Therapeutic Arteriogenesis by Ultrasound-Mediated VEGF$_{165}$ Plasmid Gene Delivery to Chronically Ischemic Skeletal Muscle

Howard Leong-Poi, Michael A. Kuliszewski, Michael Lekas, Matthew Sibbald, Krystyna Teichert-Kuliszew ska, Alexander L. Klbanov, Duncan J. Stewart, Jonathan R. Lindner

Abstract—Current methods of gene delivery for therapeutic angiogenesis are invasive, requiring either intraarterial or intramuscular administration. A noninvasive method of gene delivery has been developed using ultrasound-mediated destruction of intravenously administered DNA-bearing carrier microbubbles during their microcirculatory transit. Here we show that chronic ischemia could be markedly improved by ultrasound-mediated destruction of microbubbles bearing vascular endothelial growth factor-165 (VEGF$_{165}$) plasmid DNA. Using a model of severe chronic hindlimb ischemia in rats, we demonstrated that ultrasound mediated VEGF$_{165}$/green fluorescent protein (GFP) plasmid delivery resulted in a significant improvement in microvascular blood flow by contrast-enhanced ultrasound, and an increased vessel density by fluorescent microangiography, with minimal changes in control groups. The improvement in tissue perfusion was attributed predominantly to increases in noncapillary blood volume or arteriogenesis, with perfusion peaking at 14 days after delivery, followed by a partial regression of neovascularization at 6 weeks. Transfection was localized predominantly to the vascular endothelium of arterioles in treated ischemic muscle. RT-PCR confirmed the presence of VEGF$_{165}$/GFP mRNA within treated ischemic muscle, being highest at day 3 postdelivery, and subsequently decreasing, becoming almost undetectable by 6 weeks. We found a modulation of endogenous growth factor expression in VEGF-treated ischemic muscle, consistent with a biologic effect of ultrasound mediated gene delivery. The results of our study demonstrate the utility of ultrasonic destruction of plasmid-bearing microbubbles to induce therapeutic arteriogenesis in the setting of severe chronic ischemia. (Circ Res. 2007;101:295-303.)

Key Words: angiogenesis ■ gene therapy ■ contrast ultrasound ■ peripheral vascular disease ■ chronic ischemia

In the setting of severe coronary artery disease (CAD) and peripheral arterial disease (PAD), endogenous neovascularization represents the body’s most important attempt to restore tissue perfusion toward normal. These responses, however, are often inadequate to prevent debilitating symptoms and ischemic tissue loss. Because of the need for some form of immediate palliative therapy, there have been numerous clinical trials designed to promote new vessel growth by exogenous administration of proangiogenic genes in patients with refractory ischemic symptoms and end-organ damage.1,2 Although initial small open-labeled trials yielded promising results, subsequent larger double-blind randomized placebo-controlled clinical trials have failed to show much clinical benefit.3–5 The largely disappointing results of clinical trials of therapeutic angiogenesis may in part be explained by suboptimal delivery of genetic material to target cells or tissue.6–7 Intraarterial delivery of genes is largely ineffective because of insufficient amounts of transfection, and can also result in systemic delivery to nontargeted tissue.8 Although intramuscular delivery has been shown to more effective,8 this strategy is still limited by the inability to target certain cell types, the relatively localized nature of transfection in proximity to the injection site, and the impracticality for repetitive treatments in tissues such as the heart. The ability to noninvasively deliver genetic material to specific target tissues, such as the vascular endothelium, in a controlled manner would be an important step toward a safe and effective proangiogenic gene therapy.

A noninvasive gene delivery strategy has been developed using ultrasound-mediated destruction of intravenously (i.v.) administered DNA-bearing carrier microbubbles during their microcirculatory transit.9 This strategy has been used to amplify transfection of reporter plasmid DNA to skeletal10 and cardiac muscle.11 The mechanism by which gene trans-
fection is enhanced likely involves extravascular deposition of DNA by either transient microporation produced by high velocity microjets during gas volume oscillation in the ultrasound field, or overt microvascular rupture. Recently, this strategy has been used to deliver plasmid encoding human hepatocyte growth factor (HGF) to infarcted hearts in rats, and to deliver vascular endothelial growth factor (VEGF) to normal rat myocardium. In this study, we hypothesized that VEGF\textsubscript{165} transfection by ultrasonic destruction of plasmid DNA-bearing microbubbles administered intravenously would improve skeletal muscle perfusion in the setting of severe ischemic PAD. The study was designed to test whether this vascular-based strategy for proangiogenic gene therapy would be effective in the setting of reduced limb blood flow and whether therapy would be additive to native neovascularization. A secondary aim of our study was to determine whether gene delivery could be localized to the vasculature where therapy with VEGF\textsubscript{165} is likely to be most effective.

**Materials and Methods**

**Microbubble and DNA Preparation**

Plasmid DNA was charge-coupled to cationic lipid microbubbles as previously described. Microbubbles with a cationic (zeta potential of +60 mV) lipid shell were created, which when incubated with plasmid DNA, results in approximately 6700 plasmids on surface of each microbubble. For perfusion imaging, nontargeted lipid-shelled decafluorobutane microbubbles (MP1950) were used. Microbubble concentrations were determined by electrozone sensing with a Coulter Multisizer IIe (Beckman-Coulter). Plasmid vectors were constructed for transfection of enhanced green fluorescent protein (EGFP) alone or the cotransfection of both human VEGF\textsubscript{165} and GFP. For the latter, we constructed a bicistronic plasmid with an internal ribosome entry site (IRES) that facilitates the translation of both proteins from a single mRNA molecule, with high levels of expression of both genes within the same cell.

**Animal Preparation**

The study protocol was approved by the Animal Care and Use Committee at St Michael’s Hospital-Health Sciences Research Centre, University of Toronto. Proximal hindlimb ischemia was produced in 90 Sprague-Dawley rats. Rats were anesthetized with intraperitoneal injection of ketamine hydrochloride (10 mg kg\textsuperscript{-1}) and xylazine (8 mg kg\textsuperscript{-1}). Using aseptic technique, the left common iliac artery and small proximal branches were exposed and ligated with 4-0 suture. The incision was closed in layers and animals were recovered.

**Perfusion Imaging**

Contrast-enhanced ultrasound (CEU) imaging of the proximal hindlimb adductor muscles was performed with gated pulse inversion imaging (HDI 5000, Philips Ultrasound) at a mechanical index of 1.0 and a transmission frequency of 3.3 MHz (L7-4 transducer). Gain settings were optimized and held constant. Data were recorded on magnetic-optical disk and transferred to a computer for off-line analysis. Perfusion in the adductor muscles was assessed during continuous i.v. infusion of nontargeted MP1950 microbubbles (1 × 10\textsuperscript{7} min\textsuperscript{-1}). Background images were acquired at baseline for subtraction of tissue signal. Intermittent imaging was then performed by progressive prolongation of the pulsing interval (PI) from 0.2 to 20 s, using the internal timer. Several averaged background frames were digitally subtracted from averaged contrast-enhanced frames at each PI. PI versus signal intensity (SI) data were fit to the function, \( y = A (1 - e^{-kt}) \), where \( y \) is SI at the pulsing interval \( t \), \( A \) is plateau video intensity which is an index of microvascular blood volume (MBV), and \( k \) is the rate constant which provides a measure of microvascular blood velocity. Microvascular blood flow (MBF) was calculated by the product of \( A \) and \( k \).

**CEU perfusion data were recalculated using averaged frames at a PI of 1 s as background to eliminate signal from vessels with a transaxial plane velocity of 2.4 × 10\textsuperscript{3} m s\textsuperscript{-1}. This process eliminates signal from almost all noncapillary microvessels, with minimal loss of capillary signal, thereby yielding information from the capillary compartment alone. The relative noncapillary blood volume (NCBV) was determined by the difference of the calculated A-values. This algorithm has been previously used to measure dynamic changes in skeletal muscle capillary volume, and has been validated against assays for capillary xanthine oxidase availability.**

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For gene delivery, ultrasound transmission was performed with a phased array transducer (Sonos 5500, Philips Ultrasound) at 1.3 MHz using B-mode ultraharmonic imaging at a transmit power of 0.9W (120 V, 9 mA). Cationic microbubbles (1 × 10\textsuperscript{7}) coupled with 500 µg of CDNA were infused intravenously over 10 minutes. To allow for a wider field of delivery, the transducer was positioned transverse to the ischemic adductor muscles and ultrasound was transmitted during a slow sweep along the length of the proximal hindlimb muscles. A PI of 5 s was used to allow microbubble replenishment into the beam elevation between each pulse of ultrasound. Ultrasound transmission was continued for 10 minutes after cessation of the infusion, to destroy remaining circulating DNA-loaded microbubbles.

**Fluorescent Microangiography**

Before sacrifice, the abdominal aorta was cannulated and the distal hindlimbs flushed with 20 mL of heparinized saline. A 10% solution of fluorescent microspheres (2 µm diameter; Sigma) mixed with a 1% solution of low melting point agarose at 45°C was slowly injected into the aortic cannula. The animal was euthanized and placed in an ice bath to facilitate rapid cooling and solidification of the casting agent. Hindlimb muscle was removed and placed in 10% buffered formalin and sectioned (200 µm). Using confocal microscopy, a series of stacked images (4-µm slices) was taken and the middle 25 slices (100 µm total thickness) were projected to quantify the density of blood vessels using automated software (IPTK analysis software, Reindeer Graphics Inc). This technique has been previously described to quantify pulmonary vascular density in experimental models of pulmonary arterial hypertension.

**Immunohistochemistry**

In vivo transfection efficacy and spatial localization was determined using immunohistochemistry (see Methods supplement for specific details on tissue processing techniques, available online at http://circres.ahajournals.org). Cell surface antigens were identified using: mouse anti-human CD31 (Alpha Diagnostics Intl Inc), mouse anti-human Tie-2 (Clone Ab33, Upstate Biotechnology), UEA-1 (Sigma), and mouse anti-human Alpha-actin (Sigma). The presence of antibody was confirmed by exposure to a phycoerythrin (PE) conjugated secondary antibody, TOPO-3 (Sigma) was used as a nuclear marker.

**RT-PCR**

Semi-quantitative real-time RT-PCR for endogenous rat VEGF, angioptin-1 (Ang-1), and Angioptin-2 (Ang-2) mRNA, as well as exogenous GFP and VEGF\textsubscript{165}/GFP transcripts were performed using standard techniques in our laboratory (see online methods supplement for further details on tissue processing and specific primers used).

**Western Blotting**

Western blotting was performed to measure total VEGF\textsubscript{165} protein levels in ischemic hindlimb muscle at various time points post ligation in the VEGF-treated group, using standard techniques in our laboratory (see online methods supplement for specific details).
Experimental Protocol
CEU perfusion imaging of both hindlimb adductor muscles was performed 14 days after iliac artery ligation. Ultrasound-targeted gene delivery was then performed, according to assigned treatment group: group 1: control, no treatment; group 2: GFP plasmid; group 3: VEGF165/GFP plasmid (n/H11005 18 per group). Repeat CEU was performed at days 17 (n/H11005 6 per group) and 28 (n/H11005 12 per group). To assess late gene transfection and efficacy, an additional 36 rats (n/H11005 12 per treatment group), were studied at 8 weeks after ligation. In 4 rats per group, fluorescent microangiography (FMA) was performed immediately before sacrifice. In remaining animals, skeletal muscle tissue from the ischemic and nonischemic adductor muscles, as well as tissue from the lungs, heart, and liver was obtained for postmortem immunohistochemistry, quantitative RT-PCR, and Western blotting. Normal hindlimb muscles from an additional 6 animals without ligation were obtained for semiquantitative RT-PCR, for comparison to hindlimb muscles from ischemic animals that underwent ligation.

Statistical Methods
Data are expressed as mean±SD. Comparisons between multiple stages were made with 1-way ANOVA. When differences were found, interstage comparisons were performed using nonpaired Student t test with Bonferroni correction. Data for pre- and postgene therapy were compared by paired Student t test. Differences were considered significant at P<0.05 (2-sided).

Results
Muscle Perfusion and Vascular Density
Two weeks after iliac artery ligation, blood flow to the ischemic adductor muscles was reduced to approximately 40% of normal (Figure 1). No changes in MBV or MBF were seen at 3 days postdelivery (day 17 postligation) in any group. In group 1 control rats, there was no change in MBV or MBF over the subsequent 8 weeks. In group 2 rats receiving microbubbles bearing GFP-plasmid alone, there was a small but significant increase in MBV at 2 weeks postdelivery, but no change in MBF. At 8 weeks, MBV had returned to pretreatment levels. In comparison, group 3 rats receiving microbubbles bearing VEGF165/GFP plasmid showed significant improvements in both MBV and MBF 2 weeks after ultrasound-mediated gene delivery. By 8 weeks, MBV and MBF had decreased, however remained greater than baseline pretreatment values (Figure 1). Examples of CEU perfusion imaging of the ischemic muscle from each of the treatment groups are illustrated in Figure 2.

Image processing algorithms were applied to differentiate total microvascular flow from capillary blood flow (CBF). This analysis demonstrated that GFP-plasmid microbubble delivery increase MBV primarily by increasing noncapillary blood volume (NCBV; Figure 3), which did not translate to increases in CBF or MBF. Ultrasonic destruction of microbubbles bearing VEGF165/GFP plasmid resulted in a greater increase in NCBV, which was associated with a normalization of CBF (Figure 3). Similar to total blood flow, there was partial regression of NCBV in VEGF165/GFP muscle by 8 weeks postligation.

FMA demonstrated reduced vessel density in nontreated ischemic muscle 28 days and 8 weeks after iliac artery ligation (Figure 4). In keeping with CEU data, there was a
minor increase in vessel density in GFP plasmid–treated muscle at day 28, which was not present at 8 weeks. In VEGF<sub>165</sub>-treated ischemic muscle, vessel density at day 28 was significantly greater than both untreated and GFP-plasmid treated ischemic muscle (Figure 4), with partial regression at 8 weeks.

Efficacy of Gene Transfection
At day 17 (3 days postdelivery), a strong GFP signal was detected only in plasmid-treated ischemic muscle, with no GFP signal in control untreated ischemic muscle (Figure 5A). Although this signal was predominantly localized to the vascular endothelial layer of small to medium sized arterioles...
arterioles (50 to 150 μm) (Figure 5B), at this early time point GFP signal was also detected within capillaries and surrounding myocytes (Figure 5C).

At day 28, GFP signal was again localized to small to medium-sized arterioles (Figure 5D). In comparison to the 3 day time point, the number of vessels expressing GFP at 14 days postdelivery was ~5 fold less. In addition, there was no detectable GFP signal within capillaries or surrounding myocytes at this later time point after delivery. By 8 weeks, there was no longer detectable GFP signal within treated ischemic muscle. GFP signal was not detected in the contralateral nonischemic hindlimb, or within remote organs, including the lungs, heart, and liver, at any time point.

Figure 4. A, Representative stacked images of microvessels in ischemic hindlimb muscle after no treatment (control), GFP, and VEGF165/GFP therapy at 4 and 8 weeks postligation, using FMA. Compared with normal muscle (inset image in B), control untreated ischemic muscle showed a reduction in microvascular density by FMA that persisted over time. Ultrasound-mediated delivery of GFP-plasmid bearing microbubbles resulted in a mild increase in vessel density at 4 weeks, which did not persist to 8 weeks. After delivery of VEGF165/GFP plasmid, FMA demonstrated a marked and dense proliferation of neovessels, with an abundance of bridging arterioles. Neovascularization remained present at late time points in VEGF treated muscle. B, Quantitative vessel density by FMA of ischemic and normal hindlimb muscle. At 28 days postligation, FMA revealed a significant increase in the density of microvessels in the ischemic leg of VEGF165-treated animals vs both control and GFP treated groups, with partial regression at week 8. Scale bars 100 μm. *P<0.05 compared with normal nonischemic muscle, †P<0.05 compared with corresponding control data, ‡P<0.05 compared with corresponding GFP-treated data. (Inset image, FMA of normal hindlimb skeletal muscle).
Real-time RT-PCR data for exogenous GFP and VEGF<sub>165</sub>/GFP mRNA is shown in Figure 6. Using specific primers, robust exogenous transgene expression (normalized to the contralateral nonischemic muscle) was detected in GFP and VEGF<sub>165</sub>/GFP-treated muscles at day 17 (19.1±12.6 and 34.3±25.4, respectively), and was undetectable in control untreated animals (Figure 6). Both GFP (1.6±0.4) and VEGF<sub>165</sub>/GFP (1.8±0.6) mRNA expression persisted till day 28, but was almost undetectable (1.3±0.6) by 8 weeks in VEGF<sub>165</sub>/GFP-treated ischemic muscle.

At day 17 endogenous VEGF, Ang-1, and Ang-2 were upregulated in the ischemic limbs of all groups. Although reduced slightly, these endogenous growth factors remained upregulated in nontreated controls and GFP-treated animals at day 28 and 8 weeks (Figure 7A). In contrast, in the VEGF<sub>165</sub>/GFP-treated ischemic muscle at day 28, endogenous VEGF was reduced compared with other treatment groups (Figure 7A). At that time point, endogenous Ang-1 levels were further increased in the VEGF<sub>165</sub>/GFP-treated muscle compared with nontreated and GFP-treated ischemic muscle (Figure 7A), and had decreased by 8 weeks postligation. Endogenous VEGF, Ang-1, and Ang-2 mRNA levels in normal muscle from nonligated animals were similar to those obtained from the contralateral normal hindlimb muscle in ischemic animals (data not shown). Total VEGF protein levels by Western blotting followed a similar pattern as both exogenous and endogenous VEGF mRNA levels, with an early increase at day 17, a reduction by day 28, and near normalization by 8 weeks (Figure 7B).

**Discussion**

The potential of ultrasonic destruction of carrier microbubbles to deliver genetic material specifically to tissue that is insonified has been explored over the last decade. The therapeutic impact of this strategy has begun to be tested in animal models of disease. In this study, we have demonstrated that ultrasound-mediated delivery of VEGF<sub>165</sub> charge-coupled to microbubbles can improve resting skeletal muscle perfusion in a model of severe peripheral arterial disease.

The ultrasound-facilitated delivery of genes encoding for proangiogenic growth factors (HGF and VEGF) that have been combined with microbubbles has recently been demonstrated. At that time point, endogenous Ang-1 levels were further increased in the VEGF<sub>165</sub>/GFP-treated muscle compared with nontreated and GFP-treated ischemic muscle (Figure 7A), and had decreased by 8 weeks postligation. Endogenous VEGF, Ang-1, and Ang-2 mRNA levels in normal muscle from nonligated animals were similar to those obtained from the contralateral normal hindlimb muscle in ischemic animals (data not shown). Total VEGF protein levels by Western blotting followed a similar pattern as both exogenous and endogenous VEGF mRNA levels, with an early increase at day 17, a reduction by day 28, and near normalization by 8 weeks (Figure 7B).

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The ultrasound-facilitated delivery of genes encoding for proangiogenic growth factors (HGF and VEGF) that have been combined with microbubbles has recently been demonstrated. In these studies, improvement in tissue perfusion was implied by the histologic finding of increased capillary density, without assessment of in vivo perfusion. One novel feature of our current study is that microvascular proliferation, assessed anatomically by fluorescent microangiography, was correlated with recovery of in vivo nutritive perfusion as assessed by CEU. We also confirmed that delivery of genetic material and the biologic effects of the gene product could be accomplished in chronically and severely hypoperfused muscle. The importance of this finding should not be underesti-
mated because microbubble transit through ischemic skeletal muscle is expected to be quite low. Previous studies examined delivery of proangiogenic genes to tissue adjacent to or directly within normal myocardium which has a flow rate at least 20-fold greater than that in ischemic skeletal muscle at rest. Another unique feature of this study was the finding that exogenous VEGF<sub>165</sub> treatment suppressed endogenous rat VEGF mRNA levels, suggesting that resolution of ischemia also reversed native angiogenic growth factor upregulation.19,20

**Figure 6.** RT-PCR data showing exogenous GFP and VEGF<sub>165</sub>/GFP mRNA transcript at day 17, day 28, and 8 weeks. GFP and VEGF/GFP<sub>165</sub> mRNA expression at day 17 was high, and was significantly greater than mRNA expression at day 14 postdelivery. Exogenous transgene expression remained detectable at low levels at day 14 and was almost undetectable at 8 weeks. *P<0.001 compared with corresponding day 28 data, †P<0.05 compared with corresponding control data.

**Figure 7.** A, RT-PCR for endogenous gene expression, VEGF (solid bars), Ang-1 (open bars), and Ang-2 (hatched bars). Growth factors (normalized to the contralateral non-ischemic leg) were upregulated at day 17 in all groups, being more pronounced in the VEGF/GFP-treated animals. At day 28, there was a significant downregulation of VEGF and upregulation of Ang-1 in the VEGF<sub>165</sub>/GFP-treated group compared with both GFP and untreated control ischemic muscle, suggesting modulation of the endogenous angiogenic response. By 8 weeks, endogenous growth factor gene expression returned to near baseline levels in all groups. *P<0.05 compared with corresponding data in control and GFP-treated muscle. B, Total VEGF protein levels by Western blotting in VEGF/GFP-treated animals at day 17, day 28, and week 8. Corresponding to exogenous VEGF/GFP and endogenous VEGF mRNA levels, there was an increase in total VEGF at day 17, with a reduction at day 28, and a return to near baseline levels at week 8. *P<0.05 compared with normal hindlimb muscle.
In our study, transfection of our targeted genes was confirmed by the detection of GFP signal within tissue by fluorescent confocal microscopy and by the presence of exogenous mRNA expression on real-time RT-PCR. The former technique allowed us to localize gene transfection primarily to the endothelial layer of vessels. Early after delivery, both arterioles and capillaries expressed GFP, with a greater proportion of vessels targeted being arterioles. At early time intervals, GFP signal was also seen in myocytes adjacent to GFP-positive capillaries. At day 14 postdelivery, GFP was localized only to arterioles. These findings are consistent with previous studies showing localization of fluorescent plasmid mainly to the vascular and perivascular regions after ultrasound-mediated delivery.10 Thus, the ultrasonic destruction of intravascular carrier microbubbles targets the vascular endothelium, making this technique ideally suited to gene delivery for therapeutic angiogenesis. The latter method, real-time RT-PCR, was used to confirm the production of exogenous mRNA by our transfected genes, using an ultra-sensitive technique. The timing of mRNA expression in our study is in keeping with the findings of Bekeredjian et al11 where transgene expression peaked within the first 4 days, with a rapid decline thereafter. We did not find increases in MBF at day 3 after delivery, suggesting that the neovascularization response to gene delivery followed peak mRNA expression. Although vascular density and MBF partially regressed very late after gene delivery, they remained increased as compared with baseline pretreatment values, consistent with a persistence of neovascularization at a time point when therapeutic transgene expression had ceased. This would imply that repeated doses delivered over time will likely be required to produce a sustained angiogenic response, thus further emphasizing the importance of a noninvasive method of gene delivery.

Another novel feature of this study was the application of CEU to differentiate the functional expansion of the capillary and noncapillary microvascular compartments. These data indicated that the improvement of capillary perfusion was associated with an increase in noncapillary microvascular blood volume. It was impossible to confirm that arteriolar delivery of VEGF plasmid has a predominant effect on the same vessels. However, our findings are in agreement with the idea that remodeling of the resistance arteriolar bed is the major determining factor for reducing network resistance in the presence of a proximal stenosis and recovery of tissue perfusion.21 Ultrasound-mediated delivery of GFP-plasmid bearing microbubbles also increased noncapillary blood volume, or arteriogenesis, although to a lower degree. Previous studies have demonstrated that the biologic effects of ultrasound-mediated microbubble destruction results in the promotion of arteriogenesis in rat skeletal muscle.22,23 Postulated mechanisms for this biologic effect have included the recruitment of inflammatory cells, platelets, or bone marrow stem cells,23 the release of platelet-derived proinflammatory factors which may attract circulating endothelial progenitor cells,24 or the via the recruitment of VEGF-producing inflammatory cells.25 Unlike these studies, we found that control microbubble destruction did not result in increases in total or capillary blood flow, and are in keeping with other studies of ultrasound-mediated gene delivery.12,13 Differences in ultrasound transmit frequency and acoustic power, microbubble composition and doses, methods of blood flow determination, and vessel density measurements between studies could have accounted for these differences. With the doses and methods in our study, however, control microbubble destruction did not alter endogenous growth factor expression, and the changes in noncapillary blood volume were not sufficient enough to improve nutritive capillary blood flow. Regardless, we demonstrated that the use of plasmid encoding the growth factor VEGF, as compared with one encoding only a marker protein GFP, resulted in a substantially greater “angiogenic” effect.

RT-PCR data on endogenous growth factor mRNA expression offers unique insights into the biology of angiogenesis. VEGF is consistently elevated early after ischemia in the heart20,26 and skeletal muscle,19 and likely plays a role early in the endogenous angiogenic response to ischemia.20,26 There is increasing evidence that angiopoietins, Ang-1 and Ang-2, are also important for blood vessel formation.27,28 Ang-2 plays a synergistic role with VEGF early in the angiogenic response, with both being upregulated early after ligation in our study. In contradistinction, Ang-1 appears to be play an important role relatively later in the angiogenic process by contributing to stabilization and maturation of neovessels29,30 and opposing the actions of VEGF.31 In our study, endogenous VEGF and Ang-2 mRNA levels were reduced and Ang-1 mRNA levels further upregulated at day 28 in the group receiving VEGF165-bearing microbubbles, as compared with all other treatment groups. This finding is consistent with a reversal of early VEGF/Ang-2 upregulation with exogenous VEGF165 therapy that was not seen in other treatment groups. Western blotting for total VEGF protein levels confirmed an early elevation 3 days after VEGF delivery, and a later reduction at day 28, when exogenous transgene expression had waned. Importantly, the upregulation in Ang-1 at day 28 is consistent with the process of neovessel maturation, a late event in the angiogenic response. Taken together, these observations provide further compelling evidence for a biologic effect19,20 of our ultrasound-mediated gene delivery.

There are several limitations to our present study. We only examined a single large dose of VEGF165 at a single time point, which was highly effective. Defining the dose-response relationship for this delivery technique remains important. Given the number and complexity of the factors determining the efficacy and longevity of UM gene delivery, including (1) plasmid DNA and cationic microbubble doses and concentrations, (2) ultrasound acoustic power and pulsing interval, and (3) the effects of repeated deliveries over time, this is beyond the scope of our present study, however remains the goal of future studies. Although we believe that our findings can potentially translate into an effective noninvasive method of therapeutic gene delivery in humans, our present study only provides proof of principle. Many potential obstacles exist, including (1) determining the most safe and effective ultrasound settings and microbubble concentrations for human use, given concerns over the use of high acoustic powers, (2) defining a dose-response relationship in large animal models, in applicable organs, such as the heart, that
more closely mimics the clinical conditions, and (3) the development of ultrasound probes, designed specifically for human delivery.

In conclusion, ultrasound-mediated microbubble destruction using VEGF₁₆₅ plasmid-bearing microbubbles results in targeted transfection of the vascular endothelium, leading to arteriogenesis and improved tissue perfusion in the setting of severe chronic hypoperfusion. This noninvasive technique holds great promise as a method to target angiogenic gene therapy to ischemic tissue, in any organ accessible to ultrasound.

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Disclosures
None.

References
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EXPANDED MATERIALS AND METHODS

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For gene delivery, ultrasound transmission was performed with a phased array transducer (Sonos 5500, Philips Ultrasound) at 1.3 MHz using B-mode ultraharmonic imaging at a transmit power of 0.9W (120 V, 9 mA). Cationic microbubbles ($1 \times 10^9$) coupled with 500 $\mu$g of cDNA were infused intravenously over 10 minutes. To allow for a wider field of delivery, the transducer was positioned transverse to the ischemic adductor muscles and ultrasound was transmitted during a slow sweep along the length of the proximal hindlimb muscles. A PI of 5 s was used to allow microbubble replenishment into the beam elevation between each pulse of ultrasound. Ultrasound transmission was continued for 10 minutes after cessation of the infusion, to destroy remaining circulating DNA-loaded microbubbles.

**Fluorescent Microangiography (FMA)**

Prior to sacrifice, the abdominal aorta was cannulated and the distal hindlimbs flushed with 20 mL of heparinized saline. A 10% solution of fluorescent microspheres (2$\mu$m diameter) (Sigma) mixed with a 1% solution of low melting point agarose at 45°C was slowly injected into the aortic cannula. The animal was euthanized and placed in an ice bath to facilitate rapid cooling and solidification of the casting agent. Hindlimb muscle was removed and placed in 10% buffered formalin and sectioned (200 $\mu$m). Using confocal microscopy, a series of stacked images (4$\mu$m slices) was taken and the middle 25 slices (100 $\mu$m total thickness) were projected in order to quantify the density
of blood vessels using automated software (IPTK analysis software, Reindeer Graphics Inc.). This technique has been previously described to quantify pulmonary vascular density in experimental models of pulmonary arterial hypertension.6

**Immunohistochemistry**

*In vivo* transfection efficacy and spatial localization was determined using immunohistochemistry. Explanted tissue was cryo-embedded in OCT (Sakura, Japan) and stored at -80°C. The cryo-blocks were sectioned (15 µm thick) every 25 µm and re-hydrated in phosphate buffered saline (PBS) for 30 min, fixed in 2% paraformaldehyde (PFA) (Sigma) in PBS for 10 minutes, and washed 3 times with PBS. Cell surface antigens were identified using: mouse anti-human CD31 (Alpha Diagnostics Intl., Inc.), mouse anti-human Tie-2 (Clone Ab33, Upstate Biotechnology), UEA-1 (Sigma) and mouse anti-human Alpha-actin (Sigma). The presence of antibody was confirmed by exposure to a phycoerythrin (PE) conjugated secondary antibody. TOPO-3 (Sigma) was used as a nuclear marker.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Semi-quantitative real-time RT-PCR for endogenous rat VEGF, angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) mRNA, as well as exogenous GFP and VEGF\textsubscript{165}/GFP transcripts were performed using standard techniques in our laboratory. Tissue was homogenized using Trizol (Sigma), after which total RNA was isolated using the GenElute Mammalian total RNA kit (Sigma) and quantified by absorbance at 260 nm. Total RNA was reverse transcribed in 20 µl volumes using Omniscript RT kit (Qiagen) with 1 µg of random primers. For each RT product, aliquots (2-10 µl) of the final reaction volume were amplified by real-time PCR reactions using standardized
concentration of RNA. Rat VEGF, Ang-1, Ang-2, GFP, VEGF<sub>165</sub>/GFP and cyclophilin specific primers and SYBR green (Applied BioSystems) were then used to detect amplicon production using an ABI system.

**Western blotting**

Western blotting was performed to measure total VEGF<sub>165</sub> protein levels in ischemic hindlimb muscle at various time points post ligation in the VEGF-treated group, using standard techniques in our laboratory. Frozen tissue samples were homogenized in RIPA lysis buffer. Total protein (100 µg/line) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in 10-12% Tris-glycine gels (Invitrogen) under non-reduced condition, subsequently transferred to nitrocellulose membranes and blocked with 5% BSA in TBST buffer (10mmol/L Tris 150mmol/L NaCl pH 7.5 and 0.1% Tween 20). The membranes were probed overnight with goat anti-human antibodies to VEGF<sub>165</sub> (R&D) at concentration 0.2µg/ml in TBST with 3% BSA, followed by an anti-goat IgG secondary antibody conjugated to horseradish peroxidase (1:3000, 1hr; Promega). After being stripped, the membranes were reprobed with monoclonal antibody against Glyceraldehyde-3-Phosphatase Dehydrogenase (GAPDH) from skeletal muscle (1:5000, 2hr; Chemicon, Millipore) for the estimation of total protein loaded. Specific bands were visualized using an enhanced chemiluminescence substrate system ECL (Amersham Pharmacia Biotech). Densitometry was performed and the intensity of each band was analyzed using the Molecular Analyst software (Imaging Densitometer, Bio-Rad).

**Experimental Protocol**

CEU perfusion imaging of both hindlimb adductor muscles was performed 14 days after iliac artery ligation. Ultrasound-targeted gene delivery was then performed,
according to assigned treatment group: group 1- control, no treatment; group 2- GFP plasmid; group 3- VEGF165/GFP plasmid (n=18 per group). Repeat CEU was performed at days 17 (n=6 per group) and 28 (n=12 per group). In order to assess late gene transfection and efficacy, an additional 36 rats (n=12 per treatment group), were studied at 8 weeks post-ligation. In 4 rats per group, FMA was performed immediately prior to sacrifice. In remaining animals, skeletal muscle tissue from the ischemic and non-ischemic adductor muscles, as well as tissue from the lungs, heart and liver was obtained for post-mortem immunohistochemistry, quantitative RT-PCR and Western blotting. Normal hindlimb muscles from an additional 6 animals without ligation were obtained for semi-quantitative RT-PCR, for comparison to hindlimb muscles from ischemic animals that underwent ligation.

**Statistical Methods**

Data are expressed as mean±SD. Comparisons between multiple stages were made with one-way ANOVA. When differences were found, inter-stage comparisons were performed using non-paired Student’s t-test with Bonferroni correction. Data for pre- and post-gene therapy were compared by paired Student’s t-test. Differences were considered significant at p <0.05 (2-sided).
Reference List


