Combination Cell and Gene Therapy for Peripheral Ischemia Using Myoblasts and Stem Cells Engineered with Conditionally Silenced Genes

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### Introduction

**Peripheral limb ischemia** Peripheral arterial disease (PAD) impairs end-organ perfusion (reviewed in 1, 2) and afflicts more than 300,000 new patients annually. Intermittent claudication (muscle pain) is a common early manifestation, affecting more than 10% of people over 50 years of age. Claudication is associated with an 8-12% annual mortality. Progression to pain at rest and loss of tissue integrity in the distal limbs leads to amputation if untreated. This extreme presentation is known as critical limb ischemia (CLI). The life expectancy after amputation is less than 5 years (3). PAD is conventionally treated medically by aggressive risk factor modification, with direct intervention reserved for medical failure leading to CLI. Arterial surgery is diminishing in frequency as catheter technology has evolved. The reported USA annual procedure rates in 2003 are: 45,000 surgeries vs. 200,000 percutaneous transluminal angioplasties (PTA). The frequency of the treated lesions in the iliac, above knee, and below knee arteries are: 55,000, 60,000, and 85,000. Unfortunately, the more distal the treated vessel is, the greater the re-stenosis rate: 5%, 50%, and 75%, respectively. Re-intervention is challenging and costly. Effective pharmacotherapy for PAD is limited to pletal and trental, two drugs with limited benefit. The benefits of currently available therapies are sufficiently limited that when combined with the risk posed by co-morbid conditions, including diabetes, CAD, and renal insufficiency, amputation may be the option. Indeed one study suggested that although bypass surgery and angioplasty were increasing, there has been no parallel overall decrease in the amputation rate for PAD patients (3).

**Protein, Gene, and Stem Cell Therapy**. Risk factors for PAD include hereditary, age, diabetes, hypertension, lifestyle (smoking), and serum lipid composition. Evidence from both animal and patient studies indicate that several of these risk categories, in particular age and diabetes are associated with depressed levels of pro-angiogenic growth factors including vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) (4). Growth factor deficiency and depressed angiogenic potential may therefore contribute to the establishment and progression of arterial disease. Preclinical studies initiated in the early 1990s provided conceptual proof for therapeutic angiogenesis, and supported the implementation of clinical trials of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (reviewed in 1, 2, 5). VEGF and FGF are both multigene families. There are at least 6 distinct VEGF members and more than 22 FGF proteins. Most of the trials have focused on VEGF-A and C and FGF-1 and 2. Although these trials have generally been positive, the results are mixed in terms of therapeutic gain. There have been more than 1000 patient studies testing the different
isoforms of VEGF or FGF proteins or genes and about 100 patient studies testing bone marrow derived stem cells.

Clinical Trials of protein and gene therapy: The earliest patient studies of therapeutic angiogenesis for critical limb ischemia were reported in 1995 and 1996 (6, 7). The first of these involved transfer of plasmid encoded VEGF165 to a patient with severe CLI. The procedure promoted an increase in collateral vessels and temporary limb salvage. A similar protocol was implemented in a second larger trial involving 10 limbs, again treated with VEGF165 plasmid DNA (7). Significant improvements of ankle-brachial ratios and successful limb salvage in 3 patients were reported. These successes were short-lived and all recruits subsequently had limb amputations. These studies also revealed significant increases of plasma VEGF during the treatment that raised concern about the safety of the procedures. In the TRAFFIC study one hundred and seventy four patients with intermittent claudication received recombinant βFGF or placebo (8). At 90 days after treatment there was significant improvement in peak walking time in the treatment group but other indices were inconclusive. The RAVE trial was a large randomized double blind placebo controlled study of VEGF delivered by adenovirus (9). This study reported no difference between treatment and placebo groups and concluded that adenoviral delivery of VEGF was not a viable therapeutic strategy for treatment of CLI.

Clinical Trials of stem cells: By demonstrating that new blood vessels can be induced to grow in ischemic muscles, growth factor protein and gene therapy have provided some hope for patients with PAD. However, the perceived failure of these approaches to provide a more complete cure contributed to the search for additional factors that may more effectively re-perfuse the tissues of these patients. Using the same preclinical models as the gene transfer experiments, delivery of bone marrow derived mesenchymal cells or endothelial progenitor cells was shown to improve the recovery of hindlimb muscle in multiple models of PAD. Clinical trials have attempted to reproduce the animal studies (reviewed in (10, 11)). These trials, like the gene therapy trials, have demonstrated feasibility and safety, with significant but in most cases limited efficacy. Patient studies in Miami have demonstrated long-term limb salvage and significant although limited functional recovery (unpublished). The trans-cutaneous oxygen tension is perhaps the best endpoint to quantify the therapeutic efficacy of treatments for PAD, and has at least doubled in the Miami population within 3 weeks of treatment. A review of the available data from clinical trials of gene and stem cell therapy indicates an average increase of <0.2 in the ankle:brachial ratio (ABI) for either treatment. This number is barely above the normal standard deviation of the ABI (15%). However, the ABI is a gross measure of large vessel perfusion and is influenced by vessel calcification. Therapeutic angiogenesis may not create arteries large enough to influence the ankle brachial index.

Engineered Stem Cell Therapy: Two features may have compromised the clinical trials of gene therapy and stem cell therapy for PAD. In all of the trials where FGF or VEGF were delivered as proteins or DNA it is probable that the duration of exposure of the ischemic tissue to growth factors was insufficient to mediate the production of stable vessels. This has been demonstrated unambiguously for recombinant protein and adenovirus and is strongly implicated for plasmid delivery. Whereas several reports have demonstrated that permanent gene delivery by vectors such as AAV promote stable vessel production in animal models, these vectors have not been tested clinically because of safety issues associated with chronic unregulated expression
of genes such as FGF and VEGF (1, 2, 5). For stem cell therapy, only autologous stem cells have been used in clinical trials for PAD treatment. Multiple studies have now shown that stem cells isolated from aged or diseased patients are defective in their expression of angiogenic genes including VEGF and SDF-1 (4, 12). Therefore therapy using autologous cells may be compromised if these defective cells are unable to support an efficient angiogenic response in the target tissues.

To address these issues we profiled gene expression in the mesenchymal stem cell population isolated from the bone marrows of young, adult and old mice using Affymetrix microarrays. These screens confirmed a significant age-related loss of multiple angiogenic genes including VEGF and have served as the basis for selecting the genes for stem and myoblast bioengineering. We created a series of bi-cystronic plasmid and AAV vectors expressing both pro-angiogenic and pro-survival genes including VEGF, SDF-1, MCP-1, heme-oxygenase (HO), IGF-1, and Akt under the direction of housekeeping or tissue-specific promoters containing a combination of transcriptional silencers and hypoxia-response elements such that expression is restricted to ischemic muscle (conditionally silenced vectors (13)).

In the first series of experiments we asked whether an AAV vector expressing conditionally silenced VEGF could induce stable vessel generation in the mouse ischemic hindlimb model. Recombinant AAVs of serotype 9 where created in which human VEGF165 expression was directed by a phosphoglycerate kinase promoter containing a tandem series of hypoxia response elements and silencers (H/S). AAV9 is a newly isolated serotype that is reported to have strong tropism and highly efficient penetration in muscle (J. Wilson, personnel communication). Use of AAV9 has not been described in any ischemia model. We used laser Doppler imaging and immunohistochemistry to compare the return of blood flow in the mouse hind-limb after induction of ischemia by ligation and excision of the femoral artery and its branches (3). Conditionally silenced AAV-VEGF (rAAV-VEGF) salvaged all limbs and generated a reperfusion profile that was superior to all other forms of gene or protein delivery, in particular adenovirus and time-release gel matrices. Mature vessels were generated by rAAV treatment and VEGF expression was tightly controlled.

In the second series of experiments, autologous or syngenic mesenchymal stem cells or myoblasts were stably transduced with plasmid or AAVs containing conditionally silenced VEGF and HO. These engineered cells were tested in the rabbit ischemic hindlimb model (14). Before delivery the cells were additionally infected with an adenoviral vector expressing Akt. The latter maneuver was implemented to improve survival of the cells during the first 1-2 weeks after delivery. Relative to controls (adenovirus-VEGF; stem cells or myoblasts alone) the engineered cells produced a sharp increase in the production of stable large vessels and a corresponding decrease in the micro-capillaries. Limbs were salvaged in all cases and necrosis was not seen in the engineered cell treatment groups. The results from angiography and immunohistochemistry were consistent. Tissue VEGF165 levels again were tightly controlled.

**DISCUSSION AND CONCLUSIONS**  Our studies address two components of gene and cell therapy for PAD that may have negatively impacted the outcomes of clinical trials and can be rectified. Firstly we confirm that sustained expression of VEGF such as that mediated by a regulated AAV is essential for the production of stable vessels in ischemic skeletal muscles.
Secondly autologous stem cell therapy for PAD may be optimized by engineering the cells to express selective genes that are defective in the stem cells as a result of age or disease of the host.

ACKNOWLEDGEMENTS.

Supported by grants # HL44578 and HL69812 from the National Institutes of Health. K. A. Webster holds a Walter G. Ross Foundation Endowed Chair of Vascular Biology.

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