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Transplantation of *lac-Z*-Transduced Microvascular Endothelial Cells into the Skeletal Muscle Capillary Bed of the Rat Hindlimb Occurs Independent of the Duration of Femoral Artery Occlusion after Injection of Cells

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The skeletal muscle capillary bed may be an ideal recipient site for transplantation of genetically modified autologous endothelial cells and thus provide a basis for a technique of somatic gene therapy that would be applicable to a variety of acquired and inherited human diseases. The purpose of this study was to test the hypothesis that adhesion of *lac-Z*-transduced microvascular endothelial cells (MVEC) in the skeletal muscle capillary bed *in vivo* is dependent on the duration of arterial occlusion after injection of the transduced MVEC. MVEC derived from the abdominal fat pad of syngeneic rats (Wistar F-455) were transfected with the BAG vector, a replication-incompetent retroviral vector containing the *lac-Z* gene for β-galactosidase and the Tn5 gene for selection of the transduced cells by the neomycin analogue, G418. *lac-Z*-transduced MVEC were radiolabeled with ^{125}I-PKH-95, and, after the femoral artery was occluded for 10 min, these cells (1 to 2 × 10^6) were injected intraarterially into the rat hindlimb. In the experimental groups the femoral artery clamp was removed at 0, 60, or 120 min after injection. A control group without pre- or postinjection femoral arterial occlusion was also studied. Adhesion of MVEC in the skeletal muscle capillary bed (mean percentage of injected ^{125}I activity) was determined in groups of 4 rats at 1 day, 1 week, and 1 month after injection. Adhesion of the transduced MVEC did not increase as the duration of femoral artery occlusion after injection was increased. The highest rate was found in the group subjected to only a 10-min preclamp (32% at 1 day, 17% at 1 week, and 15% at 1 month). These results indicate that mechanical forces such as capillary shear rate and perfusion pressure may not be important determinants of adhesion and incorporation of transduced MVEC into skeletal muscle capillaries. Nevertheless, these results document significant adhesion and persistence of genetically modified MVEC transplanted into the capillary bed of the skeletal muscle. © 1994 Academic Press, Inc.

INTRODUCTION

Advances in sequencing of the human genome and development of molecular techniques for gene transfer into a target cell have made gene therapy technically feasible. One approach to somatic gene therapy is to remove cells targeted for gene transfer from an affected individual and transfer the recombinant gene into those cells *ex vivo*. These transduced cells may then be reimplanted at a specific recipient site *in vivo*. Optimal target cells and recipient sites have not been established for most forms of somatic gene therapy.

Endothelial cells have recently been shown to be suitable target cells for gene transfer [1–10]. Delivery of a gene product directly into the circulation by transduced endothelial cells may be an effective form of somatic gene therapy. However, the optimal *in vivo* recipient site for incorporation of genetically modified endothelial cells has not been determined. Although genetically modified endothelial cells have been transplanted successfully onto synthetic vascular grafts, and onto the wall of large muscular arteries after the existing endothelium has been denuded by enzymatic or mechanical techniques, the cells incorporated into the surfaces of these conduits may be too few in number to achieve an adequate recombinant protein concentration in the systemic circulation [2, 3, 9]. Since capillaries constitute more than 80% of the surface area of the circulatory system, they are a logical recipient site to transplant genetically modified endothelial cells in order to achieve systemic concentrations of a recombinant protein.

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We have recently documented short-term recombinant gene expression of *lac-Z*-transduced microvascular endothelial cells (MVEC) transplanted into the intact capillary wall of skeletal muscle in the hindlimb of the rat without the need for prior denudation of the preexisting endothelium [1]. Remarkably, transmission electron microscopy showed that certain transduced endothelial cells became incorporated within the capillary wall, while the majority persisted within the capillary lumen where they formed focal, electron-dense, contacts with host endothelium. The long-term fate of the latter cells has yet to be determined. In the aforenoted *in vivo* studies, the femoral artery was clamped for 60 min after injection of the transduced cells under the assumption that the resultant decrease in shear rates in the capillary bed would enhance adhesion of the injected endothelial cells to the capillary wall. This assumption received support from results of experiments from a parallel *in vitro* model in which the rate of adhesion of endothelial cells seeded onto confluent endothelial cell monolayers increased logarithmically with time [1]. The purpose of the current study was to test the hypothesis that the *in vivo* adhesion of *lac-Z*-transduced MVECs in the capillary bed of skeletal muscle is dependent on the duration of arterial occlusion after injection of the transduced cells.

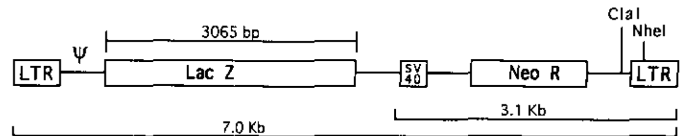

FIG. 1. Schematic diagram of the BAG vector. BAG is a retroviral-based vector containing the *lac-Z* gene. Ψ is the packaging sequence. The vector consists of the Moloney murine leukemia virus long terminal repeats (LTRs), the gene for β-galactosidase (*lac-Z*), the SV-40 early promoter, and the neomycin phosphotransferase gene (Neo ^{R}). Restriction sites are indicated as follows: *ClaI*, *NehI*. The fragment *ClaI* to *NheI* was excised, labeled, and used to probe the Northern blot. The two RNA transcripts predicted to be present in the cells transduced with BAG vector are a 7.0-kilobase (kb) mRNA transcript initiated in the LTR and a 3.1-kb mRNA transcript initiated from the SV-40 promoter.

METHODS

Rat microvascular endothelial cell derivation. Epididymal fat pads were removed aseptically from adult male rats, minced, and digested in collagenase (Sigma collagenase type 1A, 5 mg/ml, plus bovine albumin, 5 mg/ml, in Dulbecco's PBS). The flask was placed in a shaker at 125 rpm and incubated at 37°C. After a 60-min incubation, the homogenate was centrifuged at 200*g* for 5 min. The supernatant, including a thick cap of fat, was decanted and discarded. The remaining cell pellet was suspended in a washing solution (basal medium containing 2% serum) at 4°C and centrifuged again. This wash step was repeated and the cells were again resuspended. The cell suspension was filtered through a glass bead column (Sigma acid-washed glass beads, 150–212 μm in diameter) resting on a Nitex (25 μm) nylon monofilament filter (Tetko, Inc). This bead column was then rinsed with 40 ml of washing solution, resulting in trapping of blood vessel fragments in the beads and mesh, while all single cells were washed out. The blood vessel fragments were then washed off the beads and centrifuged. The resulting pellet was resuspended in trypsin–EDTA (0.05% trypsin, 0.53 m*M* EDTA-4 Na, Gibco) and incubated for 10 min at 37°C and then pelleted again. This pellet was resuspended in warm (37°C) complete medium and the cells seeded into T-25 flasks precoated with 1% gelatin.

Confirmation of identity of endothelial cells. Identity of the MVEC as endothelial cells was confirmed by their characteristic cobblestone growth pattern, the presence of receptors for the acetylated form of LDL judged by the uptake of fluorescent AcLDL [11], and binding of the lectin *Griffonia simplificolia* [12].

Endothelial cell labeling with PKH-95 and PKH-26. To each T-25 flask of confluent MVEC, 2 ml Diluent C containing 6.5 μCi of ^{125}I-PKH-95 and 1×10^{-6} *M* PKH-26 was added (Zynaxis Cell Science, Inc., Malverne, PA). Three minutes after labeling, 2 ml of bovine plasma-derived serum (BPDS) was added for 1 min. After unbound label was aspirated, each flask was washed five times with complete medium containing 20% BPDS and then was refed medium. PKH-26 is a fluorescent lipophilic dye (peak excitation 551 nm, peak emission 567 nm) that binds irreversibly within the endothelial cell membrane and does not leak into the surrounding medium or transfer to other cells [13].

Transduction of endothelial cells. T-25 flasks of MVEC at less than 50% confluence were exposed for 12 hr each day to the BAG vector (β-galactosidase after gag) murine amphotrophic retroviral vector (Fig. 1) containing the genes for both β-galactosidase and neomycin resistance (10^4–10^5 G418 resistant clones per milliliter) in polybrene (10 μg/ml), Dulbecco's modified essential media, 10% calf serum at 37°C, and 5% CO_2. Daily exposures were continued until endothelial cells reached confluence. After reaching confluence, the transfected MVEC were selected in the neomycin analog G418 (1.0 mg/ml). The percentage of cells successfully transduced was determined by histochemical staining with X-gal according to the methods of Dannenberg and Suga [14].

Northern blot analysis. *lac-Z* gene expression *in vitro* by the transduced MVEC was confirmed by Northern blot analysis (Fig. 2). Total cellular RNA was extracted and purified using method of Chomczynski and Sacci [15]. Ten micrograms of each sample underwent electrophoresis through a 1.2% agarose gel and was then blotted onto nitrocellulose membrane (Schleicher & Schuell, Keen, NH). The blots were baked in vacuum at 80°C for 2 hr. The blots were hybridized to an LTR probe at 42°C.

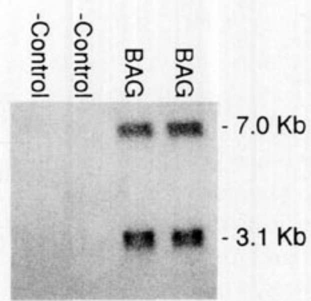

FIG. 2. Northern blot analysis of nontransduced and BAG-transduced rat microvascular endothelial cells. Total cellular RNA was isolated from nontransduced and BAG-transduced rate MVEC. Cellular RNA was fractionated in 1.2% formaldehyde-agarose gels, blotted, and hybridized to a ^{32}P-labeled LTR probe. From left to right, lanes 1 and 2 are mRNA from control nontransduced rat microvascular endothelial cells; lanes 3 and 4 are BAG-transduced and neomycin-selected MVEC.

Probes were labeled by the random primer method using [^{32}P]dCTP [16]. Washing of blots were performed at 50°C in 0.2× standard saline citrate, 0.1% sodium dodecyl sulfate (SDS). Autoradiography was performed on the blots at −70°C using Kodak XAR film (Eastman Kodak, Rochester, NY).

Experimental animal model. The fate of BAG-transduced MVEC injected intraarterially into the hindlimb of syngeneic Wistar F-455 rats (Harlan Sprague–Dawley Inc., Indianapolis, IN) was studied. Anesthesia was established with intramuscular ketamine (13 mg/kg) and zylazine (87 mg/kg). The saphenous artery branch of the femoral artery was isolated and cannulated with a 26-gauge stainless steel cannula attached to a polyethylene tube and stopcock. MVEC were labeled as noted above with a radioactive label, ^{125}I-PKH-95, for quantitative tracking after their injection into the hindlimb.

These labeled MVEC were injected into the capillary bed of skeletal muscle of the rat hindlimb through the femoral artery. Rats were then recovered and followed until completion of the study at various times: 1 day, 1 week, and 1 month later. At the time of sacrifice all the muscles of both hindlimbs were excised and counted individually in a gamma counter. Segments of the gastrocnemius and tibialis anterior muscles were removed for light microscopy to confirm recombinant *lac-Z* gene expression by histochemical staining with X-gal. The visceral organs of the abdominal and thoracic cavities were similarly studied. Animal care was in strict compliance with University of Michigan guidelines, state and federal laws, and NIH Standards 85–23.

Four groups (Groups I to IV) were studied. A control group of rats (Group I) underwent injection of transduced cells without a 10-min preclamp nor any postinjection occlusion of the femoral artery. In the three experimental groups the femoral artery was clamped 10 min prior to injection of transduced cells. This 10-min period was used to standardize precisely the duration of injection as well as to allow a sufficient time period for pretreatment of the microvascular bed in future studies. After this 10-min preclamp, 1–2 × 10^6 *lac-Z*-transduced microvascular endothelial cells were injected and the femoral artery clamp was removed immediately after injection (Group II), 60 min after injection (Group III), or 120 min after injection (Group IV).

Light microscopy. Thin slices of muscle were fixed in 1.5% glutaraldehyde in PBS for 15 min and then incubated in X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) for 18 hr at 37°C [14]. The specimens were postfixed in 2% paraformaldehyde, 2% glutaraldehyde in 0.1 *M* cacodylate buffer and stained in 1% OsO_4 in 0.1 *M* cacodylate buffer followed by staining in 0.05% uranyl acetate in 0.05 *M* maleate buffer at pH 5.2. Following dehydration in ethanol and propylene oxide the muscle sections were embedded in glycol methacrylate, and sections were stained with eosin. The X-gal reaction product appeared as blue–green granules in transduced MVEC.

Statistical analysis. Results are expressed in this report as mean percentage of injected ^{125}I activity ± 1 standard deviation. Comparisons among groups were made by analysis of variance of the mean data. Differences between groups were determined by the Newman–Keuls multiple comparisons test ($P < 0.05$ was accepted as significant).

RESULTS

Adhesion of *lac-Z*-transduced MVEC did not increase as the duration of femoral artery occlusion after injection increased (Table 1). The highest rate of adhesion occurred when the transduced endothelial cells were injected after only a 10-min preclamp, 32% of the cells were present at 1 day, 17% at 1 week, and 15% at 1 month after injection. As the duration of femoral artery occlusion increased, the rate of adhesion tended to de-

TABLE 1

Effect of Femoral Artery Occlusion Duration on Persistence of BAG-Transduced Microvascular Endothelial Cells in the Hindlimb

	Adhesion of MVEC in ipsilateral hindlimb			
	Group I	Group II	Group III	Group IV
Preclamp time:	0	10 min	10 min	10 min
Postinjection clamp time:	0	0	60 min	120 min
Study period				
1 Day postinjection	19 ± 7%	32 ± 6% *	30 ± 6%	23 ± 3%
1 Week postinjection	10 ± 1%	17 ± 5%	18 ± 12%	14 ± 2%
1 Month postinjection	9 ± 0.6%	15 ± 0.2%	13 ± 8%	9 ± 2%

* $P < 0.05$ vs Group I.

TABLE 2

Distribution of Transduced Endothelial Cells at Different Times after Injection

	1 Day	1 Week	1 Month
Injected leg	32 ± 6%	17 ± 5%	15 ± .2%
Contralateral leg	1 ± 0%	1 ± .3%	0*
Liver	58 ± 1%	76 ± 1%	54 ± 5%
Lung	5 ± .8%	4 ± .8%	3 ± 1%
Spleen	5 ± .7%	5 ± 3%	7 ± .7%
Gut	2.0 ± .1%	1.4 ± .3%	1 ± .2%
Kidney	0*	0*	0*
Stomach	0*	0*	0*
Heart	0*	0*	0*
Other parts	0*	0*	0*

Note. These distributions are for 10-min preclamp only group, but are representative of the proportions found in all groups at the different time points after injection.

* Represents less than 0.8% of injected activity, which is at the lowest limit of detection.

crease, although these differences did not reach statistical significance.

The pattern of distribution of the transduced MVEC in different organs as determined by ^{125}I PKH-95 localization was nearly identical at each time period among all four groups. The majority of the injected activity that was not located in the hindlimb was found in the liver, lungs, and spleen, all organs containing a portion of the reticuloendothelial system (Table 2). We were unable to determine if the detected activity in these organs represented viable transduced MVEC or breakdown products of these cells [1] because extensive X-gal staining occurs in these organs in control rats in whom no transduced MVEC were injected.

Histochemical staining of the muscle sections with X-gal confirmed *in vivo* expression of the *lac-Z* gene. Discrete areas of blue staining occurred in the area of the capillaries between muscle fibers (Figs. 3A and 3B). Blue staining was not seen in the walls of larger microvessels. This pattern of localization of the transduced cells in capillaries was consistent with that of our previous study in which we also confirmed *lac-Z* expression of endothelial cells within the capillary lumen as well as incorporated into the capillary wall by electron microscopy [1]. For the purposes of this histochemical analysis, three different controls were used: (a) muscle from rats in which no media or MVEC were injected, (b) muscle from rats in which media alone were injected, and (c) muscle from rats injected with nontransduced MVEC in media. In these control tissues occasional blue staining was observed, usually in macrophages located within the muscle fibers and not in capillaries between the fibers, occasionally in the region of motor endplates, and finally within macrophages circulating in the larger blood vessels.

DISCUSSION

Adhesion of *lac-Z*-transduced MVEC did not increase as the duration of femoral artery occlusion after injection the cells increased. In fact, the highest rate of adhesion occurred in the group in which only a 10-min preclamp prior to injection was used.

We hypothesized that the reduction in shear rates during femoral artery occlusion would promote adhesion of the injected transduced endothelial cells to the skeletal muscle capillary wall. Although the differences did not achieve statistical significance, the number of transduced cells found in the hindlimb 1 day after injection actually tended to decrease as the duration of femoral artery occlusion was increased. One potential explanation for this effect could be that as the duration of femoral artery occlusion after injection was increased, the magnitude and duration of reactive hyperemia increased, thereby "washing" some of the injected cells out of the capillaries. Ischemic injury to the capillary may also have played a role in this effect. Nonetheless, these results document that one can achieve significant adhesion and persistence of genetically modified MVEC injected into the capillary bed of rat skeletal muscle.

This study utilized a new endothelial cell label, ^{125}I-PKH-95, to track MVEC after their injection into the femoral artery. Under conditions of a 10-min preinjection arterial occlusion and a 60-min postinjection arterial occlusion the distribution of radioactivity after injection in this investigation was nearly identical to that of a previous reported study from our laboratory attesting to the reproducibility of this labeling technique and our model [1]. The Zynaxis cell linker, PKH, binds irreversibly within cell membranes [13]. In *in vitro* experiments, we have specifically shown that the label does not transfer from labeled to unlabeled MVEC in cultures at 24, 48, and 72 hr and 1 week after labeling. The ratio of labeled and unlabeled cells determined by FACS (fluorescent-activated cell sorter) remained constant across all time periods ($n = 2$). Furthermore, the pattern of distribution of injected transduced MVEC as shown by PKH-26, a fluorescent label attached to the Zyanxis cell linker, was the same as that observed within the histochemical stain, X-gal. These results support the published work concerning the use of PKH with other cell types, that this label binds irreversibly within a cell membrane, making it ideal for use in cell tracking studies.

The mechanism of adhesion of transduced MVEC in the skeletal muscle capillaries is not known. Mechanical factors such as the size and deformability of endothelial cells may play a role in the initial trapping of the injected cells within the capillary lumen. Adhesion of transduced MVEC to the preexisting endothelial cell monolayer of the capillary wall was unexpected, in view of the known anti-adhesive nature of endothelial cell monolayers *in*

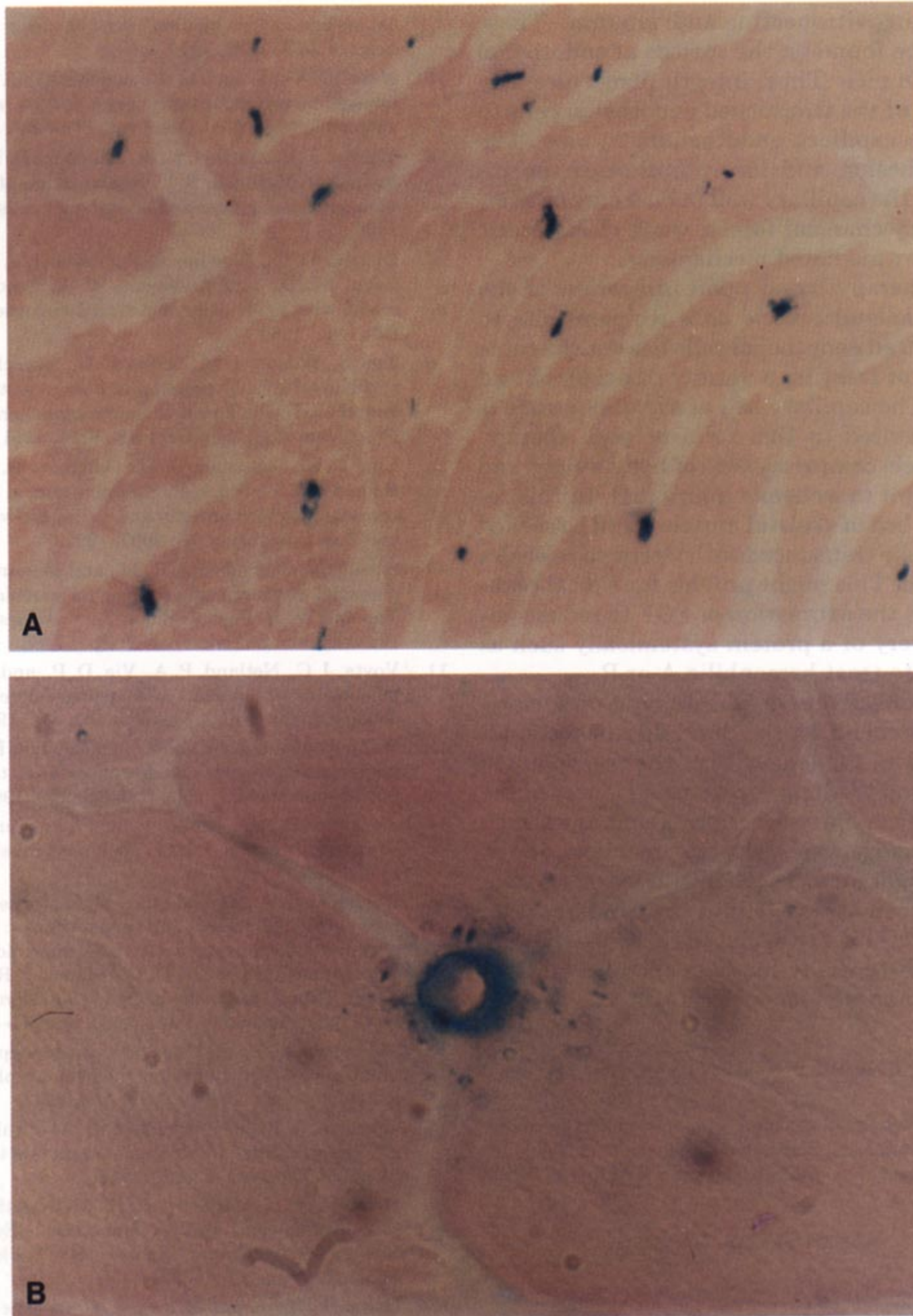

FIG. 3. Photomicrographs of X-gal-stained cross section of tibialis anterior muscles. (A) *lac-Z*-transduced MVEC are in numerous capillaries 1 hr after injection of transduced endothelial cells (140×). (B) *lac-Z*-transduced MVEC in a capillary 1 day after injection of transduced cells (800×).

vivo and the very tight regulation of endothelial cell density in the vessel wall [17–19].

Potential receptors that may mediate adhesion between the injected transduced endothelial cells and the capillary endothelium include the platelet–endothelial cell adhesion molecule (PECAM) or members of the B_1 or B_2 integrin families. PECAM is the only endothelial cell–endothelial cell adhesion molecule to mediate the *initial* adhesion between endothelial cells *in vitro* and thus would be the most likely molecule to mediate the adhesion documented in our studies [20]. The role of this adhesion molecule *in vivo* has not been established. The B_1 and B_2 families of the integrin adhesion molecules mediate adhesion between cells and matrix proteins

such as fibronectin, vitronectin, and laminin. These proteins can also be found on the surface of endothelial cells *in vitro* and *in vivo*. Thus, integrin molecules may promote adhesion of the transdcued endothelial cells to the surface of the capillary endothelium *in vivo*. The mechanism of adhesion and incorporation of the injected MVEC into the capillary wall *in vivo* may be complex and involve mechanical forces, weak electrostatic forces, and receptor-mediated mechanisms.

Somatic gene therapy based upon utilization of the capillary bed of skeletal muscle as a recipient site to transplant transduced endothelial cells has considerable merit as a means of treating a variety of acquired and genetic diseases. The capillary bed of skeletal muscle is particularly well suited to this form of gene therapy since skeletal muscle comprises 40% of body weight and is relatively resistant to ischemic injury [21]. In this regard, the capillary bed of skeletal muscle could be seeded with endothelial cells transduced with a gene to release a therapeutic protein. This might provide for a local paracrine effect such as the expression of FGF to induce angiogenesis or delivery of a protein systemically such as Factor VIII or IX to treat hemophilia A or B.

The long term effectiveness of this type of somatic gene therapy will depend on the durability of recombinant gene expression, the longevity of the transplanted endothelial cells, and the long-term consequences of the seeding process on capillary function. Techniques such as stimulating angiogenesis prior to injection of the transduced cells might increase the likelihood of incorporation of these transduced cells into the capillary wall. Complete incorporation of transduced cells into the capillary wall may substantially increase the durability of recombinant gene expression.

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