Gene transfer to atherosclerotic arteries is an important goal of gene therapy for cardiovascular diseases. We have observed previously that adenovirus-mediated β-galactosidase activity is greater in atherosclerotic than normal aorta.1 Thus, it may be possible to “target” expression of β-galactosidase to atherosclerotic arteries.

There are major differences in susceptibility of intracranial and extracranial blood vessels to hypercholesterolemia and atherosclerosis. Atherosclerotic lesions typically are less severe in the basilar artery than in extracranial arteries.2 Thus, one might anticipate that β-galactosidase activity, which is augmented in the atherosclerotic aorta,3 might be augmented in carotid artery but perhaps not in the basilar artery of atherosclerotic animals. Accordingly, the first goal of this study was to determine whether, after gene transfer, β-galactosidase activity is augmented in the basilar and carotid arteries of atherosclerotic rabbits.

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 × 10^11 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

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Atherosclerosis was produced in our previous study1 by feeding an atherogenic diet to normal rabbits. In the present study, gene transfer was examined in a genetic model of atherosclerosis—the Watanabe heritable hyperlipidemic (WHHL) rabbit,3 which has spontaneous dyslipidemia by a mechanism that is similar to human familial hypercholesterolemia.4 In contrast to rabbits which are fed a very high lipid diet and develop lesions rapidly, WHHL develop atherosclerotic lesions more slowly. Our second goal was to determine whether β-galactosidase activity is augmented in arteries from WHHL rabbits, a model of spontaneous hypercholesterolemia.

In contrast to the finding that atherosclerosis augments β-galactosidase activity after gene transfer,1 others have observed low efficiency of gene transfer to the atherosclerotic iliac artery.5 One explanation for these different findings

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could be that the adenovirus 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 adenovirus-mediated β-galactosidase activity in normal and atherosclerotic arteries, using an adenoviral vector driven by CMV or RSV promoters.

Materials and Methods

Adenovirus Vector
We used replication-deficient adenoviruses AdCMV\(\beta\text{-gal}\) and AdRSV\(\beta\text{-gal}\). 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 the CMV or RSV promoter and cDNA for bacterial β-galactosidase, preceded by a nuclear localization signal for simian virus 40 large T antigen. Recombinant viruses were grown in human embryonic kidney 293 cells that complement genes necessary for replication. There were 55 ± 2 particles/plaque-forming unit (PFU) for AdCMV\(\beta\text{-gal}\) and 35 ± 6 particles/PFU for AdRSV\(\beta\text{-gal}\).

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 exsanguination. The carotid artery and sheath and the brain were quickly removed and placed in oxygenated Krebs solution (133 mmol/L NaCl, 4.7 mmol/L KCl, 1.35 mmol/L NaHPO4, 16.3 mmol/L NaHCO3, 0.61 mmol/L MgSO4, 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. Rings from the carotid and basilar arteries were placed in a 96-well culture plate and incubated either with AdCMV\(\beta\text{-gal}\) or AdRSV\(\beta\text{-gal}\) (1 × 1011 and 3 × 1011 particles/mL) or vehicle (PBS with 3% sucrose) for 2 hours at 37°C. Rings of arteries were place in medium (Eagle’s minimal essential medium (Boehringer Mannheim) with 100 U/mL of penicillin and 100 μg/L streptomycin) for 24 hours at 37°C, in a chamber aerated with 95% O2 and 5% CO2.

Expression of β-Galactosidase
After incubation, the arteries were removed from the culture medium, rinsed with PBS, frozen in liquid nitrogen, and stored at −70°C until enzyme activity was measured. β-Galactosidase activity was measured using a chemiluminescent assay (Galacto-Light Plus, Tropix), as described previously. Tissue was minced with a scalpel blade and placed in 150 μL Galacto-Light lysis solution (100 mmol/L potassium phosphate, pH 7.8, 0.2% Triton X-100). The homogenate was centrifuged at 10,000 g for 10 minutes, and supernatant was removed. The assay was performed using 10 μL of supernatant in 200 μL Galacto-Plus substrate reaction buffer diluent (1:100 dilution). The reaction was carried out at room temperature, and light emissions were measured with a Monolight 2010 luminometer (Analytical Luminescence Laboratory). A standard calibration curve was generated with use of purified Escherichia coli β-galactosidase (Boehringer Mannheim). Protein was measured using a Bio-Rad DC protein assay. β-Galactosidase activity was expressed as mU E coli β-galactosidase per mg protein. Values for each group were calculated from an average of 2 rings from each animal.

Histochemical analysis also was performed to examine the location of expression of β-galactosidase. Following ex vivo incubation, arterial rings were rinsed with PBS and fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in PBS, as described previously. 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% formaldehyde. 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 AdCMV\(\beta\text{-gal}\) 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 AdRSV\(\beta\text{-gal}\) (data not shown).

We determined the effect of duration of exposure of carotid and basilar arteries to AdCMV\(\beta\text{-gal}\) 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 AdCMV\(\beta\)gal, there was greater \(\beta\)-galactosidase activity in WHHL than normal NZW vessels (Figure 4). At a low viral titer (1\( \times \)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 AdRSV\(\beta\)gal, there was only modest \(\beta\)-galactosidase activity, and there was no difference between normal and WHHL rabbits (Figure 5). These results indicate that there is greater \(\beta\)-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 AdCMV\(\beta\)gal, there was only modest \(\beta\)-galactosidase activity,
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 liposomes and recombinant adenoviruses, and findings were quantified in 2 studies. Less adenovirus-mediated β-galactosidase activity was observed using an RSV promoter in atherosclerotic than normal rabbit iliac arteries in vivo. In contrast, augmented adenovirus-mediated expression of β-galactosidase was observed using a CMV promoter in atherosclerotic rabbit aorta in vitro. 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 RSV versus CMV promoter.

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. The RSV promoter does not contain CRE or NFκB binding sites. 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 adenoviral 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), 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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Hereditable 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 inability to cross the blood-brain barrier. Nonetheless, the findings of Lund et al hold promise for the potential of gene therapy in atherosclerotic disease. In addition, an important finding here is that gene transfer to atherosclerotic intracranial vessels may be a viable therapeutic approach.

In the accompanying article, Lund et al provide data addressing two other questions about adeno viral 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 interestingly, 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 alter 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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Adenovirus-Mediated Gene Transfer Is Augmented in Basilar and Carotid Arteries of Heritable Hyperlipidemic Rabbits

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