivered in a DNA vector system for this use [104].

Much of the promise of cardiovascular gene therapy involves localizing gene delivery to the desired locations or cells followed by sustained or controlled protein production for the desired time frame and no longer. These capabilities could obviate some of the current limitations encountered with the direct application of growth factor proteins to tissue beds for the regeneration of the endothelium. However, since not all ECs act alike, the mere recruitment of ECs to sites of intimal injury may not prevent IH in all cases [105].

3.1. Successful gene transfer to vascular cells

Seminal concurrent publications in *Science* by Nabel et al. [106] and Wilson et al. [107] demonstrated the feasibility of seeding genetically transfected (retroviral) syngeneic ECs to areas of denuded intima or prosthetic vascular grafts and the sustained in vivo expression of the genetic product, B-galactosidase. Dichek et al. has shown that genetically transfected ECs could also be used to seed vascular stents [108]. As demonstrated by Clowes et al., genetically altered SMCs can also be used to create a substitute “endothelium.” Here long-term replication-independent gene expression was seen in vivo in retrovirally infected SMCs up to 1 year after arterial seeding in a rat model. Importantly, these SMCs did not demonstrate an altered phenotype; thus, seeding of genetically altered SMCs may be able to provide extended delivery of desired gene products to selected matrices without concomitant SMC proliferation [109].

3.2. Gene transfer strategies to promote an endothelial monolayer

The delivery of potent angiogens or genes that promote EC-specific mitogenesis or chemotaxis upon prosthetic surfaces may be able to regenerate a rapid and complete endothelium after vascular intervention. Such prosthetics could store these proteins or genes and then release these agents locally to circulating or apposing ECs in a cell demanded fashion. Ideally, these prosthetics could then be available as an off-the-shelf product.

Prostacyclin levels can be augmented by prostacyclin synthase transfection in order to enhance reendothelialization and inhibit neointimal formation in the rat model [110]. More recently, Losordo et al. were able to deliver a vascular endothelial growth factor (VEGF)-2 plasmid in a vascular stent. They found that the treatment group had an improved completeness of endothelialization, improved luminal cross-sectional area and vessel function as measured by nitric oxide production in hypercholesterolemic rabbits [111]. This approach shows much promise as a delivery system, but single gene therapy may not yield straightforward results. For example, although VEGF does improve endothelialization consistently, its effects on IH are conflicting in the literature [112].

3.3. Inhibiting thrombosis through EC gene delivery

There are a variety of ways to disrupt the activation of the blood system's coagulation cascade on thrombogenic surfaces (like stents and prosthetic grafts). Greisler et al. have described the improved patency, thromboresistance, and the absence of intraluminal graft thrombus in a heparin-analog-coated expanded polytetrafluoroethylene (ePTFE) grafts using a canine bilateral aortoiliac artery model [113]. Similarly, combined data tables of clinical data showed a decrease in subacute thrombosis with heparin-coated coronary artery stents [114].

Genetic approaches to increase thromboresistance have been achieved by multiple groups through the overexpression of thrombomodulin [115], hydrolysis of adenosine nucleotides [116], addition of an ADPase [117], plasmin induction [118] or activators [119], and hirudin [120,121]. Of course, ECs themselves secrete many of these substances endogenously [122,123], so these approaches may be no more effective than a functioning endothelium, and thus, they may be best applied to SMCs or non-cellular prosthetic systems that are not expected to endothelialize over time.

3.4. EC gene therapy to limit underlying SMC proliferation

Genetic approaches may be useful as a means to improve EC function. Kibbe et al. have shown that nitric oxide levels can be enhanced by inducible nitric oxide synthase gene transfer to vein grafts, and that these increased levels in porcine and canine models lead to decreases in IH [124–126]. Since all vascular interventions expose the vessel to an ischemic insult, another approach to improving EC function is to protect the endothelium against ischemia/reperfusion injury through the transfection of heat shock proteins. These proteins have been shown to be important in the regulation of normal vessel tone and beneficial in cardiac myocytes [127,128].

3.5. Summary

Significant inroads in gene delivery have been made to date but have failed to progress the full step needed from demonstrating the feasibility of gene delivery to supporting functional benefit from gene expression clinically. While many of the above approaches are applicable to SMCs as well as ECs, we are going to focus the next section on identifying genetic approaches specific for SMC inhibition.

4. Smooth muscle cell inhibition

One of the recurring problems of gene (and protein) therapy is the lack of cell type specificity by the agents. Thus, any given treatment usually stimulates or inhibits both ECs and SMCs either directly or indirectly. The ability to simultaneously inhibit SMC proliferation and promote EC growth through a single gene, protein or a variety of genes or proteins would logically lead to rapid clinical progress. To some degree, this has occurred through the use of cell specific enhancers, promoters, or combination chimeric genes [129–131]. Other more broad approaches attack the common denominator in the relevant signaling pathways thought to be important to the development of IH (e.g., cyclin pathway).

4.1. Inhibiting SMC proliferation and migration

One such approach is the transcriptional blockade of a selected gene or genes by introducing antisense oligonucleotides to the mRNA into the desired cells. This has been shown to systematically downregulate cytosolic gene translation in vitro and in vivo using clinically acceptable pressures [66,85]. Another approach utilizes oligonucleotides to directly block the transcription factor(s) of a selected pathway that regulate(s) gene expression by obstructing its ability to bind to its chromosomal binding sites. The cyclin signaling pathway

is essential for the progression of the cell cycle, and E2 promoter binding factor (E2F) comprises a family of transcription factors that mediate this progression. An E2F decoy oligonucleotide (Edifoligide) has been shown to limit plaque progression and IH at 6 months after operation in a hypercholesterolemic rabbit vein graft model [132]. Subsequently, this approach has been supported in a variety of other animal models [133–135], and the retinoblastoma (RB) gene as well as other cyclin antagonists have led to similar results [65,136,137].

In addition to promoting re-endothelialization, there exists evidence that certain proteins such as VEGF-C can limit IH in animal models [138], but this observation is not universal [139]. However, more potent forms of VEGF [140] are currently being evaluated for their ability to limit IH in arteriovenous (AV) fistulas for hemodialysis patients.

An approach that we are currently investigating involves differentially disrupting the coordination of integrin and growth factor signaling in vascular cells with the intention of segregating EC's and SMC's relative responses to injury. Focal adhesion kinase (FAK) is a potential target for this approach because it mediates both integrin and growth factor signaling in the activation of SMC proliferation and migration, as well as the expression of the matrix metalloproteinases that mediate migration and vascular remodeling [141–147]. Upon integrin activation via ECM or growth factor stimulation, FAK localizes intracellularly to focal adhesion complexes with its carboxyterminal FAT domain, autophosphorylates itself, and initiates various cell survival, migration, and proliferation signaling cascades [148]. FAK's carboxy-terminal region exists as a separate protein termed FAK-related non-kinase (FRNK) that lacks FAK's autophosphorylation sites (Fig. 6). FRNK can act as a dominant negative inhibitor of FAK signaling by localizing to focal adhesions, displacing FAK, and disrupting FAK signaling [149–151].

Upregulation of FRNK expression has been found to be expressed in the medial layer of injured vascular walls [152], and its upregulation after balloon angioplasty injury is concomitant to that of the ECM protein perlecan in animal models [39]. Through its heparan sulfate side chains, perlecan itself may promote EC but not SMC growth after vascular injury [40,153]. Interestingly, perlecan has been found to upregulate FRNK expression which further limits SMC proliferation [142], perhaps by promoting PTEN (phosphatase and tensin homology deleted on chromosome 10) activity directly [141,154] or indirectly through cytosolic phosphatase modulation [155]. Cumulatively, FRNK and perlecan may play a role in the downregulation of the synthetic SMC phenotype commonly found during IH.

Since FRNK is found frequently in SMCs but not in ECs in vivo, we are identifying its integrin and growth factor-mediated signaling pathways and functional role in vascular SMC hyperplasia using a three-dimensional EC and SMC co-culture assay that can incorporate FAK or FRNK gene infection to desired cell types exogenously from the surrounding media [156] (Fig. 7). This assay can be modified to deliver specific growth factors or combinations of growth factors in a temporal fashion, and it may provide guidance for testing future in vivo approaches.

4.2. Inhibiting over-exuberant SMC deposition of extracellular matrix proteins

In addition to inhibiting SMC migration or proliferation, gene therapy can be targeted to manipulate the ECM and its remodeling by SMCs. This approach is innovative and exploits the predominant role of the ECM in the composition of IH lesions [157]. Of significance, urokinase [158], plasminogen activator inhibitor-1 [159], ribozyme [160], and an elastin inhibitor [161] have all shown benefit in reducing IH in animal models. Perhaps more importantly, the interruption of matrix metalloproteinases (MMP) activity by tissue inhibitor

metalloproteinase (TIMP) infection has been shown to limit SMC proliferation and migration through various models [162–165].

4.3. Summary

Genetic approaches that directly target the SMCs in order to disrupt proliferation or favorably alter the ECM are exciting modalities that are just beginning to be understood in a comprehensive fashion. Again, it should be emphasized that integrin and growth factor stimulation are interdependent of each other, and this relationship needs to be better defined after vascular injury before we can expect a durable therapeutic benefit to patients.

5. Therapeutic angiogenesis

5.1. Therapeutic neovascularization

The ability to therapeutically control new blood vessel formation (neovascularization) could palliate a number of pathological entities and augment surgical interventions [166]. The genes of naturally occurring growth factors have been investigated as neovascularization stimuli for treatment of ischemic tissues, to promote microvascular network formation in engineered tissues, and to improve the function and survival of transplanted cells and tissues. Clinical trials using gene therapy for the treatment of myocardial and peripheral limb ischemia have shown promise [167,168], but clinical efficacy has not been proven [169].

Multiple growth factors act in a coordinated fashion to modulate invasion, network formation, and maturation of microvascular networks [166]. Many of the genes of the following proteins have been investigated as neovascularization therapies, including vascular endothelial growth factor (VEGF) [170], fibroblast growth factor-1 (FGF-1, or acidic FGF) [171], FGF-2 (also known as basic FGF) [172], FGF-4 [173], placental growth factor (PlGF) [174], angiotensin-1 (Ang-1) [175], and hepatic growth factor (HGF) [176]. Direct intramuscular or intravascular injection of genes for these proteins is able to restore blood flow in animal models of limb ischemia. Adv-VEGF gene transfer is effective in increasing blood flow in an animal model of ischemia with confounding diabetes [170]. Regardless of the factor used, current delivery strategies will likely result in heterogeneous transfection efficiencies and protein expression levels in target tissue. A recent study has shown that it is the local concentration and not the total dose of protein delivered that determines the threshold between stimulating the formation of normal versus pathologic microvascular structure [177]. Heterogeneous expression within tissue could lead to local tissue regions of edema or hypoxia. Methods for efficient and controlled transfection are needed to improve control of neovascularization.

While injection of a single gene was able to stimulate neovascularization in an animal model [170], it has been difficult to translate these results into clinical success. It is possible that the disappointing clinical results are due to limitations in the method of gene delivery. However, it may also be that these approaches oversimplify the process of angiogenesis, neglecting the complex interplay of multiple growth factors essential to the formation of a stable microvasculature.

5.2. Coordinated signaling by growth factors promotes angiogenesis

Since the blood vessel's angiogenic response to VEGF transfected cells may be transient and regress with the loss of VEGF stimulation [178], a single growth factor may not be able to coordinate the entire neovascularization cascade.

Protein delivery of placental growth factor (PlGF) has recently been shown to regulate crosstalk between VEGF receptors [174]. Combined delivery of VEGF and PlGF did not stimulate greater neovascularization than either factor alone in a wild-type animal model of myocardial ischemia. However, combined treatment was significantly more potent than either factor alone in an animal model that was refractory to either treatment alone [174]. Thus, combined therapy may be more effective in treatment of diseased or elderly patients who demonstrate an impaired response to a single gene or protein treatment.

5.3. Ex vivo gene transfection to promote angiogenesis

Effects from heterogeneous in vivo delivery can be avoided by first genetically modifying ECs ex vivo and then injecting them into the targeted tissue. ECs can be modified to deliver a growth factor or to more efficiently integrate into host tissues and form vessels. VEGF-expressing cells have been shown to stimulate angiogenesis following implantation in animal models [177,179,180]. In addition, genetic approaches can be used to enhance the delivery of transplanted cells. Bcl-2 is an anti-apoptosis protein expressed from the bcl-2 gene, originally identified as the translocated locus in a B-cell leukemia (*Bcl*), and it is upregulated during angiogenesis. When ECs transfected to over express Bcl-2 were transplanted in mice, vascular density was increased over EC transplantation alone [181].

5.4. Transcription factors can promote angiogenic gene expression

While VEGF therapy has been used successfully, it may be advantageous to use hypoxia-inducible factor 1 α (HIF-1 α). HIF-1 α is a transcription factor that activates mediators of angiogenesis, including VEGF. Vincent et al. designed a constitutively active form of HIF-1 α by deleting the C-terminal portion and replacing it with the transactivation domain of herpes simplex virus VP16 protein [182]. This HIF-1 α /VP16 transcription factor retains the DNA binding of HIF-1 α , activates VEGF gene expression, and promotes an increase in capillary density and maximal blood flow over VEGF DNA injection in a rabbit model of hind limb ischemia.

5.5. Endothelial progenitor cells as agents of angiogenesis

Endothelial progenitor cells (EPCs) present in bone marrow are known to home to sites of neovascularization and incorporate into the developing microvasculature. Recent work has shown that EPCs can be genetically modified to further enhance their therapeutic function. EPCs transfected to express VEGF stimulate a greater improvement in blood flow and angiogenesis in animal models of ischemia than progenitor cells alone [183,184]. While they have a longer life than fully differentiated cells, EPCs isolated from adults have reduced telomerase activity and regenerative capacity relative to stem cells. EPCs isolated from bone marrow and transfected to express telomerase reverse transcriptase (TERT) were more resistant to apoptosis and drastically increased neovascularization in an animal model of limb ischemia [185].

5.6. The role of arteriogenesis in relieving ischemia

While therapies focusing on stimulating angiogenesis have shown promise, it is not clear that this increase in the number of capillaries alone will be sufficient for overcoming tissue ischemia [186]. Gene therapies can also be used to stimulate arteriogenesis, which is the process of adapting pre-existing collateral arterioles into a conductance artery. As with neovascularization, a multitude of genes or proteins could be used such as VEGF, FGF-1/2, etc. [187]. Recent gene work has shown that nonviral monocyte chemoattractant protein-1 (MCP-1) gene therapies increase arteriogenesis in a rabbit ischemic limb [188]. Results from clinical trials suggest that VEGF gene therapy may also stimulate arteriogenesis [189]. Combined delivery of genes for VEGF₁₆₅ and Ang-1 has also been shown to increase

arteriogenesis. Either growth factor alone increased capillary density in ischemic mouse myocardium, but only in combination was there an increase in the arteriole to capillary density ratio [190].

Less specifically, cell delivery also can enhance arteriogenesis. Studies have shown that monocytes injected intravenously into rabbits following femoral artery ligation are able to home to sites of arteriogenesis but do not significantly increase flow. When these monocytes were genetically modified to deliver granulocyte–monocyte colony-stimulating factor (GM-CSF), collateralization was significantly increased [191]. This approach exploited the “homing” nature of monocytes to target delivery of an arteriogenic factor. While this therapy shows promise, increasing the level of monocytes in a vessel can lead to atherogenesis. Beneficial therapies should be able to stimulate arteriogenesis without concomitant atherogenesis.

5.7. Summary

Numerous studies have shown that gene therapies have the potential to stimulate neovascularization. However, questions still remain as to the best gene, or combination of genes, to use for an efficacious response. In addition, techniques need to be developed to improve the efficiency and targeting of gene delivery. These methods need to result in controlled protein levels within the targeted tissue in a time-specific pattern. Tissue engineered vascular conduits may be able to incorporate cell-specific gene delivery in a more efficient and sustained manner.

6. Tissue engineered vascular conduits

Genetic approaches have been investigated for modifying vascular cells to improve development of tissue engineered vascular grafts (TEVGs) *ex vivo*. While tissue engineering has shown promise for the development of patient-specific vascular substitutes, the use of autologous cells is limited by the decreased proliferative and functional capacity of cells isolated from many in the potential patient population, including older, diabetic, and hypertensive patients [166]. To overcome limitations in older patients, Poh et al. transfected vascular cells isolated from these patients to express human telomerase reverse transcriptase (hTERT) [192]. hTERT is thought to improve the growth of somatic cells by restoring telomerase activity. ECs and SMCs transfected to express hTERT had an increased proliferative capacity, enabling production of engineered vessels *in vitro* in bioreactors exposed to pulsatile flow at venous shear rates. Without hTERT, TEVGs engineered with cells from these patients were fragile or failed to develop entirely.

While hTERT expression overcame the decreased proliferative capacity of the cells, matrix production and the resulting mechanical strength of the TEVGs were too low to be considered for clinical application. Genetic modification has also been used to improve the function and phenotype of SMCs in TEVGs. Cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) is a downstream element in the nitric-oxide-cGMP signaling pathway. Transfection of SMCs to express PKG resulted in increased contractile protein expression, which may enhance mechanical function in TEVGs [193]. These studies indicate that gene therapies can be used to enhance the formation and function of TEVGs.

7. Status of clinical trials

Recent clinical trials have investigated a number of innovative therapies for the treatment of cardiovascular disease and its sequelae. Although not all of the trials discussed below have utilized gene therapy, the transferability of protein, stem cell, and immunologic approaches to gene therapy make them relevant to future genetic applications.

7.1. Coronary angiogenesis

In 2000 many of the leading investigators in the field of coronary angiogenesis issued a consensus statement that noted outstanding problems and potential utility of cardiovascular gene therapy (Table 1). They concluded that protein therapy is closer to practical use than gene therapy at that time [194]. Sellke et al. have developed a heparin sepharose calcium chloride-alginate beads for implantation into the epicardium and sustained release of FGF-2 to myocardial regions that cannot be revascularized operatively. They demonstrated significant relief of angina and less late perfusion defects in the treatment versus placebo group [195]. Direct injection of FGF-1 into the myocardium was also well tolerated [196], but when injected along diseased left anterior descending coronary arteries that were being perfused by a left internal mammary artery bypass graft, there was no beneficial improvement in outcome reported [197]. The FIRST (FGF Initiating Revascularization Trial) study injected FGF-2 intravascularly into the coronary arteries. These investigators found reduced angina symptoms in the treatment group at 90 days, and that this benefit was most robust in patients with more severe angina class scores. However, these benefits were lost at 180 days due to improvement in the placebo group [198]. The VIVA trial (Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis) used a combination of intravenous and intracoronary administration of high and low VEGF concentrations (50 or 17 ng/kg min). Its initial results showed no difference in symptomatic or perfusion endpoints, but at 120 days, the high-dose VEGF group did lessen the angina score of patients receiving this therapy compared to placebo [199]. As discussed by Sellke and Ruel, the lack of overwhelming success by the FIRST and VIVA trials may be attributed to the lack of specificity in localizing these growth factors due to the choice of an intravascular route of delivery [186]; coronary vein cannulation may be able to improve this specificity [200].

Rosengart et al. have used intramyocardial injection of an adenoviral vector to deliver VEGF₁₂₁ to patients and reported improvement in angina symptoms and cardiac function, but this study was only a feasibility study without placebo controls [201]. Subsequent animal model data support further investigation of this approach [202,203]. In another uncontrolled study, Losordo et al. injected plasmid VEGF₁₆₅ DNA directly into the ventricular wall through a minimally invasive thoracotomy and found they could augment VEGF levels 2 times over pretreatment values at 2 weeks, but that these levels returned to baseline by 3 months. Again, all patients reported improvement in angina symptoms over time, and these patients also had documented collateralization and/or improved myocardial perfusion scans after therapy [204,205]. Similar results were reported by this group with intravascular delivery of the VEGF-2 gene [206,207], and this approach may have a durable benefit at 2 years [208]. The AGENT (angiogenic gene therapy trial) study has looked at the benefits of intracoronary delivery of adenoviral-FGF-4 in patients with chronic stable angina. Preliminary randomized results appear to demonstrate a benefit in treadmill tolerance, perfusion scanning, and angina [209–211]. Both the VEGF-2 and FGF-4 treatments are currently being investigated in larger randomized, placebo-controlled clinical trials.

Finally, as discussed earlier, cell transplantation or re-implantation is probably the simplest way to promote cardiovascular gene therapy.

The MAGIC trial showed the potential clinical benefits of delivering stem cells to patients after myocardial infarction (peripheral blood stem cells) including increased exercise capacity, myocardial perfusion, and systolic function. However, this trial was terminated because the treatment group had a greater than 60% prevalence of in-stent stenosis [99]. Thus, caution is indicated in the rapid accession of these therapies into human trials.

7.2. Gene therapy for claudication

Claudication is a clinical manifestation of peripheral artery disease that is usually caused by occlusion or significant stenosis in one segment of the infraaortic vasculature. Here, FGF-2 delivery has been shown to improve walking time in patients when compared to a placebo control, but repeat dosing did not add a benefit [212].

However, a VEGF₁₂₁ gene trial did not confer a benefit to similar patients [213]. Thus, the promise of this approach to clinical improvement for claudication is unclear.

7.3. Preventing IH

7.3.1. Vein graft IH—Edifoligide is an E2F transcription factor inhibitor that showed promise in limiting IH in various preclinical vein graft models. It was able to be efficiently transfected in harvested saphenous vein grafts at non-distending pressures prior to implantation and was found to limit vein graft occlusions, revisions, or critical stenosis in a single center randomized and controlled phase I/II trial [214]. Recently, it has been reported that its multi-center phase III trials (PREVENT III/IV) failed to demonstrate a beneficial effect on any of these outcomes [215,216].

7.3.2. In-stent restenosis—Clowes et al. have provided evidence that IH “melting” can be facilitated through antibody blockade of growth factor receptors essential for IH response. The efficacy of this approach was improved by targeting multiple receptors. However, the durability of this approach is uncertain because of a refractory IH response over time [217,218]. Clinically, a randomized phase II trial investigating the efficacy of a single dose of PDGF-B receptor antibody failed to limit IH in patients undergoing elective coronary artery stenting [219].

The HEALING-FIM (Healthy endothelial accelerated lining inhibits neointimal growth-first in man) study used anti-human CD34 antibody-coated stents to capture circulating EPCs and facilitate rapid endothelialization following coronary artery stenting. While the investigators reported 90% endothelialization within 1 h of stenting and decreased luminal stenosis in a preclinical study [220], they did not determine endothelialization rates at early time points nor did they demonstrate an improvement in luminal stenosis clinically (out to 6 months). However, they used an abbreviated course (30 days) of clopidogrel after stenting without adverse effects [105].

7.3.3. IH in AV fistulas—AV fistulas are the preferred access operation for hemodialysis patients. However, they are known to be prone to thrombotic and restenotic complications, and although secondary patency rates have improved with endovascular approaches, restenosis remains a problem for this patient population [221]. PREVENT V is a phase I/II trial designed to study safety and efficacy of Edifoligide on limiting IH in AV fistulas, and its preliminary data are not yet available. However, PREVENT III and IV had disappointing results in phase III trials. Also, the application of VEGF-D protein to anastomotic sites of AV fistulas using a collar device is being currently being investigated. It too does not have data available yet [222]. Both of these studies bring much anticipation because of the growing need for vascular access and cost of repeat interventions in Western society. Further protein studies like VEGF-D may lead to future studies that incorporate genetic approaches of these same (or similar) proteins in order to provide transgene expression.

7.4. Safety, the perception of safety, and a role for placebo control groups

7.4.1. Safety—There are two components necessary for the successful implementation of cardiovascular gene therapy. The first is the evidentiary demonstration of safety for the various iterations of these approaches, and the second is the perception of this safety by

clinicians, patients, and society as a whole. There seems to be ample evidence defining the safe application of cardiovascular gene therapy regardless of whether a viral or non-viral vector is utilized [223,224], but there remains theoretical concerns with the gene's themselves that require long-term surveillance of participating patients. For example, VEGF has been tumorigenic [225,226], and FGF has been associated with proliferative membranous nephropathy in mouse models [227–229]. Also, the induction of hypotension after delivery of these growth factors is a concern because VEGF can cause systemic hypotension and edema of the lower extremity clinically [230,231] and FGF plays an essential role in the prevention of hypertension [232]. Most of these concerns have not been reported in clinical trials to date.

7.4.2. Perception of safety—Clinician perspective seems to be formed by the penetrance of cardiovascular gene trials into their community along with adequate satisfaction of safety concerns [233]. Improved vector systems [234] may well increase the interest levels in this treatment modality. Further patient interest in gene therapy may spur clinician participation in these studies. Finally, open and transparent governmental oversight of gene therapy through the National Institute of Health's Recombinant DNA Advisory Committee (RAC) provides the opportunity for public awareness and safe implementation of gene transfer techniques.

7.4.3. Placebo effect must be addressed in an ethically acceptable manner—

There is a difference in kind and degree between an interventional or operative placebo and a medicinal placebo, and the risk/benefit ratios for these two kinds of placebo-controlled trials are difficult to compare to each other from a regulatory viewpoint. Arguments against the use of a placebo-arm in controlled trials ignore the costs and potential harm caused by operations that do not benefit the patient in the intended manner. Indeed, many of these cardiovascular gene trials seem to echo the initially favorable clinical responses seen in patients whose angina was treated by ligation of their internal mammary arteries; this approach was eventually debunked by Beecher [235], but demonstrates the necessity of placebo controls in trials of novel techniques such as cardiovascular gene therapy.

In fact, there is some broad agreement that placebo-controlled trials can be ethically acceptable when clinical equipoise exists [236,237]. Certainly, these cardiovascular gene and protein trials meet the requirement of clinical equipoise, and future studies should be encouraged to control for the placebo effect. A recent discussion of the appropriate methods for such approaches has been described elsewhere [238], but the rush to pursue these trials should be tempered by sufficient animal model studies [239]; this will enable a safeguard against treatment without evidence as occurred for many years with regard to myocardial revascularization [240,241].

7.5. The direction of future work

As our understanding of the genome has improved substantially in the last 10 years, we have also gained a better understanding of the inter-related nature of the genome and proteome. With this realization, much interest now resides in the field of proteomics for therapeutic and biomarker advances in cardiovascular diseases [242–244]. Undoubtedly, the currently modest clinical improvements described here will improve over time, but much of the innovation may reside in a more integrated understanding of the cardiovascular genome and proteome, particularly as these therapeutic advances relate to molecular signaling pathways [245]. Still, the promise of cardiovascular gene therapy resides in its potential to deliver sustained and/or controlled expression of desired proteins in the afflicted cell types or tissue beds in a specific manner in order to improve cellular homeostasis and regeneration. Ideally, future approaches will investigate combination therapies that both promote a functional

endothelium and concomitantly limit IH. These combinations may include the application of protein(s) (for immediate activity) to and genetic transfection (for sustained expression) of ischemic tissue beds or injured vessels. Further the selective genetic transfection of ECs (to promote growth, migration, angiogenesis) and transfection of SMCs with separate genes to limit these same responses may be achievable.

In conclusion, we should not be surprised or derailed by therapeutic or commercial failure because failure brings with it attendant scientific breakthroughs. Certainly the foundation for the utility of cardiovascular gene therapy is seen in a variety of approaches reviewed here; this foundation should provide for rapid progress in the coming years, where “the good road is a-waitin’.”—Bob Dylan [246].

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A **B**

Wave	SMC activity	Growth factor mediators	Time frame after injury	ECM Production +<+++
1	Proliferation	FGF-2	24-72 hours	+
2	Migration	PDGF, FGF-2, TGF- β	1-7 days	++
3	Proliferation	PDGF, TGF- β	7-14 days	++++
4	ECM Production	FGF-2, PDGF, TGF- β , Ang II	> 2 weeks	++++

Fig. 1.

(A) Angiogram illustrating a stenosis in an in situ femoro-popliteal vein bypass graft (left). Corresponding histology demonstrates the robust myointimal growth (right). Arrow denotes IH and near occlusion. (B) Table shows a table summary of the four waves of SMC response in a rat balloon angioplasty model [36].

Fig. 2.

This is a line diagram of the most common complications after vascular interventions.

Fig. 3.

The vascular wall. (A) Graphic representation of the cross section of a small muscular artery (e.g., renal or coronary artery). (B) Photomicrograph of histologic section containing a portion of an artery (A) and adjacent vein (V). Elastic membranes are stained black (internal elastic membrane of artery highlighted by arrow). Because it is exposed to higher pressures, the artery has a thicker wall that maintains an open, round lumen, even when blood is absent. Moreover, the elastin of the artery is more organized than in the corresponding vein. In contrast, the vein has a larger, but collapsed, lumen, and the elastin in its wall is diffusely distributed. (B, Courtesy of Mark Flomenbaum, M.D., Ph.D., Office of the Chief Medical Examiner, New York City) [18]. Reprinted with permission from Kumar [18].

Fig. 4.

This is a plot demonstrating an increase in SMC proliferation with sparsely populated overlying ECs that decreases with increased percent re-endothelialization in vivo using a canine carotid artery balloon angioplasty model [93]. Reprinted from [93], with permission from the Society for Vascular Surgery.

Fig. 5.

Exogenous delivery of FGF-1 promotes endothelialization of prosthetic graft (expanded polytetrafluoroethylene [ePTFE]). Untreated graft is on the left (x117), and FGF-1 treated graft is on the right (x486). Note the capillary in-growth, cellular coverage, and the central mitotic figure [98]. Reprinted from [98], with permission from the Society for Vascular Surgery.

FAK AND FRNK

Fig. 6.

The structure of FAK is on top and its C-terminal homologue, FRNK, is on the bottom. Moving from N-terminus to C-terminus in FAK (but not FRNK) are the FERM (protein 4.1, ezrin, radixin and moesin homology) domain, which houses the autophosphorylation sites and src binding sites, and the kinase domain. The rest of FAK is remarkably similar to FRNK, and it is currently unknown if FRNK's C-terminal region has phosphorylation sites that are activated by growth factor stimulation like FAK. (PR: proline-rich regions; FAT: focal adhesion targeting sequence.)

Fig. 7.

Fluorescent images of three-dimensional SMC invasion. This figure illustrates the successful infection of vascular smooth muscle cells (500 moi, 7d) through an in vitro 3-D co-culture model of SMC hyperplasia. Here, Adv-GFP-FRNK infected SMCs (left) invasion can be compared to an adenovirus control group (adv-GFP infected SMCs) (right) to quantify SMC invasion into the matrix under clinically relevant situations (i.e., various ECM proteins or growth factors) [156].

Table 1

Summary of the challenges and practical limitations of gene versus protein therapy for stimulating coronary angiogenesis issued by experts in the field at the bequest of the Angiogenesis Foundation and the Angiogenesis Research Center at the 72nd Meeting of the American Heart Association [194]

Modality	Problems	Consensus
Gene therapy	Regulable gene vectors are not available Side effect profile is incompletely understood Length and level gene expression are unpredictable May require complicated delivery modalities for transendocardial approaches	Only option for certain genes, like transcription factors (HIF-1 α) Regulation vectors with short duration of expression are highly desirable
Protein therapy	Limited tissue half-life may require sustained-release preparation May require complicated delivery modalities	Closer to practical use than gene therapy