Pressure-Induced Vector Transport in Human Saphenous Vein

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Abstract—The efficiency of gene therapy as a pretreatment for saphenous vein coronary artery bypass grafts can be improved by increasing the transport of vector into the tunica media. The purpose of this study was to determine the effect of increasing transmural pressure on vector delivery depth in human saphenous vein segments. Specifically, we introduced adenovirus-sized microspheres luminally to observe changes in transport efficiency into the intimal and medial layers with increasing pressure. Our results indicate that transmural pressures of 100 and 400 mmHg increase the intimal concentration of microspheres as compared to 0 mmHg ($p < 0.03$), but do not significantly affect medial concentrations. We did not find increasing concentrations with increasing pressure above 100 mmHg. These results suggest that low or intermediate transmural pressures are adequate for intimal vector delivery and that techniques other than increasing pressure are required to deliver gene therapy vectors (≥ 100 nm) to medial smooth muscle cells. Also, our data support previous models designating the internal elastic lamina as the primary barrier to particle transport. Finally, our *ex vivo* microsphere perfusion experiment represents a novel way to explore functional vein permeabilities to gene therapy vectors and, ultimately, optimize vascular gene therapy protocols.

Keywords—Fluorescent microspheres, Gene therapy, Adenovirus model, CABG.

INTRODUCTION

Gene therapy designed to limit intimal hyperplasia is a promising method for improving the long-term success of coronary artery bypass graft (CABG) surgery in which saphenous vein bypass grafts are used. Genes that inhibit smooth muscle cell proliferation can be transfected into the bypass vein to prevent hyperplastic occlusion of the vein lumen.[1,8,18] Similarly, genes that limit smooth muscle cell migration, or other critical events in intimal hyperplasia, can be therapeutically administered. There are many vec-

tors that can be used for vascular gene transfer,[8,14,28] with more being designed each year. Of those currently available, adenovirus has the highest transfection efficiency in both dividing and nondividing cells,[4,14] and has been successfully transfected into human vascular tissue.[26] The complications arising from systemic exposure to adenovirus, such as an overwhelming immune response,[13] are reduced with localized catheter-based or *ex vivo* delivery strategies. However, in intact vascular tissue, the efficiency of transfection to smooth muscle cells in the media remains low.[27]

For a gene therapy vector to reach medial smooth muscle cells (SMCs), the vector must travel from the suspending fluid medium of the lumen to the vascular wall, bypass endothelial cells lining the internal elastic lamina (IEL), pass through pores in the IEL, and traverse the extracellular matrix (ECM) in the tunica media. After successful transport to target medial SMCs, infection of the cell, nuclear entrance, and transcription of the therapeutic gene must follow for complete transfection. The first steps in this process, transport through the intimal layer to target cells in the tunica media, is often the rate-limiting one, and is the focus of our investigation.

Inert and biological particle transport studies have been performed in arterial tissue;[15,16,30,33] however, venous tissue is functionally and structurally different. A more detailed understanding of particle transport in human venous tissue is required to improve delivery of genes to target cells in this tissue. Therefore, the goal of this study was to characterize the barriers to medial transport of gene therapy-like particles administered *ex vivo* to whole sections of live human saphenous vein. In particular, we studied transmural pressure for its effect on the transport of inert microspheres size-matched to adenoviral vectors (100-nm diameter).

Inert microspheres have been used successfully in a variety of gene therapy applications to model particle transport due to their ability to mimic adenoviral transport sans receptor–ligand interactions at the site of entry.[23,27,29,34] Although biologically inert microspheres cannot truly mimic receptor-mediated transfection of a gene therapy vector, the conclusions one can draw from isolating the physical

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barriers to transport are critical in characterizing the transport process as a whole. Once the barriers to transport and their susceptibility to hemodynamic perturbations are identified, new strategies for increasing transport and overall transfection efficiency may be designed and then tested using the same experimental methodology.

MATERIAL AND METHODS

Ex Vivo Experiments

Human saphenous vein specimens, ranging in length from 1.5 to 3 cm, were obtained from cardiac surgeries performed at the University of Vermont-Fletcher Allen Health Care, Burlington, Vermont. Human IRB approval was granted for the use of unused vein tissue in research. All vessels were stored at 4°C and used within 6 h of collection. When long enough sections of high quality tissue were available, vessels were sectioned into two equal segments: one experiment and one control. Otherwise, specimens from two separate surgeries were used for experiment and control. All specimens were handled in a ventilated biosafety cabinet and sutured in a sterile fashion into a custom-engineered environmental chamber. Separate chambers were used for experiment and control. Each chamber had inflow and outflow stainless steel cannulae for perfusate fluid, which were adjustable for different length specimens. Veins ranging in diameter from 2 to 5 mm were accommodated with variable size cannulae. The chamber also allowed complete submersion of the vein specimen, could be sealed closed for sterility, and had a viewing window. Two gas exchange ports with sterile 0.22 μm filters allowed incubator-controlled 5% CO_2/95% lab air to flow freely into and out of the chamber at atmospheric pressure. The experimental setup and one chamber are shown in Fig. 1.
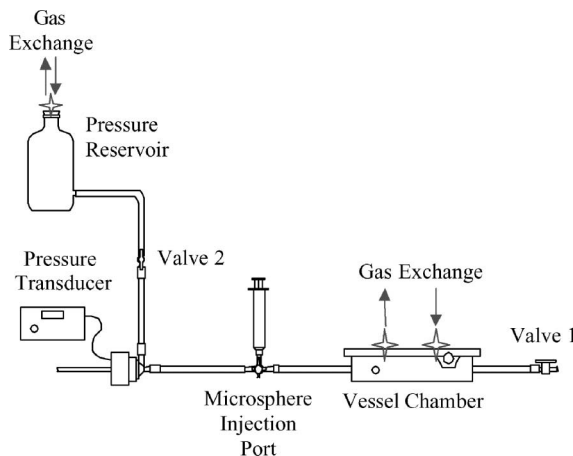

FIGURE 1. *Ex vivo* microsphere perfusion system and vessel chamber. Pressure reservoir, pressure transducer, microsphere injection port, gas exchange ports, and flow control valves shown.

Dulbecco's Modified Eagle's Medium (DMEM) (Sigma–Aldrich, St. Louis, MO) was used to fill the chamber and bathe the vessel. After veins were sutured to the cannulae, DMEM was flushed through the vessel at a flow rate of approximately 1 ml/min. Plasma proteins and growth factors were selectively excluded from the luminal and chamber media so that the effect of pressure could be investigated in the absence of receptor–ligand binding. Vessels were checked for leaks by prepressurizing them to approximately 400 mmHg for less than 1 min with valve 1 closed (see Fig. 1). If a leak was unable to be repaired, the vessel was discarded. The same steps were carried out for the control vessels. Desired pressures were obtained by raising the pressure reservoir and measured with a research grade blood pressure transducer (Harvard Apparatus, Holliston, MA). Once both vessels were successfully sutured into their respective chambers, they were brought to a baseline pressure of 30 mmHg, at which time valve 2 was closed. Chambers were then sealed and allowed to equilibrate in a temperature-controlled incubator (37°C) for 1 h.

After the equilibration time, valve 1 was opened and a solution of Texas Red-labeled carboxylate-modified polystyrene microspheres (1×10^9 spheres/ml, Molecular Probes, Inc., Eugene, OR), 100 nm in diameter, was slowly infused through the injection port. A microsphere titer of 10^9 spheres/ml was selected based on a number of gene transfer studies reporting successful transfection to vascular tissues at this concentration.[5,6,9,10,14,17,19,21,24,26] These studies tested adenoviral titers ranging from 10^8 to 10^{12} pfu/ml in a variety of environments and found the highest rate of viral transfection to occur at 10^9 pfu/ml. Valve 1 was then reclosed. By opening valve 2 and readjusting the reservoir height, experimental veins were pressurized to 100, 200, or 400 mmHg transmural pressure while the control veins remained at nearly zero transmural pressure, or the minimum pressure at which veins did not collapse (always less than 5 mmHg). The transmural pressure range selected for this experiment (0–400 mmHg) incorporates both *in vivo* physiological pressures for the long saphenous vein (5–75 mmHg[2,31]) as well as pressures in the range often used in vascular gene therapy applications (0–800 mmHg).[5,6,17,19,21,27] Finally, valve 2 was reclosed and the pressure was recorded by the pressure transducer and relayed to a computer. If the pressure dropped by more than 5% of the desired level, the experiment was stopped and the problem rectified or the vein was discarded. After a 1-h exposure, all valves were opened and veins flushed with DMEM at approximately 1 ml/min to remove microspheres remaining in the fluid but not adhered to or taken up by the vein. A small piece of vein was sectioned from the end of some specimens and placed into Karnovsky's fixative for transmission electron microscopy. Remaining tissue specimens were frozen in tissue freezing medium blocks and sectioned on a cryostat to 10-μm thickness. Slides of thin

sections were stained with a SYTOX green nucleic acid stain (Molecular Probes, Inc.).

Fluorescent Image Acquisition and Analysis

Fluorescently-labeled microspheres and cell nuclei were visualized at $10 \times$ magnification using a mercury-arc lamp and fluorescent microscope (Eclipse TE2000-S, Nikon, Melville, NY). The SYTOX-stained nuclei were excited first using a FITC filter (488/507 nm) followed by the red microspheres using a TRITC filter (535/617 nm). Three representative images were captured for each tissue section by an observer blinded to experimental condition. Digital images were relayed to a PC for semiquantitative sphere localization analysis with MetaVue Imaging software (Optical Analysis Systems, Nashua, NH). The resulting images of microspheres and cell nuclei (red and green, respectively) were then overlayed.

A square region of interest (0.8 mm^2) was drawn on to the image, isolating the vein tissue and excluding nontissue components. The autofluorescence of the IEL served as a boundary marker between the intima and media while the autofluorescence of the external elastic lamina and cell morphology changes served as a boundary between the medial and adventitial layers. The intima and media within the square region of interest were carefully traced and thresholded to generate measurements of total layer (intima or media) area (mm^2), layer area represented by microspheres (mm^2), percent layer area represented by microspheres (%), maximum intensity, minimum intensity, and average intensity.

Electron Microscopy

Transmission electron microscopy (TEM) was performed on selected specimens to confirm the location of microspheres in the vessel wall. After fixation in Karnovsky's fixative, the tissue was postfixed in 1% osmium tetroxide at 4°C for 45 min, dehydrated, cleared, and embedded in Spurr's epoxy resin as previously described.[22] Semi-thin sections were cut with a diamond knife, placed onto a copper grid, contrasted, and examined with a JOEL JEM-1210 electron microscope operating at 60 kV. These studies were carried out at the Cell Imaging Facility at the University of Vermont.

Statistics

Nine control, five 100 mmHg, five 200 mmHg, and five 400 mmHg experiments were performed. Results are expressed as mean values ± standard error of the mean (SEM). A Levene's test for homogeneity was used to check that variances were equal across groups. Mean values were compared using a two-way ANOVA for the effects of pressure and layer on microsphere area (SAS, SAS Institute, Cary, NC). Model fits to a line were also performed to assess

the continuous effect of pressure on the layer-specific data using Spearman's correlation. For the latter, the correlation coefficient (r_s), coefficient of determination (R^2), and p value (if significant) are reported. Differences between groups were considered statistically significant for values of $p < 0.05$.

RESULTS

The location of the microspheres, specifically whether situated intra- or extra-cellularly, was investigated using TEM. Images taken at $15,000 \times$ revealed small circular objects lining the lumen of the veins that were identified as microspheres in separate TEM studies on isolated microspheres. No microspheres were identified in the media using this method. A representative TEM image of microspheres lining the intima of a human saphenous vein segment is shown in Fig. 2.

Qualitative observations of fluorescent images revealed more particles along the intima of vessels perfused at high pressures than in vessels perfused at 0 mmHg (Fig. 3). Quantitatively, by image analysis, nearly double the area represented by microspheres was found along the intima of vessels perfused at 100 and 400 mmHg compared to vessels perfused at 0 mmHg. The effect of pressure on intimal microsphere area was significant ($p = 0.03$). However, tests for specific differences were only significant at 100 and 400 versus control. The percent area of microspheres along the intima measured at 200 mmHg was higher than
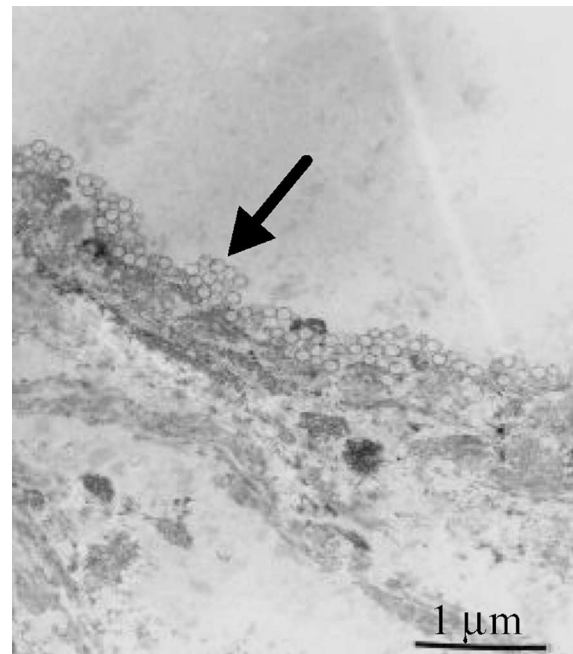

FIGURE 2. Transmission electron microscopy image of human saphenous vein perfused at 400 mmHg taken at 15,000 × magnification. Microspheres are indicated with an arrow.
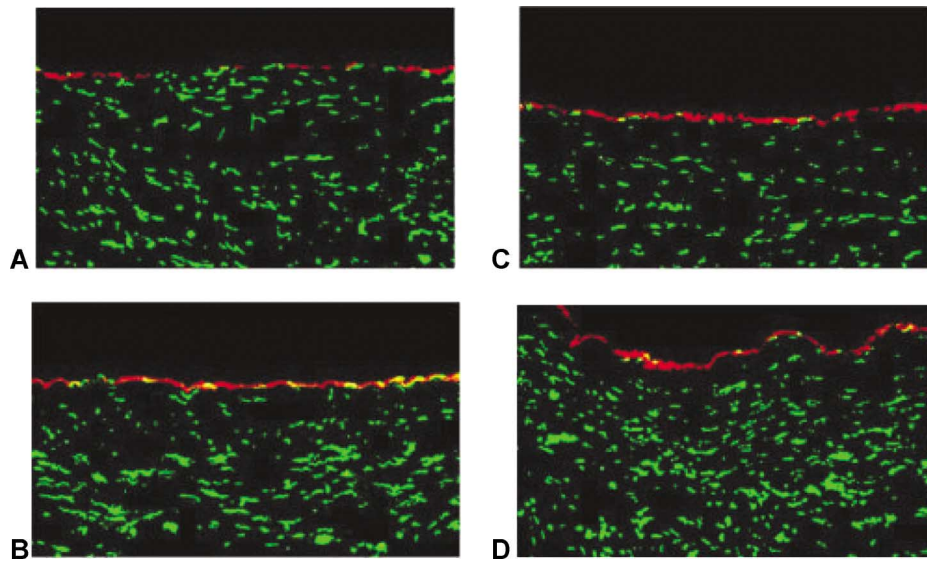
FIGURE 3. Representative fluorescent images of human saphenous vein perfused at pressures of (A) 0 mmHg, (B) 100 mmHg, (C) 200 mmHg, and (D) 400 mmHg with Texas Red-labeled fluorescent microspheres and stained with a SYTOX green nucleic acid stain. All images taken at 10 × magnification.

the control group, but not significantly ($p = 0.07$). None of the intimal microsphere percent areas for the high pressure groups were different from each other [Fig. 4(A)]. Furthermore, when considering the effect of pressure in the intima as a continuous variable, no significant relationship was found. In the intimal layer, the continuous effect of pressure on microsphere area was not well described by either a linear ($p = 0.30$, $R^2 = 0.14$) or logarithmic ($p = 0.09$, $R^2 = 0.28$) curve fit.

The areas of microspheres present in the media were less than 0.1% on average [Fig. 4(B)]. There was a notable but statistically insignificant linear correlation between increasing pressure and decreasing microsphere area ($p = 0.13$, $R^2 = 0.062$, $r_s = -0.32$). That is, it is unlikely that pressure has no effect on medial concentration, but it cannot be said with confidence that it acts to decrease the medial concentration. Note that the observer-defined intimal and medial areas measured were not significantly different as a function of pressure (Table 1).

DISCUSSION

Our *ex vivo* experiments on pressure-driven microsphere transport in human saphenous vein showed an increase in microspheres along the intima with transmural pressures of 100 mmHg and above. However, no significant increase in microsphere concentration (percent area) with increasing pressure was observed for pressures over 100 mmHg. No differences were observed in medial concentration at any pressures. To interpret these findings, we consider the effects of transcytosis, diffusion, and convection on particle transport in tissue.

Transcytosis

Transcytosis may well affect the uptake of receptor-mediated adenoviral gene therapy vectors, but is unlikely to affect transport of the microspheres used in this study for several reasons. First, carboxylate surface modifications
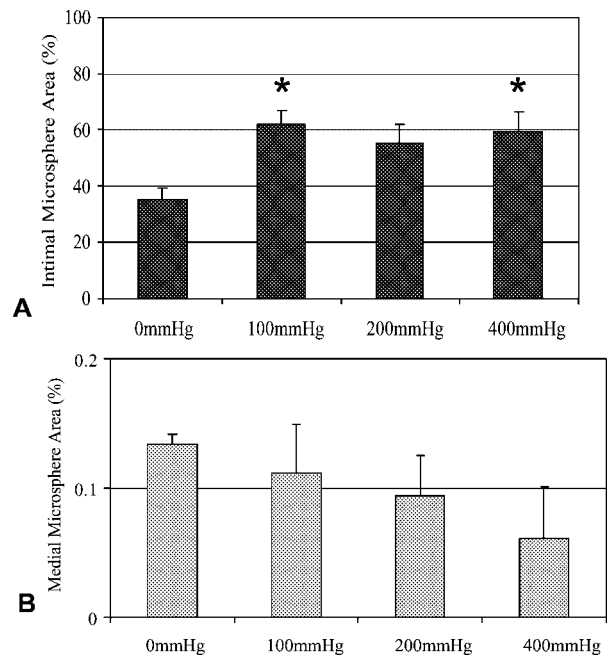

FIGURE 4. Percent area of (A) tunica intima and (B) tunica media containing fluorescent microspheres by semiquantitative image analysis. Bars show mean ± standard error of the mean. Asterisk indicates significantly different from 0 mmHg, $p < 0.03$.

TABLE 1. Average (AVG) and standard error of the mean (SEM) of intimal and medial area in human saphenous vein specimens by semiquantitative image analysis. No statistically significant differences were found between any groups for either layer.

| | Regional area (mm^2) | | | |
| | Intima | | Media | |
Transmural pressure	AVG	SEM	AVG	SEM
0 mmHg	0.007	0.001	0.22	0.03
100 mmHg	0.007	0.002	0.13	0.02
200 mmHg	0.007	0.001	0.17	0.04
400 mmHg	0.008	0.001	0.18	0.03

make these microspheres relatively hydrophilic with an approximate charge between 0.1 and 2.0 mEq/g. This hydrophilicity should limit protein adsorption to the surface of the microspheres, thus minimizing receptor-mediated recognition and interactions. Second, the suspension solution used in this experiment was serum-free. That is, few proteins were available to bind to the microspheres and react with cell surface receptors by experimental design. Note, because gene therapy to human saphenous veins can be administered *ex vivo*, the lack of serum proteins in the perfusate does not represent a limitation that would preclude this methodology from being used with adenovirus. Finally, no evidence of transcytosis in the endothelial cells was evident by TEM.

Diffusion

Diffusive flux is dependent on the surface cross-sectional area of the wall, concentration difference of vector particles across the vessel wall, and the coefficient of diffusivity of a vector within the vessel wall. Diffusive characteristics of any vector, to a first order approximation, depend inversely on size and solvent viscosity. The viscosity characteristics of the vascular wall depend on structure, composition, and solid volume fraction. These characteristics have not been measured for human saphenous vein and they are difficult to estimate *a priori*. Given the dense cellular and extracellular matrix structure of venous tissue, we would expect the effective tissue viscosity to vary between layers and to be generally very large. In the luminal fluid, which we can use as an upper limit on the diffusivity within tissue layers, the diffusivity (D) of a 100-nm fluorescent microsphere in DMEM [with viscosity 0.01 g/cm/s and temperature 37°C (310 K)] is approximately 4.5×10^{-8} cm^2/s based on the Stokes–Einstein equation for diffusivity.[3] For an approximate intimal tissue thickness $h = 0.01$ cm, the time scale for diffusion can be estimated as

$$\tau \approx \frac{h^2}{D} = \frac{(0.01\,\text{cm})^2}{4.5 \times 10^{-8}\,\text{cm}^2/\text{s}} = 2200\,\text{s} \approx 37\,\text{min} \quad (1)$$

which is less than the 1-h experiment duration. For an approximate medial tissue thickness $h = 0.05$ cm, the time

scale for diffusion can be estimated as

$$\tau \approx \frac{h^2}{D} = \frac{(0.05\,\text{cm})^2}{4.5 \times 10^{-8}\,\text{cm}^2/\text{s}} = 56000\,\text{s} \approx 15\,\text{h} \quad (2)$$

which is significantly longer than the 1-h experiment duration. Based on these approximations, diffusion indeed plays a role in particle transport in the intima, however is unlikely to affect particle transport in the media.

Convection

In contrast to transcytosis, convection should have a significant effect on vector transport within both the intimal and medial layers. In tissue layers modeled simply as thin membranes, translayer solute convection is proportional to the translayer fluid solvent flux per unit area, J_v (cm/s), described by Kedem and Katchalsky as[12]

$$J_v = L_p(\Delta P - \sigma \Delta \Pi) \quad (3)$$

where L_p is the hydraulic conductivity (L_p, cm/s/mmHg), ΔP is the transmural pressure ($\Delta P = P_i - P_e$, mmHg), σ is the osmotic reflection coefficient (σ, dimensionless), and $\Delta \Pi$ is the osmotic pressure gradient across the vessel caused by the solute ($\Delta \Pi = \Pi_i - \Pi_e$, mmHg). For a single solute, the $\Delta \Pi$ term is proportional to the concentration difference of the solute and hence represents the effect of diffusion. When the concentration of a single solute is high, or when several solutes act together to create a large osmotic pressure difference across a membrane, this pressure difference and the hydrostatic pressure difference drive fluid solvent flow. Solute or particle flux driven by the combination of fluid solvent flow (convection) and the osmotic pressure difference (diffusion) is described by Friedman[7] as

$$J_{s,c} = \bar{c}_s J_v (1 - \sigma) + \omega \Delta \Pi \quad (4)$$

where $J_{s,c}$ represents solute (microsphere) flux per unit area ($J_{s,c}$, particles/cm^2/s), $\bar{c}_s$ is the average molar concentration of solute ($\bar{c}_s$, particles/cm^3), and ω is the permeability of a layer to the solute (ω, particles/cm^2/s/mmHg). For our experiments, both the fluid flux and the particle flux driven by the osmotic pressure difference should be negligible given the relatively dilute microsphere solution in the vessel lumen and the lack of a strong ionic charge on the particles. In this case, the flux of particles across the wall reduces to

$$J_{s,c} = \bar{c}_s L_P \Delta P (1 - \sigma) \quad (5)$$

Based on this rather simple model of vector transport to and through the vessel wall, our data suggest that pressure-driven convection increases intimal particle accumulation for pressures between 0 and 100 mmHg, but is thereafter counteracted by a decrease in tissue hydraulic permeability L_p or increase in reflection coefficient σ. In all cases, the concentration difference ($\bar{c}_s$) across the wall (all layers combined) is constant. We can speculate that for transmural pressures over 100 mmHg, medial tissue

components become compacted and IEL pores collapse. This phenomenon has been shown to occur in arteries subjected to high pressures.[11] In particular, Huang *et al.* showed that in rat aorta subjected to high transmural pressures, a large pressure drop occurs across the IEL pores causing a decrease in pore entrance area and intimal cell indentations over the pores.[11]

An alternative explanation for the lack of pressure dependence in the intimal particle concentration above 100 mmHg is that pressure-dependent differences were masked by a loss of particles after the experiment due to the slow flushing of the vein with particle-free perfusate. That is, it is possible that more particles were deposited intimally at higher pressures but that this thicker layer was more vulnerable to removal by fluid shear stresses. We would note that any particles not adhered to the intima well enough to withstand this insult would likely not withstand shear stresses due to coronary blood flow after implantation as a bypass graft and thus would not effectively transfect cells.

The predominant exclusion of particles in this experiment from the media was also found in rabbit arteries by Rome *et al.* in which microspheres (93 nm in diameter) were delivered using a double-balloon catheter for pressures ranging from 100 to 400 mmHg.[27] Our results also agree with Rekhter *et al.*'s adenoviral transfection experiments in human saphenous vein rings *ex vivo* and in rabbit arteries *in vivo*.[25,26] Despite nearly 100% transfection efficiency to endothelial cells in 3–4 mm thick rings of human saphenous vein, less than 5% of subendothelial or medial smooth muscle cells successfully expressed the transgene.[26] Rekhter *et al.*'s submersion of ring specimens in an adenoviral solution most closely mimics the zero pressure condition of our perfusion study in which some intimal particle deposition and effectively zero medial delivery was found.

Finally, according to our data, the concentration of particles in the tunica media does not increase with perfusion pressure for any pressure. We can speculate that this is due to the relative impermeability of the IEL to microspheres under these conditions. The vast majority of microspheres (0.1 μm in diameter) accumulated along the intima and were not successfully transported through pores in the IEL, which have been estimated to be between 1.2 and 17.8 μm in diameter.[32] While increased circumferential stretch might be expected to open pores, we also speculate that pore compaction occurs at increased pressures based on the findings of Huang *et al.*[11] Finally, particle accumulation along the intima at increased pressures may act to block further microsphere transport into the media through IEL pores.

Once microspheres do enter the relatively spacious medial layer, particles are likely convected all the way through the media into the adventitia by conserved translayer flow. This speculation is supported by our frequent observation of more microspheres in the adventitia than in the media. Thus, we suggest that the major mechanism for particle transport in the media is via convection.

Experimental Considerations

With regard to the experiment, the determination of intimal and medial regional areas within a tissue section was subjective. The observer based the region boundaries on tissue autofluorescence, and cell shape and orientation changes. The observer was blinded to the experimental condition and defined all regions for all tissues in one sitting in an effort to increase consistency. The lack of differences in the regional area measurements for different conditions (Table 1) suggests that the observer was consistent and unbiased.

Variability in the condition of the tissue may have contributed to the large standard errors in our results. Since many veins were obtained from different patients, differences between specimens are likely. Also, the occurrence of surgical injury to human saphenous vein during explant is well known.[20] While endothelial cells were visible in all vein specimens in our study histologically (not shown), there is little doubt that injury has occurred due to either or both surgical and experimental procedures. However, for the purposes of these experiments, the advantages of using human tissue far outweighed the disadvantages of nonuniform and nonideal vein sections. Lastly, more significant differences between layer-specific particle concentrations may have been observed if we had tested within a lower pressure range. Experiments in a more physiological pressure range (5–70 mmHg) will be the subject of future work.

CONCLUSIONS

The ability of our experimental protocol to identify pressure- and layer-specific vector barriers is useful to understanding normal human venous physiology, as well as isolating both limiting and permissive factors in vascular gene therapy transfection. The *ex vivo* microsphere perfusion system represents a simple and cost-effective approach to test gene therapy protocols in human tissue in the absence of receptor–ligand interactions.

Inert, physical models of vector transport cannot replace a search for the complex biological determinants of increased gene therapy transfection efficiency, but rather could spark new ideas that take maximal advantage of both physical and biochemical mechanisms. The long-term goal of this research, therefore, is to develop a pretreatment or intraoperative protocol for administering gene therapy utilizing both physical and biological mechanisms to significantly decrease the failure rate of saphenous vein bypass grafts, and thus improve the outcome of coronary artery bypass graft surgery.

ACKNOWLEDGMENTS

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