Adenoviral Transfer of Endothelial Nitric Oxide Synthase Attenuates Lesion Formation in a Novel Murine Model of Postangioplasty Restenosis


Objective—Restenosis remains a major late complication of percutaneous transluminal coronary angioplasty (PTCA), for which the development of prevention strategies has thus far been hampered by the lack of a representative and practical animal model. We have, therefore, developed a murine model of PTCA-induced restenosis.

Methods and Results—Rigid probe angioplasty of pre-existing atherosclerotic lesions in the carotid arteries of ApoE-deficient mice was found to result in an increase in lesion size (0.14±0.04×10^5 μm^2 to 0.42±0.09×10^5 μm^2, P=0.007) with a smooth muscle cell-rich, fibrotic lesion morphology. In an additional experiment, lesions were incubated immediately after angioplasty with adenovirus bearing an endothelial nitric oxide synthase (eNOS) transgene (Ad.APT.eNOS), or an “empty” control virus (Ad.APT.empty) at a titer of 1.5×10^9 pfu/mL. Ad.APT.eNOS treatment was seen to lead to a 73.1% reduction in plaque size (0.27±0.04×10^5 μm^2 versus 1.02±0.39×10^5 μm^2, P=0.07), which translated to a significantly lowered average degree of stenosis (33.6±4.1% versus 74.6±14.0%, P=0.02). Ad.APT.eNOS also decreased lesional collagen content from 29.1% to 4.8% (P<0.001).

Conclusion—We believe that we have established a representative murine model of postangioplasty restenosis, which may serve to elucidate the mechanisms underlying restenosis and to evaluate potential antirestenotic therapies. (Arterioscler Thromb Vasc Biol. 2004;24:357-362.)

Key Words: atherosclerosis • restenosis • mouse model • gene therapy • eNOS

Since its introduction 25 years ago, the long-term outcome of percutaneous coronary revascularization procedures has been blemished by the occurrence of restenotic luminal narrowing. An extensive and diverse range of therapeutic approaches has been evaluated for their potential in the prevention of restenosis, including the technique of arterial stenting, which now constitutes a routine and efficacious prevention of restenosis, including the technique of arterial stenting, which now constitutes a routine and efficacious prevention of restenosis, including the technique of arterial stenting, which now constitutes a routine and efficacious prevention of restenosis, including the technique of arterial stenting, which now constitutes a routine and efficacious prevention of restenosis, including the technique of arterial stenting, which now constitutes a routine and efficacious prevention of restenosis, including the technique of arterial stenting, which now constitutes a routine and efficacious prevention of restenosis, including the technique of arterial stenting, which now constitutes a routine and efficacious prevention of restenosis, including the technique of 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angioplasty without and with stent implantation. The quest for improved reliability and clinical relevance of preclinical models for postangioplasty restenosis has led to the introduction of double injury protocols, in which dilatation is inflicted on pre-existing atherosclerotic lesions. Size limitations have thus far restricted such studies to larger experimental animals, including rabbits and Yucatan micropigs. Atherosclerosis-prone mice, however, including apolipoprotein E (apoE)−/− and LDL receptor (LDLr−/−) deficient mouse strains, should be considered highly suitable alternatives in view of their practicality and highly representative atherosclerotic lesion morphology. We therefore aimed to develop a practical murine model of restenosis, based on transluminal dilatation of pre-existing atherosclerotic lesions.

We have previously described the site-controlled induction of carotid atherosclerosis in apoE−/− and LDLr−/− mice by placement of a perivascular collar. The location of these plaques permits intravascular instrumentation and adenovirus instillation, using the external carotid artery as a portal of entry. In the first part of the current study, we established the feasibility of rigid-probe angioplasty of plaques obtained by this technique to effect restenotic neointima formation in ApoE−/− mice. The resulting plaques were subsequently used as a substrate for the evaluation of the antirestenotic potential of adenovirus-mediated transfer of eNOS in this model.

Methods

Adenovirus Generation and Cell Transduction

To generate the replication defective recombinant serotype 5 adenovirus expressing a human eNOS construct (Ad.APT.eNOS), the eNOS cDNA was subcloned in pAdApt, which contains a cassette for transgene expression containing the CMV promoter, polylinker, and SV40 polyA (HhaI-AvrII fragment, Invitrogen). This latter fragment enables generation of recombinant adenovirus by homologous recombination with a large cloned adenovirus fragment containing nucleotides 3534 to the right end of Ad5 after cotransfection in PER.C6 packaging cells. Large batches were grown in suspension cultures on PER.C6 cells, and viruses were purified using a two step CsCl purification. An analogous method was employed to generate the replication defective empty adenovirus vector Ad.APT.empty, which was used as a control. Efficiency of virus transduction was determined with adenovirus carrying a β-galactosidase transgene under control of a CMV promoter (Ad.CMV.lacZ).

Surgery and Gene Transfer

Bilateral pericarotid collar insertion was performed in female apoE−/− mice, aged 10 to 12 weeks, as described. Rigid angioplasty probes were fashioned by placement of epoxy resin beads (0.55 mm diameter, Perfecta International, Goes, The Netherlands) on a nylon wire (0.1 mm diameter, Silstar, Japan), in analogy with the method described by Zhou et al. Six weeks after insertion, the collars were removed and a probe was inserted into the common carotid artery through an incision in the external carotid artery. This intravascular probe was passed and retracted five times through the carotid artery through an incision in the external carotid artery. This procedure was repeated five times to achieve an initial degree of stenosis of approximately 50%. The degree of stenosis was determined by dividing the intimal area by the total area confined by the internal elastic lamina (×100%).

Tissue Harvesting and Histological Analysis

Three weeks after angioplasty, carotid artery specimens were obtained and transverse 5 μm cryosections prepared after in situ perfusion fixation with formalin as described. Cryosections were routinely stained with hematoxylin (Sigma Diagnostics) and eosin (Merck Diagnostica, Darmstadt, Germany). β-Galactosidase was demonstrated by incubation with x-gal (1 mg/mL; Eurogentec, Seraing, Belgium) at 37°C for 4 hours. Sections were stained immunohistochemically with antibodies against a macrophage-specific antigen (MOMA-2; a kind gift of Prof. Dr. G. Kraal), ASMA (clone 1A4, Sigma), eNOS, and inducible nitric oxide synthase (iNOS) (rabbit polyclonal antibodies, both from BD Biosciences Pharmingen, Franklin Lakes, NJ).

Morphometry and Statistical Analysis

Hematoxylin-eosin stained sections of the carotid arteries were used for morphometric analysis, as described. In short, the intimal surface area was calculated by subtracting the patent lumen area from the area circumscribed by the internal elastic lamina, while the degree of stenosis was determined by dividing the intimal area by the total area confined by the internal elastic lamina (×100%).

Results

Double Injury Restenosis Model

Early Effects

In preliminary experiments, passage of the rigid angioplasty catheter was found to lead to disruption of the atherosclerotic plaque in all cases, and to a break in the internal elastic lamina in the majority of animals treated, as histologically determined immediately after the procedure. One week after angioplasty, thrombus was found to be present in 2 out of 8 angioplasted vessels, accompanied by incomplete obstruction of the lumen and evidence of recanalization (data not shown).
Late Effects

Three weeks after the procedure, angioplasty was found to have led to a significant increase in plaque size compared with the untreated contralateral artery in the animals fed a western-type diet (1.00 ± 0.18 × 10^5 μm^2 versus 0.40 ± 0.12 × 10^5 μm^2, P = 0.016, Figure 1B). The effect was particularly pronounced in the chow-fed group, however, with a 3-fold increase in plaque size (0.14 ± 0.04 × 10^5 μm^2 to 0.42 ± 0.09 × 10^5 μm^2, P = 0.007). Postangioplasty plaque size (P = 0.032), intima/media ratio (P = 0.033), and intima/lumen ratio (P = 0.028) were significantly higher in the cholesterol-fed animals compared with the chow-fed group (Table 1). Histological and immunohistochemical analysis also revealed differences in plaque composition between these two groups, as depicted in two representative staining series in Figure 2. No differences in lipid content were found on oil-red-O staining, but the plaques of cholesterol-fed animals were seen to be less cellular and more necrotic, especially in the core of the lesion (Figure 2A and 2B). The ASMA content was substantially higher in choew-fed animals, particularly on the luminal side of the plaque (Figure 2C and 2D), while no significant differences were uncovered with respect to the macrophage-positive area (Figure 2E and 2F). Based on these findings, the lesion morphology observed in choew-fed animals was judged to be more representative of postangioplasty restenosis and to possess a greater discriminative power for the evaluation of antirestenotic therapies. Therefore, in all following studies, the western-type diet was discontinued after the angioplasty procedure.

Local Adenoviral Transduction

Transduction Efficiency

In vivo dose finding studies using Ad.APT.eNOS in noncolored arteries revealed that a titer of 1.5 × 10^5 pfu/mL, incubated intravascularly for 10 minutes, afforded endothelial overexpression without inducing cytopathic damage (as judged by the absence of endothelial denudation, endothelial cell swelling, or adhesion of leukocytes). One week after transluminal transduction, endothelial eNOS staining was found to have been raised significantly above background level by Ad.APT.eNOS incubation (Figure 3A and 3B), and this titer was therefore adopted for all subsequent transduction experiments. Initial adenoviral transduction studies in this model were performed immediately after angioplasty with adenovirus carrying a β-galactosidase gene instead of eNOS to facilitate the unequivocal identification of cells expressing the transgene. One week after adenovirus instillation, the transduced arteries were stained for β-galactosidase activity. In the area denuded by the angioplasty probe in the distal common carotid artery, efficient transduction of medial smooth muscle cells was found to have occurred, with efficiencies of up to 82% (Figure 3C). In the angioplastied plaque, medial transduction was minimal, but transduction was prominent in the smooth muscle cell-rich area adjoining the lumen, with maximal transduction efficiencies averaging 24.2 ± 18.4% (n = 3) of the entire plaque cell population (Figure 3D). Comparable staining patterns were achieved 1 week after transduction with Ad.APT.eNOS, both with respect to media (Figure 3E) and plaque staining (Figure 3F). Three weeks after treatment, however, expression levels of eNOS were low and comparable to those seen in the control group. Endogenous iNOS expression was demonstrated in macrophages associated with suture material used to tie off the external carotid artery, whereas iNOS expression remained below detection level in the plaque and the media in both groups at 1 week and 3 weeks after transduction (data not shown).

eNOS Transduction Studies

Lesions were incubated with Ad.APT.empty (n = 4) or Ad.APT.eNOS (n = 5) immediately after angioplasty. Body
The efficiency by Ad.APT.eNOS incubation. Determination of transduction efficiency by β-galactosidase staining one week after Ad.lacZ instillation following denudation of a nondiseased arterial segment (blue color, C; scale bar = 100 μm) or angioplasty of a pre-existing atherosclerotic lesion (D; scale bar = 25 μm). Endothelial denudation led to extensive (up to 82%) transduction of the medial smooth muscle cells, whereas incubation of the angioplastied plaque resulted in transgene expression in 24.2% ± 18.4% of plaque cells, mainly in the smooth muscle cell-rich periluminal area. Comparable levels of eNOS expression in the media (brown color, E) and the plaque (F) were seen 1 week after transduction of angioplastied vessels with Ad.APT.eNOS.

Following Ad.APT.eNOS instillation, but the difference was not significant due to excessive variation (124.8 ± 74.6 μm² and 1262.2 ± 828.5 μm², respectively [P = 0.34]).

Discussion

We have established a model of neointima formation following angioplasty of pre-existing atherosclerotic lesions, which is, to our knowledge, the first example of true restenosis in murine arteries. Angioplasty in this model leads to plaque disruption, endothelial denudation, and medial damage. When a chow diet is administered after the procedure, the resulting neointima is typified by its complex composition: rich in smooth muscle cells and collagen, yet containing residual macrophages and extracellular lipid deposits. Both the immediate and the long-term effects observed in this model are therefore reminiscent of the histological appearances that are characteristic of human postangioplasty restenosis.16,17 The neointima found in most conventional murine models of restenosis, on the other hand, is almost exclusively based on smooth muscle cell proliferation as a result of a single arterial injury, and thus typical of in-stent restenosis.16,17 A wide range of injurious stimuli has previously been employed in this context, including vascular ligation,27 endothelial denudation,12,28 perivascular cuff placement,29 and photochemical injury.30

Several of these models have been used in the evaluation of protocols aimed at the prevention of neointima formation, of which the transfer of eNOS is regarded as particularly promising.31 Thus, eNOS gene transfer has been found to reduce intimal hyperplasia after balloon injury of rat carotids32 and porcine coronary arteries.7 Notwithstanding the overtly beneficial effect of NO in these models, direct extrapolation of these data to postangioplasty restenosis on an atherosclerotic background is not necessarily warranted. Firstly, the efficiency and tissue distribution of adenoviral
transduction in most of these models differ markedly from those found after angioplasty of pre-existing plaques. For instance, we have found intact endothelium to be relatively impermeable to adenovirus suspensions, whereas endothelial denudation led to efficient transduction of medial smooth muscle cells, and angioplasty of atherosclerotic lesions enables transduction of perivascular smooth muscle cells. Secondly, in a nonoxidative milieu, gene transfer of NOS has predominantly antiatherogenic effects, including the inhibition of endothelial adhesion molecule expression and leukocyte extravasation and attenuation of smooth muscle cell proliferation and migration. In a setting of increased oxidative stress, however, as exemplified by the highly inflammatory conditions within an atherosclerotic plaque, NO may react with superoxide to form peroxynitrite (ONOO⁻). At low concentrations, the effects of ONOO⁻ are similar to those ascribed to NO, but at higher concentrations it is a powerful and toxic oxidant. Moreover, ONOO⁻ is formed from equimolar concentrations of NO and superoxide, and adequate dosing of NOS is therefore likely to be of considerable importance in an oxidative environment to avoid undesired proinflammatory effects. This likelihood is emphasized by the finding that deficiency of eNOS has been found to accelerate de novo atherosclerosis in apoE⁻/⁻ mice, whereas deficiency of the more active inducible isof orm (iNOS) attenuates atherosclerosis and reduces plasma levels of lipid peroxides. A beneficial effect of eNOS transfer to atherosclerotic lesions can therefore not be considered to be a foregone conclusion, especially because eNOS itself has been found to be capable of generating superoxide if depleted of its cofactor tetrahydrobiopterin (BH₄). To optimize conditions for the assessment of the effect of local eNOS gene transfer in our model of postangioplasty restenosis, we initially ensured that we were using a virus titer and an incubation period which did not induce cell damage, inflammation, or iNOS expression in the vessel wall. The titer thus arrived at, \(1.5 \times 10^9\) pfu/mL, is comparatively low for local vascular transduction, and we believe this to be related to the activity of the APT promoter, which has been shown to afford expression levels an order of magnitude higher than conventional CMV promoters.

Using this titer, we have shown eNOS to exert a beneficial effect on postangioplasty restenosis, both with respect to total plaque size and collagen deposition. Expression of eNOS was demonstrated in the media and plaques of Ad.APT.eNOS-incubated vessels 1 week after the procedure, but did not extend to three weeks after adenovirus instillation. This is in agreement with the temporal expression pattern afforded by most first generation adenoviruses. Expression of eNOS, however, coincided with the peak of neointimal cell proliferation (which is thought to occur approximately one week after angioplasty), a time point which could therefore be considered to be the most effective time for expression of a transgene aimed at limiting smooth muscle cell proliferation in the neointima. Nitric oxide has previously also been shown to modulate muscle cell function from a synthetic to a contractile phenotype, which is reflected by increased expression of markers of smooth muscle cell differentiation and reduction of collagen synthesis. These effects may account for the attenuation of collagen deposition and the trend toward increased ASMA expression observed in our model. Collagen-sclerotic tissue is an attribute of restenosis after percutaneous transluminal coronary angioplasty (PTCA), particularly in diabetic patients. We therefore believe our postangioplasty lesion morphology to be representative of this type of partially-fibrotic neointima, which contributes toward its validation as a model for the elucidation of molecular processes involved in nonstent restenosis after balloon angioplasty. Moreover, this model may prove to be a valuable substrate for the evaluation of antirenostotic therapies that might obviate the need for stent application. The accessibility of these lesions to intravascular application is very well suited to localized gene therapeutic approaches, whether these be viral or nonviral in nature.

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References


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