Genetic targeting for cardiovascular therapeutics: are we near the summit or just beginning the climb?

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Genetic targeting for cardiovascular therapeutics: are we near the summit or just beginning the climb? Physiol Genomics 7: 79–94, 2001; 10.1152/physiolgenomics.00073.2001.—This article is based on an Experimental Biology symposium held in April 2001 and presents the current status of gene therapy for cardiovascular diseases in experimental studies and clinical trials. Evidence for the use of gene therapy to limit neointimal hyperplasia and confer myocardial protection was presented, and it was found that augmenting local nitric oxide (NO) production using gene transfer (GT) of NO synthase or interruption of cell cycle progression through a genetic transfer of cell cycle regulatory genes limited vascular smooth muscle hyperplasia in animal models and infra-inguinal bypass patients. The results of application of vascular endothelial growth factor (VEGF) GT strategies for therapeutic angiogenesis in critical limb and myocardial ischemia in pilot clinical trials was reviewed. In addition, experimental evidence was presented that genetic manipulation of peptide systems (i.e., the renin-angiotensin II system and the kallikrein-kinin system) was effective in the treatment of systemic cardiovascular diseases such as hypertension, heart failure, and renal failure. Although, as of yet, there are no well controlled human trials proving the clinical benefits of gene therapy for cardiovascular diseases, the data presented here in animal models and in human subjects show that genetic targeting is a promising and encouraging modality, not only for the treatment and long-term control of cardiovascular diseases, but for their prevention as well.

gene therapy; angiogenesis; vascular endothelial growth factor; myocardial and limb ischemia; neointimal hyperplasia
the nation’s population) suffer from some form of cardiovascular disease (25), making it the leading cause of morbidity and mortality nationwide. The economic impact of cardiovascular disease is staggering. Expenditures through the health care system and lost productivity due to disability cost the nation nearly $300 billion each year (25). The epidemic and activity due to disability cost the nation nearly $300 billion each year (25). The epidemic and activity due to disability cost the nation nearly $300 billion each year (25). The epidemic and activity due to disability cost the nation nearly $300 billion each year (25). The epidemic and activity due to disability cost the nation nearly $300 billion each year (25). The epidemic and activity due to disability cost the nation nearly $300 billion each year (25).

In patients with cardiovascular disease, hypertension and heart disease are the primary causes of death and hospitalizations. If left unabated, hypertension can precipitate heart failure, peripheral vascular disease, coronary artery disease, renal failure, stroke, and left ventricular hypertrophy (25, 49). Management of these diseases has involved pharmacological and/or surgical interventions that treat the symptoms rather than the underlying causes. Furthermore, many cardiovascular diseases are local problems, whereas pharmaceutical therapy is systemic in nature. Although clinical data have shown these treatment regimens to be effective therapies for disease management, treatment side effects and remanifestation of symptoms remain a major concern (10, 50, 73, 85). Furthermore, noncompliance spurred by the chronic nature of the therapy and associated side effects of all therapies underlies inadequacies and limitations of currently used therapeutic regimens. Such limitations have prompted an interest in the search for better therapeutic strategies to treat cardiovascular disease. The historic sequencing of the human genome coincident with a broader understanding of the genetic basis of cardiovascular disease and improvements in gene targeting technology has inspired active investigation into genetic treatment of cardiovascular diseases. Moreover, the notion of treating local disease with localized therapy has emerged as one of the great promises of gene therapy (38).

At the 2001 Experimental Biology conference, four speakers were invited to present their findings on the current status of genetic targeting of the cardiovascular system. There was an informative and stimulating presentation of both experimental studies and clinical trials on genetic therapeutics for localized treatment of diseases such as coronary artery disease and myocardial and limb ischemia, as well as treatment of systemic cardiovascular diseases such as hypertension. Victor J. Dzau, MD, reviewed his team’s success in using genetic therapy to limit neointimal hyperplasia and to confer myocardial protection. In brief, they showed that augmenting local nitric oxide (NO) production using the Sendai virus/liposome gene transfer (GT) of NO synthase or interruption of cell cycle progression through the genetic transfer of cell cycle regulatory genes limits vascular smooth muscle cell (VSMC) hyperplasia in experimental animal models. Furthermore, Dzau presented clinical data that show this approach is feasible, safe, and effective at reducing cellular proliferation in infra-inguinal bypass patients. Douglas W. Losordo, MD, highlighted the successful application of vascular endothelial growth factor (VEGF)

Gene Therapy to Prevent Neointimal Hyperplasia

Neointimal hyperplasia of bypass grafts results in the re-occlusion in ~50% of coronary artery bypass graft (CABG) patients after 10 years. The incidence of re-occlusion in patients receiving peripheral arterial bypass grafts is even more dismal, with 20% of infrainguinal grafts occluding in the first year. Failure tends to occur in a bimodal distribution, with early failures attributable to technical issues, and late failure due to accelerated neointimal hyperplasia. Neointimal hyperplasia continues to be the leading cause of failure of percutaneous transluminal coronary angioplasty (PTCA), with 30% of grafts re-occluding after 6 mo.

The development of a neointima is critical to the proper functioning of a bypass graft, because it limits the vessel’s distensibility and, therefore, protects it from the increased intraluminal pressure and shear stress when the graft is interposed into the arterial position. It is thought to occur in response to two major stimuli. The first is manipulation and ex vivo ischemia at the time of surgery. The second is remodeling or “arterialization” of the vein graft which occurs in response to the increased intraluminal pressure and shear stress that is exerted on the venous wall by the high-pressure arterial environment.

Uncontrolled hyperplasia of the neointima, however, is detrimental to the long-term viability of the graft and is attributable to smooth muscle cell (SMC) hyperplasia. One approach to limiting SMC hyperplasia has been by augmenting local levels of NO by transfection of the cells in the vessel wall. NO 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 coding for endothelial cell NO synthase (eNOS), using the Sendai virus/liposome GT technique, has been investigated as a potential paracrine strategy to block neointimal disease. eNOS cDNA driven by a β-actin promoter and cytomegalovirus (CMV) enhancer was transfected into the VSMC of rat carotid arteries after balloon injury. This model showed no significant regrowth of endothelial cells within 2–3 wk after balloon injury. There was expression of the transgene in the vessel wall in these animals, along with improved vasmotor reactivity and a 70% inhibition of neointima formation (129).

An alternate approach to limiting the SMC hyperplasia has been by targeting genes involved in cell cycle progression. This strategy targets cell cycle activation as a result of uncoupling of a regulatory complex that involves cyclin A, Cdk2, and E2F. The transcription factor E2F transactivates a variety of genes involved in cell cycle regulation, including those encoding c-Myc and proliferating cell nuclear antigen (PCNA). This strategy has gained favor over the last 5 years, with several groups employing direct adenoviral transduction of the vessel wall with genes designed to block cell cycle progression such as p53 (8), RB2/p130 (27), ribozymes against c-myb mRNA (65), Fas ligand delivery (64), antisense cyclin G1 gene delivery (142), overexpression of human p21 (19), ras transdominant negative mutants, which interfere with ras function (46), and adenoviruses encoding a nonphosphorylatable, constitutively active form of Rb (20). The primary limitation of these approaches is that adenoviral transduction of the vessel wall itself induces a dense inflammatory response that may further contribute to neointimal hyperplasia (84). The G1/S checkpoint in cell cycle where E2F acts was, therefore, targeted as an attractive site for blocking cell cycle progression. We hypothesized that inactivation of E2F, using a decoy strategy, would prevent transactivation of E2F-responsive cell cycle genes. We observed that smooth muscle proliferation was inhibited (79) and that rabbit internal jugular vein grafts interposed into the carotid artery did not develop neointimal hyperplasia and did not develop atherosclerosis even in the presence of an atherogenic diet (68). To evaluate the safety and feasibility of E2F decoy in human venous bypass grafts, we performed the phase II randomized, controlled PRE-VENT trial of infra-inguinal bypass in patients at high risk for graft failure. Forty-one age- and sex-matched patients were prospectively randomized to one of three groups: those treated with E2F decoy, untreated, and those treated with scrambled oligodeoxynucleotide (ODN) (69). We observed a 74% reduction in cell proliferation in the treated group and a statistically significant decrease in time to primary failure (graft occlusion, need for revision, or >75% graft occlusion) in the treated group at 53 wk. There were no perioperative deaths and an even distribution of postoperative complications across the three groups (P = 0.40). Further large-scale clinical trials are needed to further validate these results.

**Gene Therapy for Myocardial Protection**

Myocardial ischemia associated with coronary artery disease is the leading cause of morbidity and mortality in the Western world (16). Recent advances in understanding the molecular mechanisms underlying the pathophysiology of ischemic heart disease coupled to improvements in cardiac GT offer the opportunity for design for gene-based therapies for both protection and rescue of the myocardium. It is now established that the injurious processes initiated by coronary ischemic events may, paradoxically, be exacerbated by reperfusion, a phenomenon referred to as ischemia/reperfusion (I/R) injury (138) and clinically manifested in patients suffering from acute coronary syndromes (95, 135, 137). Reoxygenation of the ischemic myocardium results in the formation of reactive oxygen species (ROS), leading to activation of the inflammatory cascade, myocyte injury, and endothelial dysfunction (101, 128). The accumulation of ROS during reperfusion may eventually deplete the buffering capabilities of endogenous antioxidant systems, thereby exacerbating the deleterious effects of these reactive species (128). In time, repeated I/R injury leads to progressive impairment of contractile function, culminating with hemodynamic failure (103).

A therapeutic approach aimed at potentiating endogenous antioxidant reserves could, in principle, be used as a preventive measure against I/R-induced oxidative myocardial damage. Ideally, the therapeutic strategy should confer long-term protection without the need for repeated administration of the therapeutic agent. In vivo GT of a suitable therapeutic gene(s) using a delivery method that would enable efficient and stable, sustained expression of the transgene would fulfill this strategy. Recombinant adeno-associated virus (rAAV) vectors provide a potential means to achieve long-term and stable expression of transduced genes in the myocardium (52, 107). These vectors have tropism for a variety of myocardial cell types and are poorly immunogenic, thus circumventing activation of the host immune response (24, 78). In addition, rAAV is capable of integration into the host genome, thereby providing stable expression of the transgene and, potentially, indefinite production of the therapeutic protein (78).

As a means of evaluating the feasibility of antioxidant enzyme GT as long-term first line of defense against I/R-induced oxidative injury, we used rAAV vector for intramyocardial delivery of hemeoxygenase 1 (HO-1) gene in a rat model of myocardial I/R injury induced by ligation and release of left anterior descending (LAD) coronary artery (74). Our findings indicate that rAAV-mediated delivery of HO-1 gene to the left ventricular risk area results in almost complete prevention of infarct in treated animals compared with untreated animals (≈80% reduction in infarct size), following ischemia and reperfusion at 8 wk after GT. The reduction in myocardial injury was paralleled by
sustained overexpression of the transgene and was accompanied by a decrease in oxidative stress. Comparable findings were also observed with extracellular superoxide dismutase (ecSOD) GT (2). These studies illustrate the potential of this approach as prophylactic therapy for myocardial protection. It is conceivable that the increase in basal prooxidant scavenging activity imparted by constitutive overexpression of antioxidant enzymes confers a degree of cytoprotection against future I/R episodes akin to preconditioning. Others have also demonstrated the feasibility of cardioprotective therapy using alternative targets of genetic manipulation. Using a decoy oligonucleotide against nuclear factor-κB (NF-κB), a pro-inflammatory transcription factor activated by oxidative stress (59), Morishita et al. (80) showed significant reduction of myocardial infarct in rats after coronary artery ligation. Others have demonstrated effective protection I/R injury by overexpressing stress-induced heat shock proteins such as HSP-70 (86, 106), survival genes such as Bcl-2 (23) and protein kinase B (76), as well as immunosuppressive cytokines (15, 91). These therapeutic targets are part of a repertoire of genes that are known to be induced by oxidative stress (101).

Gene therapy also offers promise as a therapeutic tool for treatment of the ischemic myocardium. Several studies have documented improved myocardial perfusion and functional recovery following GT of angiogenic factors such as VEGF (66), fibroblast growth factor (FGF) (42), and hepatocyte growth factor (HGF) (117). Recently, Su et al. (105) showed that intramyocardial delivery of VEGF by rAAV in a mouse model of coronary ischemia led to sustained neovascularization of the ischemic region, indicating that the rAAV vector is a tool for treatment of the ischemic myocardium. Rescue of contractile function in the failing myocardium is another major goal of myocardial gene therapy. The failing myocardium is characterized by alterations in calcium handling, decreased myofilament sensitivity and adrenergic receptor down-regulation and desensitization (28). Adenovirus-mediated GT of the sarcoplasmic reticulum calcium ATPase SERCA2a or antisense inhibition of phospholamban has been shown to improve contractility in aortic-banded rats (77) and in isolated ventricular myocytes (33), respectively. Similarly, adenovirus-mediated intracoronary delivery of the β2-adrenergic receptor gene led to improved contractility and hemodynamic function in rabbits (72), and cardiac overexpression of adenylate cyclase improved ventricular function in cardiomyopathic mice (100).

As further progress is made in understanding the pathophysiology of ischemic heart disease and in developing methods for safe and efficient gene delivery to the heart, a time may be envisaged when heart disease may be prevented or cured by genetic manipulation. Perhaps the ideal gene therapy for myocardial protection is one that would incorporate genetic targets for prevention (by safeguarding the myocardium against injury) and rescue from injury, if needed. The development of tissue-specific and regulatable vector systems responsive to pathophysiological stimuli would permit spatial and temporal control of transgene expression, such that the therapeutic protein would be produced only where and when needed. Such a degree of control over transgene expression would not only improve the specificity of myocardial gene therapy but could also avert potential cytotoxic effects associated with constitutive expression of the therapeutic protein.

**VEGF Gene Transfer for Therapeutic Angiogenesis in Cardiovascular Diseases**

Substantial research has focused on the administration of angiogenic growth factors (either as recombinant protein or by GT) to promote the development of supplemental collateral blood vessels that will constitute endogenous bypass conduits around occluded native arteries. This strategy is termed “therapeutic angiogenesis,” particularly in a significant population of patients with CLI or chronic myocardial ischemia who are not optimal candidates for surgical or percutaneous revascularization.

The therapeutic and pathological implications of neovascularization due to angiogenic growth factors were identified in the pioneering work of Folkman almost 30 years ago (39). Angiogenesis and vasculogenesis comprise mechanisms responsible for the development of the vascular system in the embryo (41). Until recently, vasculogenesis was considered restricted to embryonic development, whereas angiogenesis, recognized to occur in the embryo as well, was considered to be solely responsible for neovascularization in the adult. The demonstration that bone marrow-derived endothelial progenitor cells (EPCs) are increased in number in response to tissue ischemia (109), home to and incorporate into foci of neovascularization in adult animals (7), and can augment collateral development following ex vivo expansion and transplantation (51) suggests that neovascularization in the adult is not restricted to angiogenesis but involves “postnatal vasculogenesis.” In addition, a proportion of newly recognized medium-sized arteries may develop as a result of “arteriogenesis” or in situ proliferation of preexisting arteriolar connections into larger collateral vessels (4) by remodeling. It is unknown whether such remodeling occurs as a direct result of growth factor modulation or as a flow-mediated maturation of these collateral conduits.

Although many cytokines have angiogenic activity, the best studied, both in animal models and clinical trials, are VEGF and FGF. This review will discuss VEGF GT strategies for therapeutic angiogenesis in CLI and myocardial ischemia.

GT is the introduction of genetic material into somatic cells of an organism with the aim of achieving high levels of sustained gene expression without provoking adverse host reactions. Ischemic muscle represents a promising target for GT. Striated and cardiac muscles have been shown to take up and express naked plasmid DNA as well as transgenes incorporated into viral vectors. Moreover, previous studies have shown...
that the transfection efficiency of intramuscular GT is augmented more than fivefold when the injected muscle is ischemic (110, 116). Viral vectors may enhance transfection efficiency and yield higher levels of gene expression. However, in vitro (111) and in vivo (62) models have demonstrated that low-efficiency, but site-specific transfection (<1% of cells) with a gene (naked plasmid DNA) encoding for a secreted protein (e.g., VEGF) may overcome the handicap of inefficient transfection by secreting adequate protein to achieve local levels with physiologically meaningful biological effects. Thus GT of a secreted protein may achieve therapeutic effects not realized by transfection with genes encoding for proteins that remain intracellular (e.g., bFGF). Furthermore, unlike certain viral vectors, plasmid DNA does not induce inflammation.

**VEGF Gene Transfer in Critical Limb Ischemia**

The consensus statement of the European Working Group on Critical Limb Ischemia (36) states that no medical treatment has been shown to alter the natural history of CLI; patients have quality-of-life indices similar to those with terminal stages of malignancy, and amputation is often chosen as first-line therapy. Consequently, the need for alternative treatment strategies in patients with CLI is compelling.

Initial preclinical studies established that the angiogenic activity of VEGF is sufficiently potent to achieve augmentation of angiographically visible collateral vessels and histologically identifiable capillaries in rabbits with severe, unilateral hindlimb ischemia (96, 112). Subsequently, angiographic and histological evidence of angiogenesis was demonstrated following intra-arterial GT of naked plasmid DNA encoding for VEGF (phVEGF165) in humans (48). However, intra-arterial delivery has several inherent limitations that undermine successful GT for CLI. In the case of naked DNA, i.e., DNA unassociated with viral or other adjuvant vectors, cellular uptake is virtually nil when the transgene is directly injected into the arterial lumen, presumably due to prompt degradation by circulating nucleases. In addition, the diffuse distribution of neointimal thickening and/or extensive calcifying nucleases may limit GT to the SMCs of the arterial media (37). Preclinical studies subsequently established meaningful biological outcomes following phVEGF165 GT by direct injection into skeletal muscle of ischemic rabbit hindlimbs (97, 116) as evidenced by increased hindlimb BP ratio, increased Doppler-derived iliac flow, enhanced neovascularity by angiography, and increased capillary density at necropsy.

Successful VEGF GT was suggested in patients with CLI (11) utilizing intramuscular injection of 4,000 µg phVEGF165 by demonstrating regression of rest pain and/or improved limb integrity, increased pain-free walking time and ankle-brachial index, newly visible collateral vessels by digital subtraction angiography, and qualitative evidence of improved distal flow by magnetic resonance imaging. Subsequent clinical trials with phVEGF165 have utilized randomized (blinded) intramuscular injections in 55 patients with ischemic rest pain (n = 14) or ischemic ulcers (n = 41). Evidence of clinical improvement was observed in 13/14 (72%) patients with rest pain alone and 26/41 (63%) patients with ischemic ulcers over a follow-up period of 4–36 mo. Overall, a favorable clinical outcome was achieved in 65.5%; rest pain and age <50 yr were significant predictors of a favorable clinical outcome. Complications in these patients have been limited to lower-extremity edema that developed in approximately one-third of patients (12). A similar treatment strategy was used in 11 patients with Buerger’s disease presenting with CLI, 9 of which were successfully treated (47). These patients had resolution of nocturnal rest pain and healing of foot and/or leg ulcers. The ankle-brachial index increased by greater than 0.1 and newly formed collateral vessels were seen on magnetic resonance arteriography (MRA) and serial contrast angiography.

Based on preclinical studies demonstrating that VEGF-2 could also promote angiogenesis in a rabbit hindlimb ischemia model (133), randomized, double-blinded, placebo-controlled, dose-escalating trials have commenced to investigate the therapeutic potential of VEGF-2 GT in patients with CLI.

**VEGF Gene Transfer in Myocardial Ischemia**

For the purposes of myocardial angiogenesis, angiogenic cytokines have been administered via a wide variety of routes that include intravenous (44), intracoronary (138), transepipcardial at time of bypass surgery (98) or via thoracotomy (99), intra-pericardial (70) or peri-adventitial at time of bypass surgery, and most recently transendocardial by catheter (120).

Direct myocardial GT of phVEGF165 (114, 122) or VEGF-2 (124) via a minimally invasive chest wall incision in a swine model of chronic myocardial ischemia resulted in enhanced collateral vessel filling and improved perfusion to ischemic myocardium by colored microspheres. Intramyocardial injection of adenovirus encoding VEGF121 (58, 66) via thoracotomy in a porcine ischemia model improved collateral perfusion and function. Intracoronary adenoviral gene delivery produced much lower gene and VEGF levels in the myocardium with poor localization (58). Pericardial delivery of adenovirus encoding VEGF165 in a dog model did not increase collateral flow (57).

Utilizing a previously described navigation system and catheter mapping technology (NOGA) integrated with an injection catheter (Biosense-Webster, Warren, NJ) to deliver plasmid DNA encoding a reporter gene to the myocardium (121), preliminary preclinical studies in swine established that percutaneous myocardial GT could be successfully achieved in normal and ischemic myocardium in a relatively site-specific fashion without significant morbidity or mortality. Similar findings were demonstrated by a study utilizing adenoviral-assisted GT of a reporter gene (54). Safe and effective GT was also demonstrated in studies utilizing catheter-based delivery of naked plasmid DNA encod-
ing for VEGF-1 and VEGF-2 (27), as evidenced by reduced ischemia on NOGA mapping.

Published studies of VEGF GT for therapeutic angiogenesis in human subjects have thus far been limited to phase I dose-escalating, non-randomized trials involving naked plasmid DNA and adenoviral vectors. Patients in these trials generally have severe angina refractory to medical therapy, demonstrate ongoing myocardial ischemia, and are unsuitable for conventional revascularization.

A phase I, dose-escalating, open-label clinical study of myocardial GT of phVEGF165 as sole therapy (i.e., without PTCA or CABG surgery) for 30 “no-option” patients (63, 108). VEGF was administered by direct injection via a limited anterior thoracotomy under guidance of continuous transesophageal echocardiographic monitoring (35) and resulted in symptomatic improvement, increased in exercise time and objective evidence of reduced ischemia on CT(SPECT)-sestamibi myocardial perfusion scanning. Importantly, an improvement in resting defects was seen post-GT, suggesting that these preexisting resting defects constitute foci of hibernating viable myocardium (30, 102, 132), which have improved contractile activity as a result of therapeutic neovascularization. This observation was supported by the findings of electromechanical mapping (119); resting perfusion defects on the SPECT images corresponded to foci of ischemia (reduced wall motion with preserved viability) on the endocardial maps that showed significant improvement in wall motion abnormalities post-GT.

Similar favorable experience has been realized in an open-label, dose-escalating, multicenter clinical trial of VEGF-2 plasmid DNA GT in 30 patients with refractory class III or IV angina. Significant symptomatic improvement occurred in 25/29 (86%) patients who improved by two or more angina classes and increased their mean duration of exercise by more than 2 min (J. Isner, unpublished data) at 12 mo post-GT.

The only other reported study of direct myocardial VEGF GT was with adenovirus-assisted VEGF121 injection to patients undergoing bypass graft surgery (n = 15) and as sole therapy via mini-thoracotomy (n = 6). Symptoms and exercise duration improved in both bypass surgery and sole therapy groups, but stress-induced nuclear perfusion images remained unchanged. The data in this study is consistent with the concept that adenovirus VEGF121 appears to be well tolerated in patients with advanced coronary disease.

A single-blinded pilot study of percutaneous, catheter-based VEGF-2 DNA GT vs. a control/sham procedure, guided by the NOGA mapping system, was then performed in six patients with non-revascularizable symptomatic myocardial ischemia (120). VEGF-2-transfected patients reported a significant reduction in weekly anginal episodes and nitrate tablet consumption at 12 mo post-GT. In contrast, although the blinded patients randomized to the control group reported an initial reduction in these parameters, this change clinical profile was not sustained past 30 days, suggesting that the continued reduction in angina in the VEGF-2-treated group was not a placebo effect. The symptomatic improvement seen in the active treatment group was again accompanied by objective evidence of improved myocardial perfusion by both SPECT-sestamibi perfusion scanning and electromechanical mapping (120) (Fig. 1). Although the clinical findings of this pilot trial concerning efficacy are similarly encouraging, the number of patients and the single-blinded design preclude firm conclusions in this regard. Consequently, a multi-center randomized, double-blind, placebo-controlled trial of catheter-based VEGF-2 GT is underway that has thus far enrolled 19 patients. There have been no complications associated with a total of 150 injections among the 25 patients given either VEGF-2 or placebo in these two studies.

This preliminary experience thus suggests that it is feasible to replace currently employed operative approaches with minimally invasive techniques for applications of cardiovascular gene therapy designed to target myocardial function and perfusion. Such an approach may have at least three advantages compared to an operative approach. First, it potentially allows more selective delivery of the transgene to targeted ischemic zones, including sites that are less accessible by a mini-thoracotomy. Second, the catheter-based approach, because it obviates the need for general anesthesia and operative dissection through adhesions related to placement of previous bypass conduits, facilitates placebo-controlled, double-blind testing of myocardial GT. Third, the intervention can be performed as an out-patient procedure and repeated if necessary.

Potential safety concerns with VEGF. Many angiogenic factors are known to be involved in tumor growth secondary to enhancing angiogenesis. Hence, in theory, angiogenic growth factors may lead to development of tumors, and these may be too small to be recognized. Even so, there are neither in vitro nor in vivo data to suggest that VEGF increases the risk of neoplastic growth and/or metastases, although longer-term follow-up will be required to address this issue in clinical trials. Nevertheless, one must be vigilant about the possibility of cancer in patients treated with these angiogenic growth factors.

It is theoretically possible that VEGF may exacerbate proliferative and/or hemorrhagic retinopathy in patients with diabetes, in view of the high VEGF levels demonstrated in the ocular fluid of patients with active proliferative retinopathy leading to loss of vision (3). To date, this adverse effect of therapeutic angiogenesis has not been observed in more than 100 patients (½ with diabetes and/or remote retinopathy) treated with local delivery of naked plasmid VEGF-1 or VEGF-2 DNA at our institution with up to 4-yr follow-up. There was no effect on the visual acuity or fundoscopic findings as evidenced by serial fundoscopic examinations pre- and post-GT by an independent group of retinal specialists (123).

Experiments in transgenic mice engineered to over-express VEGF ± angiopoietin have been demonstrated...
lethal permeability-enhancing effects of VEGF (113). However, even though VEGF has been reported to cause local edema which manifest as pedal edema in patients treated with VEGF for CLI, it responds well to treatment with diuretics (12).

Another concern stems from the recent demonstration that inhibitors of angiogenesis tested in an apolipoprotein E-deficient mouse model of atherosclerosis inhibited plaque growth and intimal neovascularization (81) and that VEGF administration accelerated aortic plaque formation in the same model (117). However, data available from four separate animal studies (5, 6, 126, 127) and two clinical studies of human subjects (56, 125) fail to support the notion that accelerated atherosclerosis is a likely consequence of administering angiogenic cytokines. The outcome, in fact, is quite the opposite, in that administration of VEGF led to a statistically significant reduction in intimal thickening due to accelerated re-endothelialization, thereby refuting the notion that acceleration of atherosclerosis will be a consequence of VEGF-induced stimulation of angiogenesis.

Fig. 1. NOGA left ventricular EMM performed in 64-yr-old male. NOGA images in left anterior oblique (LAO) projection pre-gene transfer (pre-GT) show UpV and LLS maps; red zone, depicting abnormal wall motion, on LLS map (top right) together with preserved viability (purple/pink/blue/green) on UpV map (top left) constitute focus of electromechanical uncoupling that suggests ischemic or hibernating myocardium (arrow) in anterolateral wall. UpV and LLS maps in same projection 90 days post-GT (bottom left and right, respectively) disclose complete resolution of anterolateral ischemic zone (2.3 cm² pre-GT vs. 0 cm² post-GT). Circular brown icons in top panels represent sites of phVEGF-2 injection. Vertical and horizontal axes (X, Y, and Z) are presented by white lines. Red line represents long axis through the apex. LLS, linear local shortening; UpV, unipolar; and EMM, electromechanical mapping.
Conclusions. The current clinical strategies employed for CLI and chronic myocardial ischemia constitute an extrapolation from initial applications of GT to animal models with limb ischemia utilizing the 165-amino acid isoform of the VEGF-1 gene. These results, however, likely have generic implications for strategies of therapeutic neovascularization using alternative candidate genes, vectors, and delivery strategies; all of these are being actively studied in ongoing clinical trials. Furthermore, the relative merits of GT vs. recombinant protein administration remain to be clarified.

It is clear that site-specific VEGF GT can be used to achieve physiologically meaningful therapeutic modulation of vascular disorders and specifically that intramuscular injection of naked plasmid DNA achieves constitutive overexpression of VEGF sufficient to induce therapeutic angiogenesis in selected patients with CLI. Furthermore, at this early stage of clinical trials into myocardial gene therapy, it has been shown that direct myocardial GT utilizing different doses of naked plasmid DNA encoding for VEGF_{165} and VEGF-2 can be performed safely, and this approach augments myocardial perfusion. Ongoing clinical studies will determine the potential for neovascularization gene therapy to be performed by nonsurgical, catheter-based delivery, although early results are encouraging from a therapeutic standpoint.

For the most part, clinical studies of therapeutic angiogenesis have been restricted to patients with myocardial or limb ischemia who have no other options. Although this is the group to target in the near future, it is not difficult to foresee a time when the sizeable population of patients who undergo bypass surgery, but are not optimal candidates for that procedure, may be eligible for therapeutic angiogenesis, which might be performed at an earlier stage of disease and thus provide a greater possibility of a successful outcome.

Kallikrein Gene Therapy for Cardiac and Renal Diseases

Tissue kallikrein cleaves low-molecular-weight kininogen substrate to produce the vasodilator kinin peptide (13). Kinin is then cleaved by kininase I or II. Intact kinins bind to the bradykinin (BK) B_2 receptor, whereas kinin’s metabolites of kininase I, such as des-Arg^{5}-BK or des-Arg^{10}-Lys-BK, bind to the BK B_1 receptor (94). Binding of kinins to their respective receptors activates second messengers in target tissues and triggers a wide spectrum of biological effects such as vasodilation, vasoconstriction, and inhibition or stimulation of cell growth (13). The kallikrein-kinin system can be blocked at several steps. The activity and function of tissue kallikrein can be inhibited or regulated by kallistatin, a novel serine proteinase inhibitor (18, 141). The B_2 receptor can be blocked by the specific B_2 receptor antagonist, icatibant (Hoe-140), and the B_1 receptor can be blocked by des-Arg^{5}-[Leu]^{8}-BK, a specific B_1 receptor antagonist (67, 93). The vasodilatory action of the kallikrein-kinin system counterbalances the vasoconstrictive action of the RAS. These two systems are linked by angiotensin-converting enzyme (ACE), a dipeptidase, which is kinase II. Therefore, ACE has dual functions: it not only converts angiotensin I to vasoconstrictor peptide angiotensin II (ANG II), but also degrades the vasodilator kinin to release a dipeptide fragment, Phe-Arg, from the carboxyl end of the kinin peptide. The beneficial effects of ACE inhibition in hypertension and cardiovascular dysfunction could also be attributed to kinin accumulation, as icatibant can partially abolish these effects (60, 61, 71). Using somatic GT approaches to achieve a continuous supply of kallikrein/kinin, we have demonstrated that tissue kallikrein plays a protective role in cardiovascular remodeling and renal damage mediated via NO-cGMP and/or PGE-cAMP signaling pathways (Agata J, Chao L, and Chao J, unpublished observations; 21, 22, 31, 82, 83, 134, 136, 139, 140). Moreover, transgenic mice overexpressing the genes encoding tissue kallikrein or B_2 receptor gene in mice causes salt-sensitive hypertension and a dilated, failing cardiomyopathy (34, 75, 104, 130, 131). Taken together, these findings indicate that the kallikrein-kinin system plays an important role in cardiac function.

Kallikrein gene delivery in cardiac protection. The role of the kallikrein-kinin system in cardiac function was investigated using hypertensive animals induced by pressure overload or volume overload and in normotensive animals with ischemic heart disease. To achieve this goal, human tissue kallikrein cDNA was placed under the control of CMV promoter/enhancer. Expression of the human tissue kallikrein gene after transfection of the plasmid DNA into HEK-293 cells was identified in culture medium by enzyme-linked immunosorbent assay (ELISA). The plasmid DNA was then introduced into replication-deficient adenovirus. Expression of human tissue kallikrein mRNA in rats after adenovirus-mediated kallikrein gene delivery was detected in heart, kidney, and aorta by RT-PCR followed by Southern blot analysis. In addition, immunoreactive human tissue kallikrein levels were measured in the circulation and urine of rats receiving the human kallikrein gene but not in control rats receiving the luciferase gene (21, 22, 31, 134, 136, 140).

ANG II formation in the heart increases after myocardial infarction. Elevation of ANG II levels causes cardiac hypertrophy, fibrosis, and apoptosis and leads to cardiac remodeling and heart failure. ACE inhibition leads to reduced ANG II formation and also increased kinin levels. Similar to ACE inhibitors, delivery of the kallikrein gene results in a continuous increase of kallikrein-kinin that could counteract the action of ANG II. Consequently, kallikrein-kinin can reduce cardiac hypertrophy, fibrosis, and apoptosis and provide protective effects in cardiac remodeling and heart failure. Our studies indicate that adenovirus-mediated kallikrein gene suppresses cardiac remodeling in chronic heart failure after myocardial infarction.
The protective effects of kallikrein GT include: 1) improved cardiac function and cardiac reserve in response to stress, 2) reduced cardiac hypertrophy, fibrosis, and left ventricular enlargement, 3) improved endothelial function by increased blood flow and decreased vascular resistance, 4) increased capillary density in the heart, 5) reduced oxygen consumption, and 6) reduced myocardial apoptosis. Kallikrein GT increases cardiac kinin, NO, cGMP, and Akt/protein kinase B (Akt/PKB) levels. These results indicate that kallikrein gene delivery delays the progression of heart failure and improves cardiac reserve through enhancement of endothelial function. Activation of kinin-Akt-NO signaling pathway leads to inhibition of myocardial apoptosis and cardiac remodeling by reduction of cardiomyocyte size, cardiac fibrosis and increased capillary density (Fig. 2).

The cardioprotective effect of kallikrein GT in animal models is summarized in Table 1. A single injection of the human tissue kallikrein gene in adenoviral vector or naked DNA prolongs BP reduction for weeks in hypertensive rats such as spontaneously hypertensive rats (SHR), Dahl-salt-sensitive (Dahl-SS), deoxy-corticosterone-salt, 2-kidney 1-clipped (2K1C), and 5/6 nephrectomy rats (21, 22, 31, 134, 136). Kallikrein gene delivery also reduces aortic thickening, cardiac hypertrophy, and fibrosis in these hypertensive rats and in normotensive animals subjected to gentamycin-induced nephropathy or myocardial infarction-induced chronic heart failure (Agata et al., unpublished observations; 21, 22, 31, 82, 83, 134, 136, 139, 140). In acute myocardial I/R and chronic heart failure, kallikrein gene delivery reduces cardiac apoptosis, and the effect is abolished by icatibant. Kallikrein GT increases capillary density in the heart in SHR and in normotensive rats with chronic heart failure. These results indicate that a continuous supply of kallikrein-kinin from GT attenuates cardiac remodeling in both hypertensive animals and normotensive animals. In addition, these results suggest a direct role of the kallikrein-kinin system in cardiac protection independent of BP regulation.

Kallikrein gene delivery in renal protection. Adenovirus-mediated kallikrein GT also protects against renal injury. Table 2 summarizes the protective effect of kallikrein gene delivery in the kidney. Kallikrein GT enhanced renal function by increasing glomerular filtration rate and renal blood flow in hypertensive (including Dahl-SS, 2K1C, 5/6 nephrectomy) and normotensive (gentamycin-induced nephropathy) rats (21, 22, 31, 82, 134, 136). Furthermore, kallikrein gene delivery also reduced salt-, pressure-, or drug-induced renal damage in these animal models. Previous reports by other investigators showed that kallikrein protein therapy via the minipump reduced renal damage without affecting the BP of Dahl-SS rats fed with a high-salt diet (118). Taken together, these results indicate that kallikrein gene delivery could have a direct action in renal protection independent of its BP-lowering effect. Moreover, we have shown that kallikrein gene delivery not only protects against salt-induced renal damages but also could reverse these damages in Dahl-SS rats (21, 22). Adenovirus-mediated kallikrein gene delivery is also effective in reversing salt-induced cardiac hypertrophy in Dahl-SS rats (22). The beneficial effects of kallikrein gene delivery in attenuating salt- and drug-induced renal injury include: 1) increased renal blood flow and glomerular filtration rate; 2) attenuation of glomerular sclerosis, tubular damage, loss of brush border, and protein cast formation; 3) in-

Table 1. Protective effects of kallikrein gene delivery in the heart

<table>
<thead>
<tr>
<th>SHR</th>
<th>Dahl-SS</th>
<th>2K1C</th>
<th>5/6 Nephrectomy</th>
<th>Gentamycin-induced nephropathy</th>
<th>Myocardial I/R</th>
<th>Chronic heart failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Pressure</td>
<td>Cardiac Hypertrophy</td>
<td>Cardiac Fibrosis</td>
<td>Apoptosis</td>
<td>Infarct Size</td>
<td>Capillary Density</td>
<td></td>
</tr>
<tr>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

SHR, spontaneously hypertensive rat; Dahl-SS, Dahl salt-sensitive rat; 2K1C, two-kidney one-clip rat; I/R, ischemia/reperfusion; ND, not determined. Arrows indicate increase (↑), decrease (↓), and no change (↔).
hibition of renal cell proliferation; and 4) reduced blood urea nitrogen and urinary protein and albumin levels.

Potential mechanisms of kallikrein gene delivery in cardiac and renal protection. It has been well established that binding of kinin to its receptor activates NO-cGMP and/or PGE-cAMP signaling pathways. Systemic or local delivery of the human tissue kallikrein gene results in expression of human kallikrein in rats and thus increased kinin levels. Binding of kinin to its receptor activates the B2 receptor and leads to increased cardiac and urinary (or renal) AMP, NO, and cGMP levels in rats after kallikrein gene delivery (Agata et al., unpublished observations; 21, 22, 31, 82, 83, 134, 136, 139, 140). In addition, kallikrein GT results in an increase in the phosphorylated form of Akt/PKB (without changes in total Akt kinase) and a decrease in superoxide production and collagen accumulation in heart. The phosphorylated Akt may inhibit apoptosis by phosphorylating the Bad component of the Bad/Bcl-Xl complex to enable cell survival. Another possibility for attenuation of apoptosis by kallikrein gene delivery could be due to increased NO-cGMP formation that can prevent the degradation of the pro-survival members of the bcl-2 family and the release of cytochrome c from mitochondria and inhibition of caspase activity (53). Since kallikrein GT increases kinin, NO, and cGMP levels in heart, these results indicate that kallikrein gene delivery may inhibit myocardial apoptosis through the Akt-Bad or Akt-NO-cGMP pathways. Moreover, increased cAMP and NO may also attenuate cardiac fibrosis via inhibition of transforming growth factor-β (TGF-β) expression and thus reduce extracellular matrix protein (collagen, fibronectin) accumulation (29, 32). The effect of kallikrein gene delivery on superoxide formation in the heart during acute myocardial I/R injury could be attributed to NO-mediated inhibition of NADH/NADPH oxidase activity (26). Suppression of ROS may also lead to attenuation of cell proliferation via the mitogen-activated protein kinase (MAPK) pathway. It is also likely that kallikrein gene delivery could suppress plasminogen activator inhibitor (PAI-1) expression via NO-cGMP (14).

Multiple roles of kallikrein-kinin in cardiovascular and renal disease. Our studies have demonstrated that kallikrein gene delivery produces multiple beneficial effects in cardiac and renal function in animal models. The protective effects following delivery of the tissue kallikrein gene include BP reduction; attenuation of renal injury, cardiac infarction, cardiac remodeling, and heart failure; inhibition of neointimal formation in blood vessels after balloon angioplasty; reduction of stroke-induced mortality; and increased angiogenesis in the ischemic heart and hindlimb. These results indicate that kallikrein GT may have a therapeutic potential for cardiovascular and renal diseases.

Antisense strategy for a long-term control of hypertension. Dr. Raizada’s research group has been involved in providing “proof of concept” for the antisense-based gene therapy for hypertension. The group has selected the RAS as a target for antisense therapy to regulate the hyperactivity of this system in certain forms of hypertension. The basic principle of the antisense approach is that it blocks the formation of targeted proteins rather selectively, either at the transcriptional or translational level. The rationale for targeting the RAS are multiple: 1) the role of the RAS in hypertension is well established, 2) the RAS is widely distributed in the body, thus providing an ideal target for systemic gene delivery, and 3) traditional pharmacological agents that target the RAS are proven antihypertensive medications. As a result, there are well-developed protocols that can be used to compare the outcomes and efficacy of antisense gene therapy with traditional pharma-cotherapy.

First conceptual support that antisense targeting of the RAS could be effective in the treatment of hypertension was derived from the experiments with the use of antisense oligonucleotides to angiotensinogen and the ANG II AT1 receptor subtype (AT1R) (43, 90). The BP-lowering effect was reproducible and persisted for days. The next breakthrough came when the research groups of Drs. Raizada and Phillips each independently established experimental protocols for systemic delivery of antisense by viral vector-mediated gene delivery system. For example, use of the AAV prolonged the BP-lowering effects for weeks (90). However, retroviral vector turned out to be an ideal vector for providing conceptual evidence for the antisense gene therapy (40, 87). This is based on the fact that one could produce large quantities of the vector with high titers that has the ability to integrate into the genome for prolonged antihypertensive effects. Data from these studies are summarized below.

Intracardiac administration of a single bolus of a retroviral vector containing AT1R-AS-cDNA in 5-day-old SHR produced life-long antihypertensive effects. High BP was prevented from developing throughout life and was comparable with daily administration of losartan, an AT1R antagonist (87). No BP-lowering effect was observed in the normotensive rat (87, 92). The prevention of cardiac and vascular pathophysio-

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Table 2. Protective effects of kallikrein gene delivery in the kidney

<table>
<thead>
<tr>
<th></th>
<th>Glomerular Filtration Rate</th>
<th>Renal Blood Flow</th>
<th>Glomerular Sclerosis</th>
<th>Tubular Damage</th>
<th>Renal Cell Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dahl-SS</td>
<td>↑</td>
<td>↓</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2KIC</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>5/6 Nephrectomy</td>
<td>↑</td>
<td>↓</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gentamycin-induced nephropathy</td>
<td>↑</td>
<td>↑</td>
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<td>ND</td>
<td>ND</td>
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</tbody>
</table>

See legend of Table 1 for description of symbols.
Hypertension. However, these data also raise many questions: 1) Can one use other components of RAS (e.g., ACE) as a target for antisense therapy to produce a similar long-term antihypertensive effect? The answer appears to be affirmative based on our studies (87). 2) Are antihypertensive effects of the AT1R-AS specific? Existing data demonstrates that the expression of AT1R-AS has no significant effects on the AT2R and vasoconstrictor responses to phenylephrine (87). Further studies must be carried out to compare other physiological parameters such as renal functions. 3) What is the AT1R-AS transduction efficiency and cellular localization of the antisense transcript? It is surprising that sufficient cardiovascular cells are transduced by a single bolus of viral vector administration that long-term antihypertensive effects are produced. The high rate of transduction could, in part, be a result of multiplication of cells that are initially infected since the vector is administered in the developing rat. This must be confirmed. In addition, the cellular localization of the transcript must be examined to support this view. 4) Is the expression of the AT1R-AS of any consequence in the normotensive rat? Initial data suggest that its expression is of little or no consequence as far as the basal BP is concerned (87). This is consistent with the pharmacotherapy in which both AT1R antagonists and ACE inhibitors have little effect on normal individuals (9). This further supports a long-held view that the RAS is of little relevance in the control of normal BP (55, 115). However, the system comes into play when the RAS is challenged such as in hypertension. Our recent observation supports this hypothesis (88). AT1R-AS expression normal rats, when challenged with chronic-low dose ANG II, were completely protected from developing hypertension.

Future Perspectives

The studies so far have established that genetic targeting of the RAS prevents hypertension for life in the SHR. They are consistent with data from the other groups (89, 90). Thus it appears that the strategy is technically feasible and innovative and could constitute the basis for further developments. The following issues must be addressed to take this antisense gene therapy approach to the next level toward its possible use in the treatment of human hypertension.

We must test the antisense strategy with other animal models of hypertension. Both genetic (i.e., ren-2, transgenic, etc.) and nongenetic acquired (i.e., Goldblatt, DOCA-salt, etc.) models of hypertension must be tested.

We must determine whether this strategy would reverse established hypertension. So far, our approach has been to prevent development of hypertension. It holds little promise for the treatment of human hypertension, due to lack of reliable genetic markers for early detection of the disease. Thus we must determine whether the reversal strategy is feasible. New viral vectors that have higher transduction efficiency to infect nondividing cells should be tried. AAV and lentivirus-based vectors have shown great promise in preliminary experiments (87).

Safety and a better route of delivery of viral vectors need to be established. It is imperative that the genomic site of integration of the viral vector is known and its influence on the neighboring genes is established. In addition, the vector must undergo a proven safety analysis, and a germ line transmission of transgene by the vector must be determined. Finally, we have used the intracardiac route for systemic delivery of the vector. This is not the most efficient route, because the hepatic and pulmonary vascular beds would extract most of the vector before it reaches the cardiovascular-relevant tissues. Tissue-specific targeting must be attempted.

We must establish a regulated gene expression system in which the expression of the RAS antisense could be regulated by exogenous means. This is critical to turn off the therapeutic response as a result of any unforeseen side effects.

It would be interesting that other hypertension-related genes must be targeted by the GT approach. Although antisense GT targeting the RAS has provided encouraging results, other genes such as Ca2+ channels, signaling molecules-related genes, or genes relevant to matrix proteins may prove to be more effective sites for hypertension gene therapy.

In conclusion, it appears that antisense gene therapy strategy is an exciting pharmacotherapeutic approach that holds great potential for permanent control of hypertension.

General Conclusions

In answer to the question posed in the title concerning whether we are “near the summit or just beginning the climb” with regard to genetic targeting for cardiovascular therapeutics, it is obvious that we are somewhere in the middle of the climb. It is clearly evident from the data presented in this review that genetic targeting seems to be an extremely promising and
encouraging therapy not only for the future of the treatment and long-term control of cardiovascular disease but its prevention as well. However, much more research must be conducted to move this field from the benchtop to the bedside and possibly to genomic therapeutics. For example, basic understanding of causative genes in the pathophysiology of cardiovascular disease must be explored. In addition, fundamental research to improve the safety profile and route of gene delivery vectors must continue. It is also imperative that a regulatable gene expression system is developed in the event of unforeseen side effects or once genetic therapy is no longer necessary. This is an exciting time for physiologists. We are on the frontier of using innovative functional genomics methodologies to not only treat, but to potentially cure, the debilitating effects of cardiovascular disease.

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