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RESEARCH ARTICLE

A direct mechanical method for accurate and efficient adenoviral vector delivery to tissues

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We describe a mechanical method for delivery of adenoviral vector to the adventitial surface of arteries and to other tissues. Our goal was to characterize, principally in intact carotid artery, the morphological, biochemical, and functional effects of mechanical delivery of a recombinant β-galactosidase-expressing adenoviral vector following its direct application using a small paintbrush. Our ex vivo and in vivo data demonstrate efficient, accurate, and rapid transduction of arteries without compromise of their morphological, biochemical, and functional integrity. We also demonstrate the general applicability of this technique in vivo via transduction of skeletal muscle, fibrotendinous tissue, peritoneum, serosal surface of bowel, and wounded skin. We conclude that direct mechanical delivery of an adenoviral vector to tissues using a suitable paintbrush represents an intuitive, accurate, and effective means of augmenting gene transfer efficiency, and may be a useful adjunct to other delivery methods.
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Introduction

Experimental studies have demonstrated successful transfer of a variety of recombinant genes in the cardiovascular system both *ex vivo* and *in vivo* in animals and humans[1–13] suggesting that gene transfer technology may, in future, offer an effective therapeutic option for the spectrum of human cardiovascular diseases.[14–18] Most vascular gene transfer studies, to date, have utilized replication-incompetent adenovirus as a delivery vehicle for recombinant cDNA. *Ex vivo* delivery of this vector has been carried out by immersion of arterial tissue in a chamber containing vector-solution,[3,5,11] while *in vivo* delivery has been carried out through the lumen of an artery, that is, by direct injection, or via its outer "adventitial" layer, that is, perivascularly.[2,4,7,8,10,13,19–21] A number of groups have demonstrated the ability of perivascular (adventitial) gene delivery to favorably and profoundly modify vasomotor function in a variety of cardiovascular models[3–5,11–13,19,20,22] and this approach, which has been reported to minimize the proinflammatory effects of adenoviral vectors,[10] can be used as an effective adjunct to the deployment of intravascular gene delivery stents and catheters. *In vivo*, current vascular gene delivery techniques have limited ability to produce specific targeting and selective localization of vector in a desired arterial territory or individual vessel. To address this limitation, several gene transfer studies have been carried out using novel molecular and chemical methods in cultured cells and intact tissues,[23–28] and using a microprojectile method in a variety of tissues.[29] Overall, however, relatively little attention has been given to mechanical techniques which, owing to their more direct approach, may be beneficial from the perspectives of both accuracy and efficiency of gene transfer.

Heistad and colleagues[2,14] have described the use of mechanical head-tilt to assist in targeting adenoviral vectors to the adventitia of intracranial arteries following vector injection into the cerebrospinal fluid in small animals. While this method was shown to be helpful in similar *in vivo* gene transfer studies carried out in a large-animal model,[4,13] vector delivery was still relatively indirect and nonfocal. In a more direct method of mechanical delivery to the carotid artery described by Kullo *et al*,[19] sequestration of vector around the vascular adventitia was carried out to facilitate more focal gene transfer. However, in addition to requiring carotid artery sheath incision and insertion of a perivascular catheter for vector delivery, this method, like others, requires prolonged vector incubation times, typically 30 min to 2–3 h.[3,5,9,11,23,30,31] In the case of nonreaspirated adventitial injection of vector, the transduction time is "indefinite", that is, till the time of sacrifice of the animal.[10,19] Several groups have used viral titers of 10^9–10^{10} plaque forming units (PFU) per "target" (ie, vessel segment of vascular territory within a living host) to facilitate effective expression of recombinant proteins in the vessel wall.[2–5,9–13,19,20,23,32] To explore the possibility of effective mechanical delivery of adenoviral vector to tissues, we postulated that a small paintbrush could be used to

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rapidly, efficiently, and accurately apply an adenoviral vector directly to a desired tissue target *in vivo*. This work suggests that our mechanical vector delivery method facilitates enhanced concentration and localization of vector at the tissue surface to which it was directly applied, enabling reduction of vector incubation time and titer *in vivo*.

Materials and methods

Animal tissue

An animal model involving intact carotid artery was chosen as the focus for this study in the context of our ability to determine the effects of mechanical vector delivery in this tissue preparation morphologically, biochemically, and functionally, and because of our previous experience with vascular gene transfer in dogs[3,4,13,22,31] and rabbits.[19,20] *Ex vivo* experiments were carried out using arteries harvested from mongrel dogs. Canine carotid artery (5 mm outer diameter (OD)) was chosen for its robust size and ease of surgical access. On account of the ready availability, ease of handling, and previous studies from our group involving *in vivo* gene transfer to carotid arteries of this species,[19,20] *in vivo* experiments were carried out in adult New Zealand white rabbits. Rabbit carotid artery (2 mm OD) was chosen for ease of surgical access, translatability of data from *ex vivo* canine carotid artery experiments, and because this artery was more delicate than its robust canine counterpart, thereby allowing further testing for the physical effects of mechanical vector delivery in smaller vessels. Where specified, arterial "ring" refers to an isolated segment of carotid artery of length 1–2 cm (dog) or 3–4 mm (rabbit). In certain *in vivo* experiments, the effects of mechanical vector delivery were studied in rabbit skeletal muscle, tendon, peritoneum, serosal surface of bowel, and wounded (surgically incised) skin. All procedures were performed in accordance with institutional guidelines.

Adenoviral vectors

Replication-incompetent recombinant adenoviral vectors encoding genes for β-galactosidase (AdLacZ) and endothelial nitric oxide synthase (AdeNOS; used mainly as a control vector in this study) were used as described elsewhere.[3,11,13] Procedures and handling of tissues exposed to recombinant vectors were approved by the Institutional Biosafety Committee. The native vehicle used to suspend adenoviral vector consisted of 140 mmol/l NaCl, 10 mmol/l Tris buffer, 2 mmol/l $MgCl_2$, and 10% glycerin; pH 8.0. Where specified, vector titer refers to the number of PFU of AdLacZ or AdeNOS per tissue target (eg, whole artery or arterial ring), while vector concentration refers to the number of PFU per microliter of transduction solution.

Gene transfer

In the present work, "control" or "nontransduced" arteries or rings refer to those not exposed to vector. *Ex vivo*, control rings were incubated in phosphate-buffered saline (PBS; Gibco-BRL, Rockville, MD, USA) for 10 min at room temperature prior to transfer to fresh Dulbecco's modified Eagle medium (DMEM; Gibco-BRL). They were then incubated at 37°C in a CO_2 incubator for a further 24 h prior to functional experiments or 48 h prior to morphological and biochemical experiments. Other rings referred to as "transduced" were incubated in a similar manner in parallel with, but separate from, control rings following exposure to adenoviral vector diluted in PBS to a final titer of 10^7 or 10^9 PFU/ring. *In vivo*, intact rabbit carotid arteries were exposed in parallel either to dripped or brushed PBS alone (control) or to PBS-suspended AdLacZ (10^7 or 10^9 PFU/artery) applied perivascularly by dripping or brushing (see below). Rabbits were killed 48 h later and tissues harvested for further studies. The tissue transduction time, that is, the time of direct exposure of tissue to vector prior to PBS-rinsing *ex vivo* or PBS-irrigation-aspiration *in vivo*, was 10 min for each ring or artery (determined from technical optimization experiments; see below). Care was taken to avoid direct contact between pipettes or irrigation-aspiration devices and transduction sites. Vector titers and overnight incubation times were chosen in the context of previous studies.[3–6,11,13,19,20]

Transduction techniques. Two techniques were used to transduce carotid artery *ex vivo* and *in vivo*. The first ("vector-dripping" or "drip-immersion") was a conventional method involving drop-by-drop placement of vector-containing solution onto the artery or ring until its complete immersion was achieved using the lowest volume of vector-solution for canine carotid rings *in vitro* (requiring 150 μl of vector-solution/ring) or intact rabbit carotid arteries *in vivo* (requiring 400 μl of vector-solution/artery). The dripping procedure was typically completed within 10 s for each specimen. The second technique ("vector-brushing") involved use of a small paintbrush (Richeson brush, Series-9151, comprised of horse-hair natural bristle; flat-tip diameter=1/4"; Carson Art Supply, Rochester, MN, USA) to physically apply vector directly to the outer arterial surface by lightly brushing vector-solution into the tissue (see optimization data, below). Vector-solution was first pipette-dripped onto the upward-facing arterial surface and then immediately brushed into the tissue using a total of 12 unidirectional light brush strokes. *Ex vivo*, arterial rings were handled gently using forceps applied only to their extreme ends, and carefully turned over to facilitate circumferential application of vector; *in vivo*, carotid arteries were brushed linearly and unidirectionally along the surgically exposed upward-facing surface only. The brushing procedure was typically completed within 1 minute for each specimen. Arterial compression associated with brushing is defined as reduction of the vertical diameter of the arterial ring as determined visually following linear ruler measurements before and during brushing. "Light" compression refers to a stroke pressure causing <30% vertical compression of the arterial lumen. For brushing, 20 μl of solution/cm^2 of arterial tissue was found to be the minimum volume required for complete coverage of the artery or ring. In this study, arteries or rings subject to the above techniques were studied in parallel using vector-containing and vector-free solutions. Certain *in vivo* experiments in rabbits involved mechanical transduction of skeletal muscle, tendon, peritoneum, serosal surface of bowel, and wounded skin with PBS-suspended AdLacZ at a titer of 10^7 or 10^9 PFU/tissue target. These experiments,

conducted in parallel with appropriate controls, were carried out in accordance with the methods detailed above.

Fibrin glue. Certain *ex vivo* gene transfer experiments involved the use of fibrin glue, a clinically relevant adhesive-hemostatic composition,[33] as a vehicle for vector suspension. For these, 10 µl of vector was first mixed with 15 µl of human donor fibrinogen (St Marys Hospital Transfusion Medicine Service, Rochester, MN, USA) instead of PBS. Immediately following dripping of the vortexed vector-fibrinogen composition onto the upward-facing arterial surface, 15 µl of recombinant bovine topical thrombin (Jones Pharma, St Louis, MO, USA) was dripped onto the same surface and then brushed circumferentially into the wall. Mixing of fibrinogen and thrombin in a 1:1 ratio by volume caused the fibrin glue to set within 5 s. These rings, studied in parallel with rings exposed to PBS with and without vector, were transduced and incubated as described above.

Histology and histochemistry

For histochemical staining of recombinant β-galactosidase, tissues were stained with BluoGal, a destaining-resistant chromogenic compound (Gibco-BRL). Specimens were first rinsed gently in PBS and placed separately, but in parallel, in BluoGal staining solution (2 ml/specimen). Specimens *in vitro* were shielded from light and incubated at 37°C in a CO_2 incubator for up to 20 h, observed every 2 h for the first 8 h, then at 12 and 20 h. To control for nonspecific staining, AdLacZ-transduced arteries were incubated in BluoGal in parallel with, but separate from, BluoGal-incubated nontransduced (negative control) and AdeNOS-transduced (vector control) arteries. To facilitate accurate comparability between transduction techniques, brushed and dripped tissues were stained in parallel. Following fixation in 10% buffered formalin, certain arterial rings were embedded in paraffin and cut into serial 5 µm cross-sections. To control against the possibility of destaining during paraffin embedding, brushed and dripped AdLacZ-transduced rings were processed for histology in parallel, along with the appropriate brushed and dripped negative-control and control vector (AdeNOS)-transduced rings; nuclear fast red was used for counterstaining. The following terminology is used to describe BluoGal staining of tissues viewed macroscopically: "maximal staining" refers to an entirely dark-blue-stained artery or arterial ring; "extensive staining" refers to the presence of predominantly contiguous dark-blue-regions while "moderate staining" refers to the presence of only blue-stained patches (predominantly noncontiguous); "mild staining" refers to the presence of only a light-blue hue in tissues; and "no staining" refers to the complete absence of blue staining. Where specified, the term "focal" refers to the presence of predominantly small areas of stained tissue (stain width $\leq 1/5$ of the arterial OD) while "nonfocal" refers to the presence of predominantly large areas (stain width $\geqslant 1/2$ of the arterial OD). To minimize observer error in interpretation of histochemical data, all experiments were multiply repeated and all specimens were viewed by at least two investigators.

β-Galactosidase assay

Canine carotid artery rings incubated for 48 h following a 10-min *in vitro* exposure to PBS only (negative control), AdeNOS (10^7 or 10^9 PFU/ring; vector control), AdLacZ (10^7 or 10^9 PFU/ring) dripped, AdLacZ (10^7 or 10^9 PFU/ring) brushed alone, or AdLacZ (10^7 or 10^9 PFU/ring) brushed in the presence of fibrin glue, were homogenized in 300 µl of lysis buffer and assayed for β-galactosidase activity using a chemiluminescent assay system (Galacto-Light™, Tropix, Bedford, MA, USA) described elsewhere.[6] Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA), and normalized β-galactosidase activity is expressed as relative light units (RLU)/µg of total protein.

Cyclic 3,5-guanosine monophosphate (cGMP) assay

Rabbit carotid arteries were transduced for 10 min *in vivo* with AdeNOS either via dripping (5×10^9 PFU/artery; 400 µl transduction volume) or by brushing (10^9 PFU/artery; 80 µl transduction volume) as described above. Paired carotid arteries were transduced in parallel by each method, and the final vector concentration was the same (1.25×10^7 PFU/µl) for each artery. Forty-eight hours after transduction, rabbits were killed and their carotid arteries harvested for measurement of cGMP. As previously reported by members of our group in rabbit carotid artery,[19,20] a radioimmunoassay RIA technique was used to determine the content of cGMP. From each harvested artery, rings were immediately immersed in a solution containing 3-isobutyl-L-methylxanthine (IBMX; 10^{-3} mol/L) which was used to inhibit the degradation of cGMP by endogenous phosphodiesterases. Rings were then incubated at 37°C for 30 min before being snap-frozen in liquid nitrogen and stored at −70°C until the time of RIA. The cGMP assay was carried out using a kit from Amersham (Piscataway, NJ, USA), while protein assay was carried out using the DC Protein Assay Kit (Bio-Rad).[19,20] Tissue cGMP levels are expressed as pmol/mg protein.

Isometric force recording

Vascular reactivity was studied by isometric force recording as described elsewhere.[3–6,11,13,19,20] Vasomotor responses were compared between brushed-*versus*-dripped rings in the presence of AdLacZ (10^9 PFU/artery) or its absence (PBS alone). To evaluate relaxation responses, rings were contracted with phenylephrine (10^{-6} mol/l) before addition of relaxing agents. At the conclusion of each recording, rings were contracted with 10–60 mmol/l KCl. Concentration-responses and median effective concentration (EC_{50}) values were obtained as described elsewhere.[3,11,13] Contractions to KCl are expressed as percentage of contraction induced by 10^{-6} mol/l phenylephrine (100%), while relaxations to acetylcholine and bradykinin are expressed as percentage of relaxation induced by 3×10^{-4} mol/l papaverine (100%). Acetylcholine, bradykinin, papaverine, and phenylephrine were obtained from Sigma (St Louis, MO, USA). Drugs were prepared and diluted as described elsewhere.[3,11,13] Drug concentrations are expressed as final molar concentrations in solution.

Data analysis

Results are expressed as mean ± standard error of the mean (s.e.m). In all experiments, *n* indicates the number of animals from which arteries were harvested. Statistical analysis of dose–response curves and EC_{50} values by two-way repeated measures analysis of variance (RM-ANOVA) and Student's *t*-test, respectively, is described elsewhere.[3,11,13] For analysis of data from β-galactosidase assays, mean background enzymatic activity (31.3 ± 6 RLU/μg; detected in blank chambers and non-transduced rings; $n=8$ dogs) was first subtracted from mean activities measured in all other groups assayed in parallel. Data were then analyzed by unpaired *t*-tests between treatment groups. Data from cGMP RIA were also analyzed by an unpaired *t*-test between treatment groups. For all experiments, probability values of $P<0.05$ are considered statistically significant.

Results

Technical optimization

Several technical parameters were varied in order to optimize our mechanical method for AdLacZ transduction as indicated by maximal recombinant β-galactosidase staining (ie, an entirely blue-stained artery) using BluoGal histochemistry (data summarized in Table 1). Maximal staining was observed at an AdLacZ titer of 10^9 PFU/arterial ring only when vector was suspended in PBS and applied to the outer arterial surface via 12 light strokes of a stiff-bristle paintbrush made from natural horse-hair (representing an "optimized" brushing technique). Notably, during *ex vivo* technical optimization experiments, relatively low volumes of transduction solution (20 μl/cm^2 of target tissue) were found to be associated with maximal staining, using our mechanical technique. Further, independent of AdLacZ transduction titer and volume, no staining was seen in brushed rings transduced for only 5 min, while the amount of staining noted in brushed rings transduced for 10 min was macroscopically similar to the staining noted in brushed rings transduced under the same conditions for 20, 30, and 45 min. This 10 min adventitial transduction time using our mechanical technique was confirmed *in vivo* during studies in rabbits (see below).

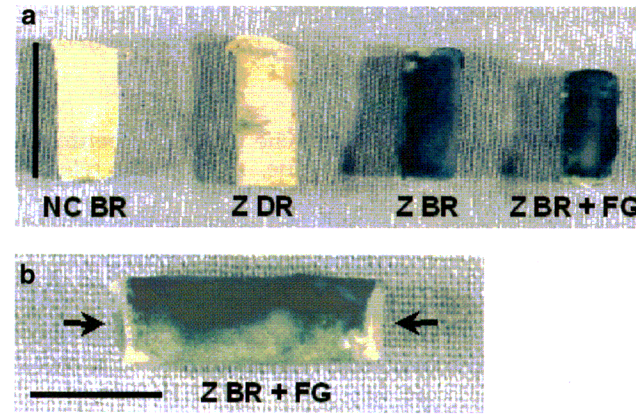

Figure 1 *Morphological study of canine carotid artery transduced ex vivo. (a) The figure shows four carotid artery rings obtained from the same animal. The negative control ring (NC BR) was brushed with saline only (ie, no AdLacZ) and shows no staining with BluoGal. The ring exposed to AdLacZ via conventional dripping of virus (Z DR) shows only patchy staining. Rings exposed to the same titer of AdLacZ via brushing of the vector into the arterial wall in the absence (Z BR) or presence (Z BR+FG) of fibrin glue show intense staining. Titer of vector=10^9 PFU/ring. Bar=1.8 cm. (b) A side-on view of the ring brushed with vector suspended in fibrin glue (Z BR+FG). Note the linear distribution (arrows) of the vector across the brushed (upper) surface of the artery, localized by the rapidly setting "glue". Bar=1 cm.*

Accuracy and efficiency of gene transfer

Morphological study. Intense histochemical staining was observed in vector-brushed compared with vector-dripped arterial rings transduced *ex vivo* in parallel at the same titer (10^9 PFU/ring) and studied 48 h later macroscopically (Figure 1) and microscopically (Figure 2). As determined from multiple cross-sections of AdLacZ-brushed rings, the level of adventitial transduction noted histologically (Figure 2c) was consistent throughout the entire ring lengths. This was not the case for AdLacZ-dripped rings in which the presence and extent of adventitial transduction was more irregular (see Figure 2b for a representative appearance of transduced regions in these rings). The microscopic findings were consistent with the different patterns of transduction observed macroscopically (Figure 1a). Pertaining to *in vivo* studies carried out in rabbits 48 h after a 10-min transduction time, the linearity of recombinant gene expression along

Table 1 Optimization of mechanical vector delivery technique in canine carotid artery *ex vivo*[a]

Parameter	*Variation*	*Maximum*[b]
Vector titer per ring	10^7–1.5×10^9 PFU/ring	10^9 PFU/ring
Transduction volume	10–100 μL/cm^2 of ring	20 μl/cm^2
Transduction solution	DMEM, PBS	PBS
Transduction time	5–45 min	10 min
Paintbrush bristle	Stiff (horse hair), non-stiff (camel hair)	Stiff
Number of brush strokes	2–12 strokes/ring	12 strokes/ring
Stroke pressure	Ultralight, light, heavy	Light

[a]Data from $n=7$ dogs (140 carotid artery rings); carotid arteries transduced *ex vivo* with AdLacZ (10^7–1.5×10^9 PFU/ring) or nontransduced (control; ie exposed to vector-free solution only).[b]Maximum staining using BluoGal histochemistry (ie resulting in an entirely blue artery).Note that no BluoGal staining was observed in nontransduced or AdeNOS-transduced brushed and nonbrushed arteries (all $n=7$) studied in parallel with AdLacZ-transduced arteries. Abbreviations: AdeNOS, adenoviral vector encoding recombinant endothelial nitric oxide synthase gene (a control vector in this study); AdLacZ, adenoviral vector encoding recombinant β-galactosidase gene; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; PFU, plaque forming unit.

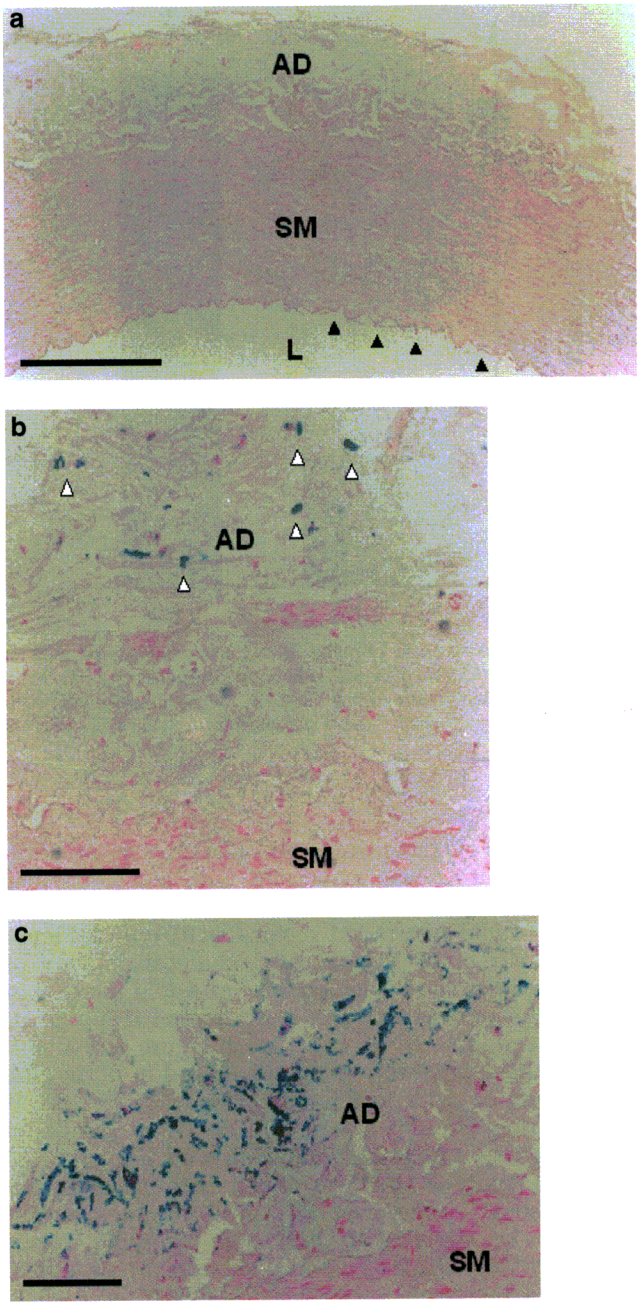

Figure 2 *Morphological study of canine carotid artery transduced ex vivo. Cross-sections of the negative control (NC BR; a), vector-dripped (Z DR; b) and vector-brushed (Z BR; c) rings shown in Figure 1. Note the absence of BluoGal staining in the negative control, some scattered foci of staining (arrow heads) in the adventitia of the Z DR ring, and considerably enhanced presence of staining in the Z BR ring. Preserved endothelium in (a) (a brushed ring) is indicated by arrow heads. AD, adventitia; L, lumen; SM, smooth muscle. Bar, 300 μm (a) and 150 μm (b, c). Nuclear fast red counterstain. Magnification= × 25 (a) and × 50 (b, c).*

the exposed arterial adventitial surface (consistent with the linear brushstrokes used for vector delivery) was evident macroscopically (Figure 3). Notably, focal mild-to-moderate staining was observed on the adventitial surface of vector-brushed arteries transduced with as little as 10^7 PFU each, while no staining was seen at this titer in vector-dripped carotid arteries transduced in parallel in the same animals (n=4; Figure 3c). Under the

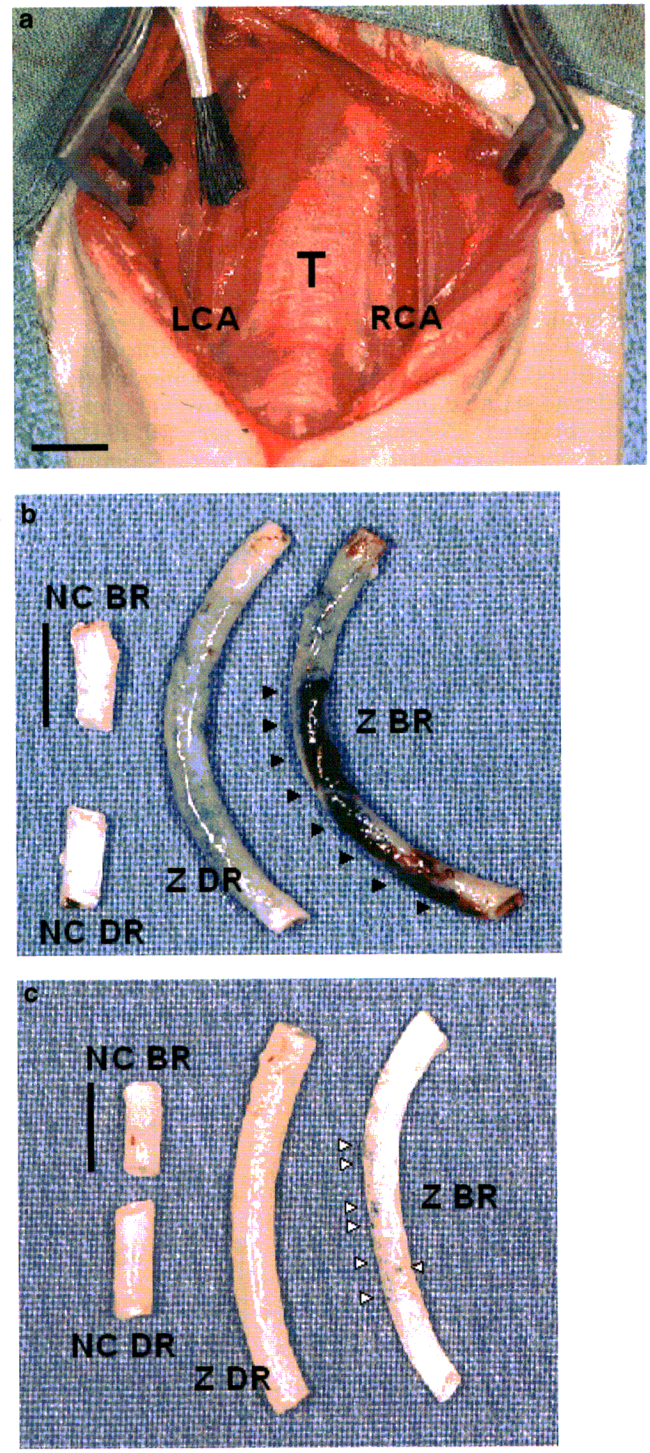

Figure 3 *Morphological study of rabbit carotid artery transduced in vivo. (a) The intraoperative photograph shows the paintbrush technique for application of vector to a target tissue, in this case the left carotid artery (LCA). T, trachea; RCA, right carotid artery. Bar=1 cm. (b ,c). The panels show isolated AdLacZ-transduced and nontransduced (control) carotid arteries, at vector titers of 10^9 PFU/artery (b) and 10^7 PFU/artery (c). In (b), note the considerably enhanced, linear expression of β-galactosidase in the vector-brushed artery (Z BR; arrow heads) compared with the patchy expression of recombinant protein in the vector-dripped artery (Z DR). Bar=1 cm. As shown in (c), at an AdLacZ titer of 10^7 PFU/artery, no expression of recombinant protein could be visibly detected in the Z DR artery, although linear expression of β-galactosidase could still be seen in the Z BR artery (arrow heads). Bar=1 cm. In all cases, respective negative control (NC) arteries, brushed (BR) or dripped (DR), did not show any positive staining with BluoGal.*

same conditions, except at a higher titer (ie, 10^9 PFU/artery), the amount of staining observed in vector-brushed arteries was also considerably greater than that observed in vector-dripped arteries (n=4; Figure 3b). PBS vehicle-brushed and vehicle-dripped control arteries exposed to BluoGal in parallel with transduced arteries did not stain positively for β-galactosidase at either titer (Figures 3b and c).

Morphologically, arterial brushing of 10^9 PFU/ring AdLacZ suspended in a viscous vehicle, fibrin glue, resulted in enhanced gene transfer compared with application by dripping 10^9 PFU/ring AdLacZ suspended in PBS, respectively, producing nonfocal extensive BluoGal staining compared with nonfocal mild-to-moderate staining (n=4; Figure 1a). However, compared with brushed PBS-suspended vector (10^9 PFU/ring) which produced circumferential BluoGal staining in arterial rings, brushed fibrin glue-suspended vector (10^9 PFU/ring) localized linearly along the upper halves of ring surfaces onto which the fibrinogen-plus-vector and thrombin were first dripped prior to circumferential brushing (Figure 1b). This qualitative reduction in gene transfer associated with brushing vector suspended in fibrin glue, compared with brushing vector suspended in PBS, was confirmed and quantified biochemically (see below).

Biochemical study

As determined by β-galactosidase chemiluminescence assay using canine carotid artery rings transduced *ex vivo* with AdLacZ, there was an approximately 40-fold lower level of β-galactosidase activity measured in rings transduced at 10^7 PFU/ring (n=4) compared with rings transduced under the same conditions except at 10^9 PFU/ring ($P<0.001$; n=4; Figure 4). Notably, at a

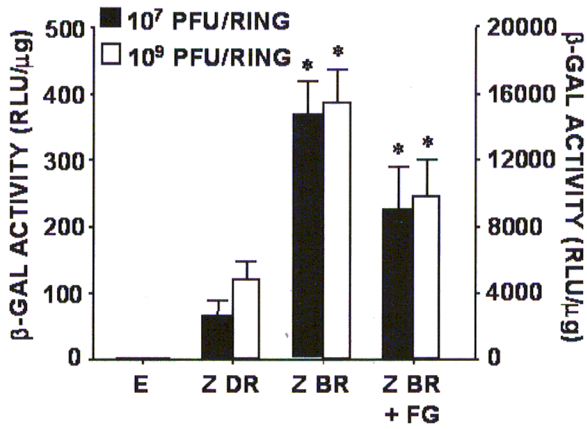

Figure 4 *Biochemical study of canine carotid artery transduced ex vivo. The graph shows the level of β-galactosidase activity in rings exposed to control vector AdeNOS (E) alone, or AdLacZ vector dripped onto (Z DR) or brushed into (Z BR) the ring. Certain rings were brushed with AdLacZ in the presence of fibrin glue (Z BR+FG). Note the difference in scale between the left and right y-axes: the left y-axis shows β-galactosidase activity measured in rings exposed to vectors at a titer of 10^7 PFU/ring (filled columns); the right y-axis shows activity measured in rings exposed to vectors at a titer of 10^9 PFU/ring (unfilled columns). For both titers, there are significant increases in enzyme activity (three-fold at 10^9 PFU/ring and six-fold at 10^7 PFU/ring) in rings brushed with vector in the absence of fibrin glue compared with vector-dripped rings (*$P<0.01$; n=4 each). PFU, plaque forming unit; RLU/μg, relative light unit per microgram of total protein.*

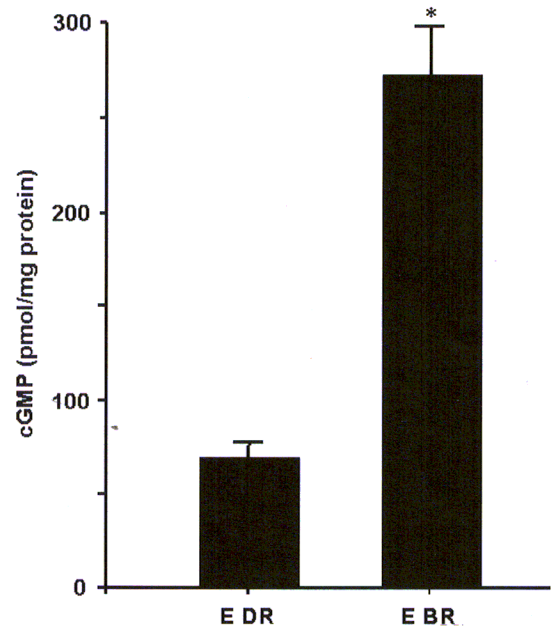

Figure 5 *Biochemical study of rabbit carotid artery transduced in vivo. The graph shows the level of cGMP to be significantly elevated in arteries transduced by AdeNOS-brushing (E BR) compared with AdeNOS-dripping (E DR) at the same vector concentration of 1.25×10^7 PFU/μL (*$P<0.05$; n=3).*

titer of 10^9 PFU/ring, mechanical delivery of PBS-suspended vector was associated with a 3.3±0.4-fold increase in transduction compared with conventional dripping of PBS-suspended vector ($P<0.01$; n=4; Figure 4). At a titer of 10^7 PFU/ring, mechanical delivery of PBS-suspended vector yielded a 5.7±0.5-fold increase in transduction compared with conventional dripping of PBS-suspended vector ($P<0.01$; n=4; Figure 4). At both AdLacZ titers, brushing vector suspended in fibrin glue was associated with less transduction (38±7% less at 10^7 PFU/ring; 37±8% less at 10^9 PFU/ring) compared with brushing vector suspended in PBS ($P<0.05$ and n=4 each; Figure 4). However, at both titers, transduction from brushing vector suspended in fibrin glue was still found to be superior to that from dripping vector suspended in PBS (2.1±0.2-fold superior at 10^9 PFU/ring and 3.5±0.3-fold superior at 10^7 PFU/ring; $P<0.05$ and n=4 each; Figure 4). It should be noted that some β-galactosidase activity (31.3±6 RLU/μg) was detected in control (nontransduced; n=8) dripped and brushed rings which were assayed in parallel with dripped and brushed rings transduced with AdLacZ at titers of 10^7 and 10^9 PFU/ring. However, this level of activity was significantly less than β-galactosidase activity observed in all AdLacZ-transduced groups ($P<0.001$; n=4 each), and not significantly different from levels detected in AdeNOS (control vector; 10^7 and 10^9 PFU/ring)-transduced groups also studied in parallel ($P>0.05$; n=4 each; Figure 4). Finally, as determined by cGMP RIA using rabbit carotid arteries transduced *in vivo* by brushing or dripping the same concentration of AdeNOS (1.25×10^7 PFU/μl), there was a 4.0±0.5-fold increase in cGMP levels in AdeNOS-brushed compared with AdeNOS-dripped arteries ($P<0.05$; n=3; Figure 5).

Safety

No evidence was found for tissue damage associated with our mechanical method. At the outset, it should be noticed that in our arterial cross-sections, the appearance of adventitial ruffling was frequently noticed both in nonbrushed (Figure 2b) and brushed (Figure 2c) sections alike, suggesting that it was an artifact of tissue sectioning and processing (more likely to occur in carotid arteries whose adventitia is comprised of loosely packed connective tissue) rather than an effect associated specifically with brushing. Macroscopically, in brushed-*versus*-dripped arteries in the presence and absence of AdLacZ, there was no difference in the patency of the arterial wall *ex vivo* (Figure 1A) or *in vivo* (Figures 3b and c). Preserved endothelium was observed in all sections obtained from bushed arterial rings (see, eg, Figure 2a). Lastly, we found no impairment of vascular reactivity (including endothelium-dependent vasomotor function tested with bradykinin and acetylcholine, and endothelium-independent vasomotor function tested with KCl) in brushed-versus-dripped arteries studied in parallel in the presence and absence of AdLacZ *ex vivo* ($P>0.05$ between groups; $n=4$ dogs; Table 2) or *in vivo* ($P>0.05$ between groups; $n=12$ rabbits; Table 2).

Generality

To determine if our mechanical transduction technique could be generalized to tissues other than arteries, we examined its applicability *in vivo* in neck skeletal muscle, abdominal wall tendon, peritoneum, outer bowel surface, and wounded (incised) skin. As observed using BluoGal histochemistry, mechanical delivery resulted in extensive transduction at 10^9 PFU/tissue target and mild-to-moderate transduction even at a low titer of 10^7 PFU/target, findings observed across the range of rabbit tissues tested (four duplicate sets of morphological data; total $n=12$ rabbits; Table 3). Matched nontransduced brushed and dripped tissues stained in parallel showed no positive staining except in segments of bowel and peritoneum where a mild blue hue from endogenous β-galactosidase was observed. Macroscopically, this staining was considerably less than that seen in corresponding AdLacZ-brushed tissues (four duplicate sets of morphological data; total $n=12$ rabbits; Table 3).

Discussion

As relatively little attention has been given to mechanical methods for vector delivery which, owing to their more direct approach, may be beneficial from the perspectives of both accuracy and efficiency of gene transfer, the principal objective of this study was to characterize mechanical delivery of adenoviral vector to tissues via direct application using a small paintbrush. The key findings of this study are that our mechanical vector delivery technique: (1) is associated with morphological and biochemical evidence for accurate and efficient gene delivery and expression in carotid artery even at a relatively low AdLacZ titer of 10^7 PFU/arterial ring; (2) is compatible with the use of adenoviral vector suspended either in saline alone or fibrin glue, a compound used clinically; (3) can be used to safely transduce even the most delicate of tissues (ie, small arteries) without structural or functional compromise; and (4) can be used to rapidly and effectively transduce a variety of tissues *in vivo*. To our knowledge, the use of a paintbrush to directly apply a vector to a target tissue represents a novel form of mechanical gene delivery. However, it should be noted that Yu *et al*[34] carried out a study to optimize cutaneous gene transfer in mice in which a coarse nylon-bristle toothbrush was first used to abrade dorsal skin epidermis via 100 brush strokes prior to topical application of a plasmid. In our study, the mechanism of augmented gene transfer efficiency likely relates to: (1) our ability to first locally sequester vector at the tissue surface by topical application (facilitated by lower transduction volumes, and reflecting the same general principle underlying certain chemical methods which have been shown to augment viral transduction by concentrating vector at cell and tissue surfaces[23,25]); and (2) subsequent direct massaging of infectious particles into the substance of the tissue itself (ie, improving access of vector to cells in the target tissue).

Table 2 Summary of vasomotor function data in carotid artery following dripping or brushing with or without vector[a]

	Ex vivo				*In vivo*			
	Bradykinin		*KCl*		*Acetylcholine*		*KCl*	
	Max. relax. (%)	EC_{50} (*−log mol/l*)	*Max. contract.* (%)	EC_{50} (*mmol/l*)	*Max. relax.* (%)	EC_{50} (*−log mol/l*)	*Max. contract.* (%)	EC_{50} (*mmol/l*)
Control								
Dripped	80±2	7.7±0.2	320±49	17.1±2.3	100±0	7.3±0.1	227±23	19.3±0.3
Brushed	84±2	7.7±0.3	306±73	16.4±1.8	99±2	7.4±0.1	221±27	19.3±0.3
AdLacZ								
Dripped	79±5	7.7±0.4	329±42	16.5±2.2	100±0	7.2±0.1	223±29	19.2±0.4
Brushed	82±2	7.9±0.2	341±69	16.5±2.1	97±2	7.0±0.1	237±33	19.0±0.5

[a]Data from four dogs (*ex vivo*) and 12 rabbits (*in vivo*) transduced with AdLacZ (10^9 PFU/tissue site) or nontransduced (control; ie exposed to PBS only). Responses are expressed as mean±s.e.m. For each agent, differences between groups are not significant ($P>0.05$). *Ex vivo*, relaxations are expressed as a percentage of relaxations to papaverine (3×10^{-4} mol/L; 100%=6.4±0.2 g for each group) while contractions are expressed as a percentage of contractions to phenylephrine (10^{-6} mol/l; 100%=5.7±0.2 g for each group). In vivo, relaxations are expressed as a percentage of relaxations to papaverine (3×10^{-4} mol/l; 100%=3.0±0.2 g for each group) while contractions are expressed as a percentage of contractions to phenylephrine (10^{-6} mol/l; 100%=2.9±0.2 g for each group).

Table 3 Summary of data indicating generality of mechanical vector delivery technique in vivo[a]

Tissue	*Histochemical staining*[b]		
	Negative control	*AdLacZ titer*	
		10^7 *PFU*	10^9 *PFU*
Carotid artery	—	+	+++
Incised skin	—	+	++
Neck skeletal muscle	—	++	+++
Abdominal wall tendon	—	++	+++
Parietal peritoneum	±	++	+++
Bowel outer surface	±	++	+++

[a]Data from n=12 rabbits mechanically transduced *in vivo* with AdLacZ (10^7 or 10^9 PFU/tissue site; n=4 each) or nontransduced (control; ie brushing or dripping of PBS only; n=4).[b]−, no staining with BluoGal; +, mild staining (light-blue peppering); ++, moderate staining (blue patches); +++=extensive staining (contiguous deep-blue regions); ±, nonspecific staining (light-blue hue due to the presence of endogenous β-galactosidase).

It should be reiterated that, as expected with the gentle brushing action associated with our technique, there was no morphological, biochemical, or functional evidence for any damage to even delicate tissue such as rabbit carotid artery.

Reduced transduction time

An important finding in this study was that our mechanical delivery technique facilitated reduction of transduction time down to 10 min which, compares favorably with transduction times of 30 min to 3 h using nonmechanical vascular gene transfer techniques described elsewhere.[3,5,9,11] In this context, our technique also compared favorably with the *in vivo* mechanical adenoviral vector delivery technique described by Kullo *et al*[19] involving incision of the rabbit carotid artery sheath, insertion of a perivascular catheter for pericarotid infusion of AdLacZ, followed by removal of the perivascular catheter and suture-closure of the newly vector-filled carotid sheath in order to maintain approximation of AdLacZ vector-solution against the adventitial surface of the carotid artery. In addition to the relative complexity of their experiments, in order to obtain effective expression of recombinant protein, Kullo *et al*[19] used higher titers of AdLacZ (2×10^9 PFU/artery) and prolonged transduction times (4 days). From our study, one benefit of reduced transduction time for *ex vivo* paradigms pertains to contributing to reduced overall experiment time while, for *in vivo* models, reduced transduction time may be helpful in lowering risks associated with prolonged anesthesia and tissue exposure.

Enhanced accuracy and efficiency of gene transfer

Morphological evidence. That accurate and efficient transfer of AdLacZ to the vascular wall had occurred following mechanical delivery was indicated not only by the intense histochemical staining observed in the walls of vector-brushed compared with vector-dripped arterial rings transduced *ex vivo* in parallel at the same titer (10^9 PFU/ring; see Figures 1 and 2), but also by the linearity of recombinant gene expression observed *in vivo* along the exposed arterial adventitial surface, a finding consistent with the linear brushstrokes used for mechanical vector delivery (Figure 3). Notably, focal mild-to-moderate staining was observed on the adventitial surface of vector-brushed arteries transduced with as little as 10^7 PFU each (Figure 3c), that is, at a relatively low AdLacZ titer in cardiovascular gene transfer paradigms.[2–5,9–13,19,20,23,32] To our knowledge, adenovirus-mediated gene expression has not been previously demonstrated morphologically in arteries transduced with as little as 10^7 PFU each, nor has maximal staining (as demonstrated herein at a titer of 10^9 PFU/artery) been observed following a transduction time of only 10 minutes. These findings, which provide key pieces of morphological evidence for enhanced gene transfer efficiency associated with our mechanical method, were confirmed biochemically. It should be noted that using immunoelectron microscopy for recombinant protein, we have previously shown that adventitial fibroblasts are the principal cellular targets for adenoviral entry and recombinant protein synthesis following conventional (drip-immersion) perivascular delivery;[11,22] the morphological data in the present study are consistent with those findings. Further, in the light of previous adenovirus-mediated perivascular gene transfer studies,[3,4,11,13] the level of adventitial transduction observed in this study following mechanical delivery of adenovirus (see Figure 2c) is consistent with that required to profoundly alter vascular reactivity using a vasomodulatory vector such as AdeNOS which enters adventitial fibroblasts and enhances local production of a diffusible relaxant gas, nitric oxide.[3,4,11,13]

With respect to experiments involving fibrin glue, brushed PBS-suspended vector produced circumferential BluoGal staining in arterial rings, while under the same conditions, brushed fibrin glue-suspended vector localized linearly along the upper halves of ring surfaces onto which the fibrinogen-plus-vector and thrombin were first dripped prior to circumferential brushing. This finding was likely due to rapid setting of the glue which, occurring within 5 s of contact between fibrinogen and thrombin, may have precluded circumferential vector distribution by brushing. This qualitative reduction in gene transfer associated with brushing vector suspended in fibrin glue compared with brushing vector suspended in PBS was confirmed and quantified biochemically. Despite the approximate 40% reduction in mechanical transduction of AdLacZ in the presence of

fibrin glue compared with mechanical transduction in the presence of PBS at the same titer, our morphological and biochemical data indicated that mechanical transduction in the presence of fibrin glue was up to 3.5-fold superior to conventional nonmechanical transduction involving dripping PBS-suspended vector at the same titer. For this reason, we believe that fibrin glue, an alternative composition to those used by other investigators to augment vector localization and gene transfer efficiency in cells and tissues,[23–25,27] can be used with the paintbrush technique to effectively transduce arteries.

Biochemical evidence. The 40-fold lower level of β-galactosidase activity measured in rings transduced at 10^7 PFU/ring compared with 10^9 PFU/ring was consistent with the overall 2-log reduction in titer. One possible explanation for the relatively increased benefit of mechanical transduction at a titer of 10^7 PFU/ring (5.7-fold increase in transduction with vector-brushing compared with vector-dripping under the same conditions) compared with 10^9 PFU/ring (3.3-fold increase in transduction with vector-brushing compared with vector-dripping under the same conditions) is that the true advantage of direct physical application of vector to tissues is more likely to be unmasked at a lower titer where less saturation of arteries by vector particles following conventional vector-dripping may occur. Interestingly, *ex vivo* transduction experiments carried out at 10^9 PFU/ring showed greater augmentation of mechanical transduction efficacy morphologically (see Figure 1A) than found biochemically at this titer (see Figure 4). Perhaps one explanation for this disparity is that despite a paucity of BluoGal staining in rings transduced by dripping AdLacZ, some degree of biochemically detectable recombinant β-galactosidase protein synthesis may still have occurred albeit below the level of visible detection.

As an *in vivo* correlate of mechanical gene transfer using a potentially therapeutic vector, namely AdeNOS,[3–5,11,13,16] we showed a four-fold increase in transduction (as measured biochemically using cGMP RIA) in rabbit carotid arteries transduced via brushing compared with dripping. Further, these transductions were carried out at the same concentration of vector, namely 1.25×10^7 PFU/μl, indicating that our *in vivo* mechanical gene transfer technique had an advantage in transduction efficiency independent of vector concentration. Based on our data, we believe that superior transduction occurs when vector particles are directly and gently massaged into the target tissue at relatively low transduction volumes (eg, at 20 μl/cm² of tissue).

Safety and generality

The findings of our *ex vivo* and *in vivo* vasomotor experiments were consistent with those of previous vascular gene transfer studies involving adventitial delivery.[3–5,11,13,19,20] In accordance with our morphological findings which showed intact vessel walls and sublayers macroscopically and microscopically independent of delivery technique, our vasomotor data indicated that both the smooth muscle layer and endothelium (the latter of which is one of the most delicate cell monolayers known) were functionally intact following brushing, even in a small-caliber artery such as the rabbit carotid. Results from other *in vivo* experiments, which demonstrated that mechanical delivery of adenovirus could be used to effectively transduce skeletal muscle, tendon, peritoneum, serosal surface of bowel, and wounded skin, provide evidence that the technique is not limited to vascular transduction alone, but rather may have a broader utility. Taken together, our data suggest that mechanical methods for adenoviral vector transduction such as the one described herein should be considered as useful adjuncts to other established gene delivery techniques.

Clinical applications of this technology

Based on the findings of this study, we believe that our mechanical delivery technique will be compatible with future application of appropriate, therapeutic clinical-grade vectors directly to the surfaces of diseased tissues or organs. From both surgical and interventional perspectives, it is conceivable that a small paintbrush-like device can be modified for use either directly by a surgeon at the time of open surgery or endoscopic "keyhole" surgery, or by an interventionalist at the time of bronchoscopy, arthroscopy, intracranial ventriculoscopy, or gastrointestinal endoscopy. Pathologic lesions that may be amenable to this technology include inflammatory, infective or fibrotic foci, wounded skin, neoplastic and dysplastic lesions, and post-resection tumor beds. With regard to potential endovascular applications of this technology, either alone or in combination with a vascular stent, an appropriate catheter device equipped with a paintbrush-like tip may be used to brush a therapeutic anti-atherosclerotic and/or vasorelaxing vector onto the endoluminal surface of a stenotic atheromatous plaque or vasospastic artery. In this setting, any molecule expressed and secreted would most likely need to diffuse or be targeted to affect the smooth muscle layer of a diseased artery.

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