Uniform Scale-Independent Gene Transfer to Striated Muscle After Transvenular Extravasation of Vector

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Background—The muscular dystrophies exemplify a class of systemic disorders for which widespread protein replacement in situ is essential for treatment of the underlying genetic disorder. Somatic gene therapy will require efficient, scale-independent transport of DNA-containing macromolecular complexes too large to cross the continuous endothelia under physiological conditions. Previous studies in large-animal models have revealed a trade-off between the efficiency of gene transfer and the inherent safety of the required surgical and pharmacological interventions to achieve this.

Methods and Results—Rats and dogs underwent limb or hemibody isolation via atraumatic tourniquet placement or myocardial isolation via heterotopic transplantation. Recombinant adeno-associated virus (10^13 particles per kilogram) or recombinant adeno-associated virus (10^{14} genome copies/kg) encoding the lacZ transgene was delivered through pressurized venous infusion without pharmacological mediators. Muscle exhibited almost 100% myofiber transduction in rats and dogs by X-galactosidase staining and significantly higher β-galactosidase levels compared with nonpressurized delivery. No significant difference was seen in β-galactosidase levels between 100- or 400-mm Hg groups. The <50-mm Hg group yielded inhomogeneous and significantly lower transgene expression.

Conclusions—Uniform scale- and vector-independent skeletal and cardiac myofiber transduction is facilitated by pressurized venous infusion in anatomic domains isolated from the central circulation without pharmacological interference with cardiovascular homeostasis. We provide the first demonstration of uniform gene transfer to muscle fibers of an entire extremity in the dog, providing a firm foundation for further translational studies of efficacy in canine models for human diseases. (Circulation. 2005;112:1780-1788.)

Key Words: endothelium ■ gene therapy ■ muscles ■ veins ■ muscular dystrophy, Duchenne

Cardiovascular homeostasis in vertebrates depends on the physical integrity and restricted permeability of the microvascular endothelium and its encasing basement membrane. In terrestrial vertebrates, selected physiological and morphological parameters vary in rough proportion to the vertical distance from the heart, reflecting gravitational effects on hydrostatic pressure. The development of any therapeutic interventions that rely on the transport characteristics and rheological properties of the vasculature mandates investigation of scale dependence to avoid the pitfalls of “mouse-to-man” extrapolation. Delivery of drugs targeting sites in the extravascular space from the central circulation requires at least focal if not global permeation of the microvascular barrier. In gene therapy for systemic diseases, the “drug” is a DNA molecule at least 20 times the molecular weight of the encoded protein. Duchenne muscular dystrophy defines an extreme paradigm for this challenge: the target tissue approximates half the total body mass, and the underlying genetic defects can be fully complemented only by in situ synthesis of a high-molecular-weight structural protein.

We and others have previously shown that efficient permeation of the endothelial barrier in larger rodents and dogs requires transient alteration of the functional integrity of this cell layer. The combined use of (1) complete circulatory isolation with the use of tourniquets and proximal arteriovenous cannulation, (2) systemic heparinization to prevent thrombosis, (3) peripheral vasodilation to optimize perfusion of muscle capillaries, and (4) histamine to produce physical gaps between adjacent endothelial cells has achieved widespread, homogeneous, vector- and scale-independent gene transfer to muscles of entire extremities. These studies began with marker transgenes and have been applied to
therapeutic transgenes in several disease models in rodents and dogs. Greelish et al.⁴ described the first somatic gene transfer approach to achieve supraphysiological levels of transgene expression in muscle fibers throughout a limb, as desirable to maintain the highest possible ratio of vector to total myonuclear DNA throughout life.

Despite this progress, the 4 essential components of this vector delivery technology, as listed above, have stymied clinical translation because of the inherent risks of hemorrhage and hemodynamic instability. A better understanding of the microvascular response to histamine might suggest alternative, safer pathways to therapy in systemic diseases. Thus, we focused attention on the mechanism of action of histamine as an endothelial permeabilizing agent. Histamine receptor activation rapidly and reversibly modulates junctions between adjacent endothelial cells.¹⁴–¹⁹ The pattern and distribution of histamine receptors are inhomogeneous and specifically parallel the abundance of loosely organized junctions in the postcapillary venules. It has been proposed that this localization of receptors to the postcapillary venules, a vascular site with inherently low wall stress, promotes the rapid reversibility of the inflammatory response.¹,³,²⁰–²²

In resting muscles, precapillary vasoconstriction restricts orthograde access to the postcapillary venules.²³ Retrograde perfusion of venules from a more central vascular access site is precluded in larger mammals by the presence of valves in the larger peripheral veins.²⁴ We hypothesized that centrifetal or afferent flow from a distal and superficial venous access site would follow the orientation of the peripheral valves while promoting locally retrograde flow through smaller valveless tributaries into postcapillary venules, as long as the pressure applied exceeded that on the arterial side of the circuit. We further hypothesized that a distending transmural force in the venules would mechanically substitute for the transient effect of histamine on endothelial permeability and, if applied rapidly, would overcome homeostatic venoconstriction that might otherwise limit access to the entire vascular tree. Finally, on the basis of observations that homeostasis is rapidly restored after apparent “stress failure” of the microvasculature in both the pulmonary⁵⁵ and mesenteric⁶⁶ circulations, we hypothesized that similar resiliency of the cardiac and skeletal muscle microvasculature would protect against the development of clinical signs of microangiopathy after forced extravasation.

In testing these hypotheses sequentially in small- and large-animal models, we have established an afferent transvenular retrograde extravasation (ATVRX) method for somatic gene transfer to muscle that achieves scale-independent, limb-wide transduction of virtually 100% of muscle fibers, while avoiding the risks of histamine, papaverine, heparin, and arterial access. The findings suggest that the mechanical support provided by the venular basement membranes promotes rapid resealing of the endothelial cell junctions at this anatomic site despite forced extravasation of fluid and macromolecules into the interstitium. The demonstrated safety of this approach should facilitate its adoption for clinical studies in diseases ranging from hemophilia to muscular dystrophy and cardiomyopathy.¹⁰,¹³,²⁷–²⁹

**Methods**

**Animal Studies**

Our selection of appropriate models for these experiments reflected the comparative anatomy and morphology of the vascular tree in rodents, carnivores, and primates. We used 1 small-animal model (Sprague-Dawley rats, male, 0.20 to 0.25 kg) and 1 large-animal model (mixed hounds, male, 5 to 11 kg). Animal studies were approved by the Institutional Animal Care and Use Committee.

**Evans Blue Dye Studies**

Five percent albumin mixed with 10 mg/mL of Evans blue dye (EBD) was infused into adult rats (0.05 mg/g body wt). Six percent
of this solution was diluted to a volume of 20 mL/kg and infused at 400 mm Hg through the greater saphenous vein (GSV; see below) into a tourniquet-isolated hindlimb, whereas the remaining 94% was infused slowly into the GSV of the contralateral hindlimb. Thirty minutes after the start of infusion, animals were euthanized and necropsied.

To study the effects of forced extravasation on postprocedural microvascular function, rats underwent hindlimb tourniquet placement with pressurized infusion of sterile PBS (20 mL/kg) into the isolated limb. After the infusion, the tourniquet was released, and 0.05 mg/g body wt of EBD/albumin was delivered slowly via a jugular vein catheter at 0, 5, and 20 minutes after tourniquet release, followed by necropsy.

Preparation of Recombinant Vectors Derived From Adenoviruses and Adeno-Associated Viruses
All vectors were obtained through the University of Pennsylvania Vector Core. All vectors used encoded the transgene lacZ coupled to the constitutive CMV promoter. Multiple lots with variable titers of vector were used for experiments with the goal of delivering \( \sim 10^{13} \) genome copies (GC)/kg of recombinant vector derived from adenovirus-associated viruses (rAAV) and \( 10^{12} \) particles per kilogram of recombinant vector derived from adenoviruses (rAd) per subject. rAAV serotype 2/1 was used for all experiments except for preliminary studies in the rat in which rAAV serotype 2/1 was used. The vector was diluted in sterile 1× PBS for infusion.

ATVRX Delivery of Vector and EBD in Rats
After administration of xylene and ketamine anesthesia, rats underwent tourniquet isolation as described before with the exception that heat-tapered polyethylene tubes (PE 10; Becton Dickinson) were placed and secured into the GSV, which was then ligated distally. Tourniquets were tightened to isolate the limb circulation, and a total volume of 20 mL/kg of sterile PBS and vector was infused at varying pressures of 100, 200, and 400 mm Hg with a dwell time of 30 minutes. To study the effect of dwell time on the efficiency of delivery, a series of rats were infused at 100 mm Hg ATVRX with dwell times of 0, 5, 15, and 30 minutes. After the tourniquets were released and the catheter was removed, the GSV was ligated. We performed slow continuous infusions with the same volume and dwell times of vector to obtain the \(<50\text{-mm Hg}\) pressure data set. The 0.5-mL infusions without tourniquets illustrated the results of a simple intravenous infusion without ATVRX.

In the hemibody infusions, rats underwent overnight fasting to minimize risk of aspiration from the increased intra-abdominal pressure due to the placement of the infraumbilical tourniquet. The vector was diluted in 10 mL of sterile PBS and delivered via simultaneous infusions through cannulation of bilateral saphenous vein cannulation, similar to above, at 200 mm Hg. All rat experimental groups consisted of 2 rats, and the approximate weight of all rats used was 0.20 to 0.25 kg. Necropsy was performed at day 14.

ATVRX Delivery of Vector in Rat Hearts
Donor rats were exsanguinated through the abdominal aorta and inferior vena cava (IVC) and perfused with ice-cold sterile normal saline. The donor heart was explanted, and all pulmonary veins and left superior vena cava (SVC) were ligated. The heart was immersed in ice-cold normal saline. In the control group (n = 3), rAd was diluted in 2 mL of Plegisol and infused into the explanted hearts through a catheter in the aorta, maintaining anterograde flow at 80 to 100 mm Hg for 10 minutes. In the experimental group (n = 3), the same amount of vector and Plegisol was delivered through the coronary veins via cannulation through catheters traversing the IVC and SVC, maintaining retrograde flow at 60 to 80 mm Hg. The SVC and IVC were ligated with silk ties after the completion of delivery, and the donor heart underwent heterotopic transplantation. The donor aorta and pulmonary artery of the explanted hearts were anastomosed to the recipient’s aorta and IVC below the renal vessels with 7-0 Prolene. The rats were necropsied on day 7.

ATVRX Delivery of Vector in Dogs
Dogs were infused with rAAV (10^{14} \text{GC}) and rAd (10^{12} \text{particles}) diluted in 500 mL of sterile PBS at a delivery pressure of 300 mm Hg. After intravenous sedation with medetomidine and butorphanol, dogs underwent GSV cannulation with a 20-gauge catheter.
angiocatheter similar to the rats. An atraumatic tourniquet was placed at the level of the groin and tightened until the femoral pulse could no longer be palpated. Standard intravenous tubing and pressure bags were used to administer the infusion. The total time of infusion was 20 minutes. The tourniquet was then released, and the cannula was removed. The GSV was ligated. Necropsies were performed at day 14. The same procedure was performed without tourniquet placement to compare the ATVRX data with those of a simple intravenous infusion with rAAV. The rAAV ATVRX group had 3 subjects, the rAd ATVRX group had 2 subjects, and the simple intravenous infusion group had 1 subject. These procedures were performed with the use of mild, rapidly reversible sedation, without the need for endotracheal intubation or mechanical ventilation.

Data and Statistical Analysis

Tissue samples were snap-frozen in isopentane cooled in liquid nitrogen at the time of necropsy. Five- to 8-μm sections and whole-mount sections were stained for X-galactosidase (X-gal) activity as described in our previous publication. Tissues were counterstained with hematoxylin and eosin, and no bluing agent was used. Tissues samples were assayed for β-galactosidase (β-gal) activity with the use of a luminometric kit (Applied Biosystems) and measured for protein concentration with the Dc Protein Assay Kit (Bio-Rad) (see Tables I to III in the online-only Data Supplement at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.105.534008/DC1). All data were analyzed with a 1-way Tukey-Kramer HSD analysis (JMP IN version 4.0.4, SAS Institute, Inc) with α=0.025 and a difference considered to be significant at P<0.05 (see Figures I to IX in the online-only Data Supplement).

Results

Rapid infusion of EBD/albumin against a proximal tourniquet revealed homogeneous fluid and albumin extravasation throughout the limb (Figure 1), creating a volume of tissue edema several times greater than that of the blood volume in the extremity. Microvascular integrity is fully restored by 20 minutes after tourniquet release after ATVRX, as illustrated by the confinement of EBD-albumin to the capillaries of the ATVRX-treated muscles (Figure 1). To address the size dependency of the forced extravasation process, we next substituted recombinant vectors derived from adenoviruses (rAd) and adeno-associated viruses (rAAV).

Both the pattern of tissue transduction and the total amount of the lacZ transgene product detected depended entirely on the method of infusion used. Simple low-pressure infusion of vector into a peripheral vein resulted in tissue β-gal levels similar to levels of the muscles of untreated control animals (Figure 2A). In contrast, successful afferent transvenular retrograde extravasation of vector was achieved with pressurized infusions of the same total dose of vector, in conjunction with tourniquet occlusion at a proximal site (Figure 2A). Muscle tissue from the contralateral limb, outside of the isolated ATVRX regions, exhibited β-gal activity similar to that seen in untreated tissue (Figure 2A). ATVRX with low pressure (<50 mm Hg) had lower levels of transduction in an inhomogeneous pattern (Figure 2B), whereas there was no significant difference in the level or efficiency of transduction of myocytes with variation of the dwelling time (0, 5, 15, or 30 minutes [Figure 2C]). Use of moderate (100 mm Hg) and high pressure (400 mm Hg) resulted in uniform transduction of muscle fibers at an efficiency approaching 100% (Figure 3A and 3B; 400 mm Hg, see online-only Data Supplement Figure X).
Hemibody ATVRX infusion successfully transduced muscles of both hindlimbs and muscles surrounding the pelvic girdle (eg, gluteus maximus; Figure 3C and 3D; 200 mm Hg) with similar efficiency (Figure 4A). β-Gal activity levels were significantly higher than background in the ATVRX-treated muscles, whereas intra-abdominal and intrathoracic tissues of the treated animals (kidney, liver, spleen, testis, heart, lungs, and diaphragm) did not show β-gal levels higher than those of negative control animals (Figure 4B). All animals tolerated the procedures well and gradually mobilized the interstitial fluid loads without signs of cardiopulmonary compromise as they rapidly returned to their preprocedural weights. Normalized to the titers of input vector, the absolute values of tissue X-gal were similar in animals infused with rAAV vectors bearing capsids of serological classes 1 and 2 (see online-only Data Supplement Figure XI).

In in vivo studies in transplanted rat hearts, ATVRX infusion into the coronary vein with myocardial isolation yielded significantly higher β-gal activity compared with rat cardiac myocytes treated via anterograde infusion into the aortic root (Figure 5A and 5B). The majority of the myocardium demonstrated positive X-gal staining on cross section (Figure 5C) as well as at higher magnification (Figure 5D to 5F).

Similar peripheral limb infusion studies were done in the dog. Figures 6 and 7 show that the successful gene delivery with ATVRX is scale independent. Despite the readily discernible increase in the histological thickness of the canine perimysial and epimysial fascia, the pattern and extraordinary efficiency of transduction reflect uniform vector distribution and extravasation throughout the entire hindlimb (Figure 6 and online-only Data Supplement Figure XII). Similar histochimical results were obtained with rAd and rAAV (Figure 7 and data in the online-only Data Supplement). All dogs tolerated this procedure well, generally returning to full, symmetrical weight bearing within minutes of completion of the procedure. None of the dogs in this series demonstrated any clinical signs of muscle dysfunction, hemodynamic instability, or peripheral venous valvular insufficiency after the procedure.

**Discussion**

Collectively, the findings of the present small- and large-animal studies have important implications for vascular biology and gene therapy. From the standpoint of basic research, this study addresses issues at the levels of the macrovasculature and microvasculature. The clinical spectrum of venous stasis disease has provided historical rationale for the detailed study of the anatomy and physiology of valves in the peripheral circulation. Although it is clear that peripheral valvular aplasia and acquired valvular dysfunction predispose to venous stasis pathology, little is known of the signal transduction pathways that dictate the anatomic location of valves during ontogeny. If there were valves to prevent retrograde flow to the tributaries draining blood from individual muscles into the named veins, it is unlikely that the procedure used in the present studies would achieve the observed uniformity of gene transfer throughout the limb in the dog. Our dissections at necropsy in adult dogs and in human cadavers in the anatomy teaching laboratories support the textbook assertion that vessels <2 mm in diameter do not have valves. We therefore model the pathway for flow during the infusion as afferent or anterograde through the valve-rich larger veins but retrograde through the valveless tributaries (Figure 8). Our catheterization of superficial branches of distal veins also provides for flow in the appropriate direction through the perforating veins that connect to the deep system. Thus, the described approach allows widespread distribution of vector through the normal vascular anatomy without the need for damaging retrograde flow across the venous valves. None of the animals in this study developed clinical evidence for postprocedural peripheral valvular incompetence.

At the microvascular level, 2 distinct issues are addressed. First, this study provides a convincing demonstration of scale-independent, widespread extravasation of DNA-protein complexes from sites accessed by way of the venous side of
the circulation. From the standpoint of time-honored physiological models of the microvasculature, the effective diffusion diameters of these “nanoparticles” can be approximated as 100 nm and 25 nm, respectively. These dimensions suggest that regardless of net charge, both Ad and AAV vectors are vastly too large to be accommodated by the smallest and most abundant of the proposed “pores” regulating mural permselectivity under normal circumstances. Our earlier studies using high-pressure infusion from the arterial side showed that only in the presence of histamine was there detectable transport to the interstitium, supporting a model in which even the larger pores were too small to accommodate these vectors under physiological conditions. The present experimental findings are consistent with our hypothetical model for mechanical distention and perturbation of the endothelial sheet. Although the precise site and the mechanism of vector transport across the endothelium are below the anatomic resolution of our studies, the infusion approach was developed on the basis of a plausible mechanism of rapidly reversible alteration in the morphology of individual cells (intracellular/transcellular openings) and/or the strength of adhesive contacts between adjacent endothelial cells (intercellular openings) (Figure 8). The locally high concentration of histamine H2 receptors along with the weakest intercellular contacts along the entire endothelial sheet identifies the postcapillary venules as a likely point of particle extravasation in our present studies. Pressure-induced transcellular extravasation of red blood cells has been documented at the level of single venular capillaries in the frog mesentery, providing an alternative or parallel route for vector transport in the present studies.

Neither species studied, rats or dogs, exhibited any clinical evidence of postprocedural angiopathy. Coupled with the EBD studies, this suggests that the involved vascular segment(s) has an inherent ability to rapidly recover normal function after the transient pressure-induced elevation in mural permeability. The extravasation of virus-based vectors will likely require significantly lower pressures than those required for the extravasation of red blood cells. Nonetheless, recent studies show that even the transmural pressures required for red cell extravasation are well tolerated in selected vascular beds, suggesting that endothelial “sensing” provides a pressure pop-off mechanism to protect against irreversible injury.
Numerous functions have been attributed to the microvascular basement membrane. This viscoelastic supporting structure probably also plays a major role in the ability of the postcapillary venules to undergo rapidly reversible changes in permeability without permanent compromise in mural function. It has been suggested that in its static role supporting the endothelial sheet, the thickness of the basement membrane varies as needed to accommodate regional differences in transmural pressure.6 Ultrastructural studies have provided ample evidence of the barrier function of the intact basement membrane when skeletal muscle microvascular beds are studied in the context of transmural pressures in the normal physiological range.38 In both our previous and present studies, the dependence of vector transport on supraphysiological transmural pressures suggests that the rapid convective flow of water into and across the venular basement membrane is sufficient to overcome the barrier function of this layer. In larger mammals, the relatively increased thickness of this layer is expected to augment its barrier function with regard to particle transport.9 This has important implications for translational studies of this technology and strongly supports the role for large-animal studies. In the dog, our control experiments have revealed negligible gene transfer from the vasculature to muscle when Ad or AAV vectors are infused with microvascular transmural pressures in the physiological range.

We have used AAV vectors of serotypes 1 and 2 in these studies with virtually indistinguishable findings. This contrasts sharply with the results of studies in which these and other related vectors have been evaluated after direct intramuscular injection (eg, AAV6, which appears to have evolved from an AAV1 on 2 homologous recombination39). Our data suggest that some of the serotype-specific patterns of gene transfer reflect differences in diffusivity, differences that recede in the context of forced convective transport. Although recent studies demonstrate widespread gene transfer after simple tail vein injection in mice and hamster pups with AAV6 and AAV8,40,41 the exact mechanism of vector transport across endothelium remains to be elucidated. Similar intravenous infusions in rats and dogs using AAV1 and AAV2 suggest size-dependent limitation of the smaller rodent models (eg, thin basement membranes and complete absence of peripheral venous valves). Early studies with adenovirus vectors in the mouse42 also underestimated these barriers to systemic gene transfer in larger species, suggesting that relevant aspects of vascular biology are dictated by animal size.

In the present studies we applied transmural pressures over a range from <50 to 400 mm Hg and with varying dwell times. Statistical analysis of this data suggests that pressures as low as 100 mm Hg without any need for dwell may saturate the vector transport effect with this approach, although much higher pressures with dwell times up to 30 minutes were well tolerated by the animals even during infrarenal tourniquet occlusion. This “dose-response” relationship should be specifically titrated in the large animal before clinical application. The bipedal stance of humans imposes a uniquely high ratio of venous pressure in the distal pelvic limb to total body weight.7 Only adult, large-breast dogs and larger primates, in particular the great apes, approximate this aspect of the relevant vascular biology, as the larger hoofed quadrupeds have specific adaptations in the distal limbs that may affect edema prevention.6,8 In diseases in which regional gene transfer to a single limb might provide a durable therapeutic effect, upper limb infusion may provide an opportunity to achieve adequate gene transfer at lower infusion pressures.

In the muscular dystrophies, this approach could be applied sequentially to all 4 extremities as long as central venous pressures were appropriately monitored. As noted, our single limb infusions in the healthy dog are rapidly performed under light sedation with minimal monitoring. Studies in the dystrophic dog will be essential to address the possibility of...
unforeseen, disease-specific issues in the application of this technology. The present study provides the first demonstration of scale-independent muscle transduction of essentially 100% of the muscle fibers in a vertebrate limb, as is desirable for single-dose transduction at saturating levels to provide the most durable therapeutic effect. Ultimately, the therapeutic end point in the muscular dystrophies is stable transduction of the limb, limb-girdle, truncal, respiratory, and cardiac muscle mass. Although our experimental findings have provided a welcome alternative to the need for pharmacological manipulation of the endothelium, they do not directly address the need for comprehensive, systemic gene delivery. Extrapolation of this approach to the systemic circulation will require ongoing protection of the heart and lungs from the physiological consequences of transiently elevated transmural pressure, a focus of our ongoing studies.

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**References**


CLINICAL PERSPECTIVE

The circulatory systems of terrestrial vertebrates must achieve homeostasis in the presence of scale-dependent hydrostatic pressure differences induced by the gravitational field. Several anatomic correlates of this functional constraint are evident in comparisons between small and large mammalian species. At least 3 such anatomic differences have the potential to complicate the “mouse-to-man” extrapolation of results from drug and gene delivery studies. In the present study the authors test several interrelated hypotheses about macromolecular transport in the rat and dog and apply the findings to the problem of vascular delivery of vectors for gene therapy. Iteration of the approach results in a general method that is both scale and vector independent for gene delivery from the vascular space to striated muscle. The demonstration of limb-wide transduction of virtually 100% of muscle fibers in the dog represents an important technological breakthrough and provides a platform for further development toward gene therapy in muscular dystrophy and other genetic diseases (eg, hemophilia). Additional data show that the method may be generalized to the coronary circulation, as will be critical to address both the genetic cardiomyopathy of muscular dystrophy and acquired forms underlying congestive heart failure.
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