Background and Purpose—There are major differences in susceptibility of intracranial and extracranial arteries to atherosclerosis. The goal of this study was to examine adenovirus-mediated gene transfer to basilar and carotid arteries of Watanabe heritable hyperlipidemic (WHHL) rabbits, which have spontaneous hypercholesterolemia and atherosclerosis, and normal New Zealand White (NZW) rabbits. We used 2 different adenoviral vectors, driven by either cytomegalovirus (CMV) or Rous sarcoma virus (RSV) promoters.

Methods—Basilar and carotid arteries were removed from WHHL and NZW rabbits and cut into rings. The arteries were incubated with an adenoviral vector that expresses β-galactosidase and is driven by either a cytomegalovirus (CMV) or Rous sarcoma virus (RSV) promoter (AdCMVβgal or AdRSVβgal). Arteries were incubated with virus for 2 hours, and then incubated in medium for 24 hours to allow expression of transgene. Transgene expression was assessed by enzyme activity (Galacto-Light assay) and by a histochemical method after X-Gal staining.

Results—After gene transfer, β-galactosidase was expressed in endothelium and adventitia but not media. There were moderately severe atherosclerotic lesions in carotid arteries and early lesions in basilar arteries. Enzyme activity after gene transfer with AdCMVβgal (3 × 10³ particles/mL) was greater in the basilar artery of WHHL than NZW (137 ± 40 versus 25 ± 10 mU/mg protein, \( P < 0.05 \)) (mean ± SE) and in the carotid artery (133 ± 27 versus 34 ± 11 mU/mg protein, \( P < 0.05 \)). After gene transfer with AdRSVβgal, transgene expression was similar in arteries from WHHL and normal NZW rabbits.

Conclusions—This is the first study to examine gene transfer to intracranial and extracranial arteries from atherosclerotic animals. The findings suggest that an adenoviral vector with a CMV, but not RSV, promoter provides greater transgene expression in the basilar and carotid arteries from spontaneously atherosclerotic rabbits than from normal rabbits. (Stroke. 1998;29:120-125.)

Key Words: atherosclerosis ■ cerebral arteries ■ promoter regions ■ gene transfer ■ hypercholesterolemia ■ rabbits
could be that the adeno virus was driven by a cytomegalovirus (CMV) immediate early promoter in our studies, while the Rous sarcoma virus (RSV) promoter was used in other studies. The third goal of this study, therefore, was to compare adeno virus-mediated β-galactosidase activity in normal and atherosclerotic arteries, using an adeno virus vector driven by CMV or RSV promoters.

Materials and Methods

Adeno virus Vector
We used replication-deficient adenoviruses AdCMVβgal and AdRSVβgal. The recombinant virus was amplified and purified in the University of Iowa Gene Transfer Vector Core. The DNA constructs comprise a full-length copy of the adenovirus genome of approximately 36 kb. The early region 1 (E1) genes have been deleted and replaced by either a CMV or an RSV promoter and cDNA for bacterial β-galactosidase, which can encode β-galactosidase activity (mU/mg protein) in the carotid and basilar arteries from NZW rabbits. There were 55% particles/plaque-forming unit (PFU) for AdCMVβgal and 35% to 4% particles/PFU for AdRSVβgal.

Experimental Preparation
Adult WHHL and normal NZW rabbits of either sex were studied. Experimental protocols were approved by our institution’s animal care committee. Rabbits were euthanized by injection of sodium pentobarbital (50 mg/kg) into the marginal ear vein followed by pentobarbital (5 mg/kg) and 0.2% glutaraldehyde in PBS, as described previously. Tissue was minced with a scalpel blade and placed in 150 mmol/L NaHCO3, 0.61 mmol/L MgSO4, and 7.8 mmol/L glucose, and 2.52 mmol/L CaCl2). The carotid and basilar arteries were then isolated and cut into segments 2 to 3 mm in length.

Vascular rings were then incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Sigma) solution for 2 hours at room temperature. Vessels were rinsed in PBS and postfixed with 4% paraformaldehyde and 0.2% glutaraldehyde in PBS, as described previously. Vascular rings were incubated in 3-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Sigma) solution for 2 hours at room temperature. Vessels were rinsed in PBS and postfixed with 4% paraformaldehyde. The fixed rings were then embedded in paraffin and sections were cut from the block, placed on slides, and counterstained with nuclear fast red. Sections of arteries were examined for positive staining (blue nuclei) with light microscopy.

Statistical Analysis
All data are expressed as mean±SEM. Overall, intergroup comparisons were made using 1-way ANOVA, and individual group means were compared using the t statistic for least significant differences. Statistical significance of differences among means of groups was determined using Student’s paired t test. Differences were considered to be significant at P<0.05.

Results
Total serum cholesterol was 66±14 mg/dL in NZW rabbits and 537±40 in WHHL rabbits. There were moderately severe atherosclerotic lesions in the carotid artery of WHHL rabbits and early lesions, particularly at branch points, in basilar artery of WHHL.

Effects of Viral Titer and Duration of Exposure to Virus
Studies were performed to determine the effect of viral titer on β-galactosidase activity (mU/mg protein) in the carotid and basilar arteries of NZW rabbits (Figure 1). Arteries were incubated for 2 hours with AdCMVβgal and 24 hours in medium. There was a dose-dependent increase in β-galactosidase activity for both the carotid and basilar arteries (Figure 1). Dose-dependent expression also was observed with AdRSVβgal (data not shown).

We determined the effect of duration of exposure of carotid and basilar arteries to AdCMVβgal on β-galactosidase activity in NZW rabbits (Figure 2). After incubation of the vessels with a submaximal concentration of virus (1×1011 particles/mL), the arteries were incubated for 24 hours in medium. There was a time-dependent increase in β-galactosidase activity for both the carotid and basilar arteries. Based on these findings, we studied the carotid and basilar arteries in the following experiments after 2 hours of exposure to 1 or 3×1011 particles/mL of virus.

Histochemistry
Staining for β-galactosidase was observed in adventitial and endothelial cells of carotid and basilar arteries of WHHL (Figure 3) and NZW rabbits (not shown). No staining was observed in vehicle-treated vessels.
Transgene Expression in the Carotid Artery
After incubation of rings of carotid artery in AdCMVβgal, there was greater β-galactosidase activity in WHHL than normal NZW vessels (Figure 4). At a low viral titer (1 × 10^{11} particles/mL), transgene expression tended to be greater (not statistically significant) in WHHL than normal rabbits (Figure 4).

In contrast, after incubation of rings of carotid artery in AdRSVβgal, there was only modest β-galactosidase activity, and there was no difference between normal and WHHL rabbits (Figure 5). These results indicate that there is greater β-galactosidase activity in carotid arteries from WHHL than those from normal NZW rabbits, using a CMV promoter but not RSV promoter.

Transgene Expression in the Basilar Artery
After incubation of the basilar artery in AdCMVβgal, there was greater β-galactosidase activity in arteries from WHHL than normal NZW vessels (Figure 4). At a low viral titer (1 × 10^{11} particles/mL), transgene expression tended to be greater (not statistically significant) in WHHL than normal rabbits (Figure 4).

In contrast, after incubation of rings of carotid artery in AdRSVβgal, there was only modest β-galactosidase activity, and there was no difference between normal and WHHL rabbits (Figure 5). These results indicate that there is greater β-galactosidase activity in carotid arteries from WHHL than those from normal NZW rabbits, using a CMV promoter but not RSV promoter.
than normal NZW (Figure 6). There were not sufficient tissue samples to examine gene transfer with AdRSVβgal in all basilar arteries. Nevertheless, after incubation of the basilar artery with AdRSVβgal, β-galactosidase activity clearly was not greater in WHHL rabbits (1 ± 0.4 mU/mg protein, n=6) than in normal NZW (2.3 ± 0.9 mU/mg protein, n=4).

In all of the experiments described above, arteries were incubated in medium for 24 hours after incubation in virus. We also incubated the carotid artery for 48 hours in medium, after 2 hours of exposure to virus. The goal was to determine if, after a longer incubation time, expression of β-galactosidase would continue in normal vessels so that transgene expression might be similar in WHHL and normal NZW rabbits. Using AdCMVβgal, after incubation for 48 hours, there again was significantly more β-galactosidase activity in the carotid artery of WHHL compared with NZW (Figure 7). After incubation with AdRSVβgal, there again was no difference in β-galactosidase activity in the carotid artery of WHHL versus NZW (Figure 7).

Discussion

This study represents the first report of gene transfer to intracranial arteries from atherosclerotic experimental animals. The major finding of the study is that there is greater activity of β-galactosidase in intracranial and extracranial arteries from atherosclerotic (WHHL) than normal rabbits, when an adenoviral vector driven by a CMV promoter is used. Greater β-galactosidase activity in WHHL was observed in carotid arteries, which demonstrate moderately severe lesions, and basilar arteries, which have early lesions. These studies also demonstrate that in WHHL, adenovirus-mediated expression of β-galactosidase is greater when driven by a CMV promoter than an RSV promoter.

β-Galactosidase Activity in Atherosclerotic Arteries

These findings indicate that expression of β-galactosidase is greater in cerebral arteries from atherosclerotic than normal animals. Other studies have examined gene transfer to non-cerebral vessels from atherosclerotic rabbits using liposomes10 and recombinant adenoviruses,1,5,11,12 and findings were quantified in 2 studies.1,5 Less adenovirus-mediated β-galactosidase activity was observed using an RSV promoter in atherosclerotic than normal rabbit iliac arteries in vivo.5 In contrast, augmented adenovirus-mediated expression of β-galactosidase was observed using a CMV promoter in atherosclerotic rabbit aorta in vitro.1 The difference in findings could be attributed to the method of gene transfer (in vivo versus in vitro), the different vessels (iliac artery and aorta), or the use of different promoters (RSV versus CMV) to drive β-galactosidase activity after gene transfer. Our results indicate that differences in enzyme activity after gene transfer to atherosclerotic vessels can be attributed at least in part to use of an RSV5 versus CMV promoter.1

One explanation for greater expression of β-galactosidase in arteries from WHHL when the CMV promoter is used is that, in the CMV promoter, there are CRE (cAMP response element) and NFκB binding sites, enhancer regulatory regions that positively regulate the promoter.13,14 The RSV promoter does not contain CRE or NFκB binding sites.15 Thus, enhanced expression of β-galactosidase in arteries from WHHL may be produced by activation of either transcription factor, CREB (cAMP response element binding protein), NFκB, or both.

The transcription factors CREB and NFκB are induced by several physiological and pathophysiological stimuli in blood vessels. CREB may be stimulated by minimally oxidized
LDL in cultured aortic endothelial cells and by an increase in intracellular cAMP. Activation of NFκB has also been demonstrated in endothelium of atherosclerotic lesions. In addition, reactive oxygen species in atherosclerotic tissue may activate NFκB. Atherosclerotic lesions contain macrophages and neutrophils that release proinflammatory cytokines, which also activate NFκB. Finally, oxidized LDL in mice that are fed an atherogenic diet activates NFκB in arteries in vivo. Thus, there are multiple mechanisms by which CREB and NFκB in the CMV promoter might be activated by atherosclerotic lesions.

**WHHL Rabbits**

In a previous study, atherosclerosis was produced by feeding rabbits an atherogenic diet. When rabbits are fed an atherogenic diet, arterial lesions form rapidly, with accumulation of lipids in macrophages and formation of lesions with foam cells. In contrast, atherosclerosis occurs spontaneously in WHHL rabbits, because they lack LDL receptors and consequently fail to clear LDL from their plasma. WHHL animals have more gradual formation of atherosclerotic plaques, without marked accumulation of lipids in macrophages, and lesions have fewer foam cells.

Our results, in carotid and basilar arteries, are consistent with those in our previous study, in which increased β-galactosidase activity was observed in atherosclerotic aorta from rabbits that were fed a high-lipid diet. Although atherosclerosis develops more gradually in WHHL than fat-fed rabbits, WHHL also exhibited enhanced β-galactosidase activity in the carotid and basilar arteries. These studies suggest that there is augmented expression of β-galactosidase using a CMV promoter in atherosclerotic arteries, even though the cause, rate of progression, and severity of the disease differ.

We explored the possibility that intracranial vessels may differ from extracranial vessels in expression of CMV-driven transgenes following adeno viral gene transfer, especially because intracranial vessels are relatively resistant to atherosclerosis. In this study, we observed early lesions in the basilar artery of WHHL and greater activity of β-galactosidase in basilar artery from WHHL than normal rabbits. Thus, hypercholesterolemia and early lesions in the basilar arteries of WHHL rabbits are sufficient to augment β-galactosidase activity.

A histochemical method was used to examine the site of expression (adventitia or endothelium) of β-galactosidase. β-galactosidase activity in the basilar artery was observed in both adventitia and endothelium in this and a previous study. Histochemical staining underestimates transfection efficiency, and thus may not be appropriate for precise quantitation of β-galactosidase expression in vessels. In this study, all quantitation was performed by measurement of enzyme activity, not by histochemical measurements.

These studies demonstrate gene transfer of a reporter gene to cerebral vessels. Transfer of genes that produce changes in function of vessels will be of great interest both for studying vascular biology and potentially for therapy. We and others have observed functional changes after gene transfer of eNOS to the carotid and basilar arteries. Superoxide dismutase (SOD) may play a critical role in protection of cerebral vessels against oxidative stress, and the importance of Cu/Zn-SOD, MnSOD, and ECSOD can be addressed using gene transfer. Finally, we speculate that transfer of a gene that encodes a potent vasodilator, such as calcitonin gene-related peptide, may prove to be of therapeutic values in prevention of vasospasm following subarachnoid hemorrhage.

In conclusion, it is likely that atherosclerotic arteries will be a major target for vascular gene therapy. There is greater β-galactosidase activity in basilar and carotid arteries of atherosclerotic rabbits, with minimal or moderate lesions, than in normal arteries, when β-galactosidase expression is driven by a CMV promoter. We speculate that adenoviral gene transfer, driven by a CMV promoter, may be useful in therapy for complications of cerebral vascular atherosclerosis.

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**References**

Heritable diseases are the manifestation of alterations at the protein level. For some, the abnormality is the result of disruption of a single gene, as is the case with sickle cell disease. For others, the genetic abnormalities are less apparent, involving a number of genes and their protein products to display a complex phenotype, as in coronary and cerebral vascular disease. Yet in both of these circumstances, the prospect of gene therapy holds great promise, whether to replace the defective gene or to provide a protein that will ameliorate the underlying abnormality.1

The explosive growth in the understanding of the molecular mechanisms of cardiovascular disease over the past two decades has led to a rapidly expanding number of potential targets for therapeutic intervention. Examples of this phenomenon can be seen in the design and use of thrombolytic agents and, more recently, platelet glycoprotein IIb/IIIa receptor antagonists. One potential downfall of newer therapeutic agents is that they are frequently proteins, hence their effective local gene delivery, obviating the need for systemic administration of potentially harmful agents.

In atherosclerotic cerebral vascular disease, the blood vessels themselves (the endothelium and underlying media) would be the targets of treatment. Atherosclerotic diseases are uniquely suited to gene therapy for several reasons: (1) the endothelium is the first line of defense between the vasculature and the tissues; (2) adenoviral vectors efficiently infect the endothelium and therefore appear well-suited to deliver therapeutic genes to the vasculature; and (3) catheter-based approaches may provide effective local gene delivery, obviating the need for systemic administration of potentially harmful agents.

Clearly, though, many of the specifics of gene therapy approaches remain to be worked out.1 3 For example, are adenoviruses the best vectors to use? If so, what regions of the viral genome might be harmful or beneficial? What mode of delivery is best, and will catheter-based methods work? And perhaps one of the most important questions, what target genes will provide the best treatment for cerebral vascular disease?2

In the accompanying article, Lund et al provide data addressing two other questions about adenoviral vectors, namely, is gene transfer equally effective in normal and atherosclerotic vessels, and do different promoters provide similar rates of gene transfer? Interestingly, they found that, of two promoters that should both direct constitutive expression of target genes, a CMV promoter yielded greater transgene expression than an RSV promoter in both carotid and basilar artery segments. More importantly, transgene expression, as measured by β-galactosidase enzyme activity, appeared to be enhanced by the presence of atherosclerosis in both types of vessels. These findings have implications not only for the optimal design of adenoviral gene therapy vectors, but also for the efficacy of such therapy in cerebral vascular disease. In addition, an important finding here is that gene transfer to atherosclerotic intracranial vessels may be a viable therapeutic approach.

However, several important points should be noted. First, the findings of Lund et al are from ex vivo, rather than in vivo, gene transfer, a situation that may greatly affect the efficiency of gene delivery. Second, while the use of reporter genes such as β-galactosidase is an important first step in such experiments, the efficacy of gene transfer of appropriate target genes (eNOS, SOD, VEGF, FGF) may vary greatly and needs to be tested in similar models. Finally, as has so often been observed with new experimental therapies, humans may respond quite differently than animals. In this case, atherosclerosis in spontaneous hyperlipidemic rabbits may be pathogenetically distinct from human atherosclerosis. Nonetheless, the findings of Lund et al hold promise for the potential of gene therapy in atherosclerotic cerebral vascular disease.

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Editorial Comment

Adenovirus-Mediated Gene Transfer Is Augmented in Basilar and Carotid Arteries of Heritable Hyperlipidemic Rabbits
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