Adenovirus-Mediated Gene Transfer of Macrophage Colony Stimulating Factor to the Arterial Wall In Vivo

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Abstract—Macrophage colony stimulating factor (MCSF) is believed to play a key role in one of the earliest events in atherosclerosis, ie, monocyte to macrophage differentiation in the arterial intima. The aim of this study was to examine the biological effects of vascular wall expression of MCSF. A recombinant adenovirus vector encoding human MCSF (AdMCSF) was generated by standard techniques of homologous recombination in 293 cells. The rabbit carotid artery was transduced with AdMCSF. As negative controls, carotid arteries were transduced with either an adenoviral vector encoding β-galactosidase, an adenoviral vector encoding apolipoprotein E, or diluent alone. Intima-media thickness ratio was calculated 5 and 21 days after transduction. The cell type present in intimal infiltrates was analyzed by immunohistochemistry. MCSF expression was demonstrated in the vessel wall of AdMCSF-transduced vessels by reverse transcription–polymerase chain reaction and immunofluorescence. In contrast to control vessels, adenovirus-mediated MCSF expression was associated with an intimal cellular infiltrate consisting of smooth muscle cells and small numbers of macrophages. Whereas the intima-media thickness ratio was greater in AdMCSF-transduced vessels at 5 days, this difference was no longer statistically significant at 21 days. These results suggest that MCSF may play a role in recruitment of monocytes and macrophages to the vessel wall and may contribute to smooth muscle cell proliferation and migration. (Arterioscler Thromb Vasc Biol. 1998;18:1157-1163.)

Key Words: macrophage colony stimulating factor ■ monocytes ■ atherosclerosis ■ gene transfer ■ smooth muscle cells

One of the earliest features of atherosclerosis is the adherence of monocytes to the endothelium, followed by their migration into the intima.1,2 In the intima, monocytes differentiate into macrophages, and subsequent exposure to modified lipoproteins leads to the formation of foam cells. In addition, foam cells may also originate from SMCs.3 There is evidence that in early atherosclerosis, SMCs migrate from the media to the intima of the arterial wall, where they proliferate and endocytose various lipoproteins, including modified LDL,4 resulting in the formation of foam cells.5 Further evidence of a role for SMCs in this process is the observation that SMCs from atherosclerotic but not normal arteries express the receptor for MCSF, the proto-oncogene c-fms.6 Thus, it appears that both macrophages and SMCs contribute to the formation of foam cells characteristic of fatty streak lesions.

MCSF, an 85-kDa protein that is produced by cells of monocyte-macrophage lineage, endothelial cells, and fibroblasts, is known to stimulate the differentiation and proliferation of monocytic progenitor cells and may play a role in the differentiation of monocytes to macrophages in the arterial wall.7 MCSF activates various functions of mature macrophages through a specific receptor encoded by proto-oncogene c-fms.8 SMCs also express c-fms8 and thus, MCSF may also be involved in the migration and proliferation of these cells in atherosclerosis.

MCSF is expressed in atherosclerotic but not normal human arteries.9 Furthermore, MCSF deficiency results in significantly reduced atherogenesis in mouse models of atherosclerosis.10 The role of MCSF in atherogenesis may be addressed by overexpressing MCSF in the arterial wall by using gene transfer techniques. Recently, adenoviral vectors have been used to efficiently transduce cells of the vasculature.11 In this study, the effect of adenovirus-mediated gene transfer of the human MCSF gene to the rabbit carotid artery is examined.

Methods

Measurement of Cholesterol and MCSF in Plasma
Cholesterol levels were measured by standard enzymatic techniques. MCSF levels were measured by an ELISA assay (Quantikine, R&D Systems).

Generation of Recombinant Adenoviruses
In brief, cDNA for human MCSF or human apoE3 was subcloned into pMAL123 (kindly provided by Dr Jeff O’Brian, Dupont Merck...
Plasmid pMAL123 was obtained by inserting 3 linkers at EcoRI, XhoI-BglII, and BglII sites. The resulting plasmid was linearized and cotransfected with XhoI-restricted adenovirus strain d109 DNA into 293 cells by conventional calcium phosphate precipitation. Recombinant vectors were generated by homologous recombination (Figure 1B). Plaques containing recombinant adenovirus vectors were picked, and viral DNA was isolated by a modified Hirt extraction.25 Plaques were screened by restriction mapping, and the virus was amplified in 293 cells from plaques that contained inserts of the appropriate size. Positive plaques underwent 2 more rounds of plaque purification. Plaques were again picked and screened by restriction endonuclease mapping. The resulting recombinant adenovirus vectors were expanded in 293 cells and purified by double cesium-gradient ultracentrifugation as previously described.14 The isolated viral band was dialyzed against 140 mmol/L NaCl, 10 mmol/L HEPES (pH 7.2), and 1 mmol/L MgCl₂ and stored at −70°C in 10% glycerol. The viral titer was determined by plaque assay. Cells (293 and human umbilical vein endothelial) transduced with AdMCSF expressed human MCSF as detected by RT-PCR (data not shown). The adenoviral vectors encoding MCSF and apoE were under the control of the major late promoter. An adenoviral vector encoding β-galactosidase under the control of the cytomegalovirus promoter was used as a further control and as a means to examine the distribution of transgene expression (AdβGal was a kind gift of Dr James M. Wilson, University of Pennsylvania, Philadelphia).

**In Vivo Gene Transfer**

All experimental protocols were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care. Male New Zealand White rabbits weighing 3.0 to 3.5 kg were anesthetized with intramuscular ketamine (68 mg/kg), xylazine (Rompun, 9 mg/kg), and acepromazine (2.3 mg/kg). After bilateral paramedian incisions on the anterior neck, the common carotid arteries were exposed usingatraumatic surgical technique. Side branches, 5 to 8 mm away from their origin, were coagulated or tied off with 5–0 silk. The adventitia was cleaned off at the proximal and distal ends of the exposed segment. After the administration of heparin (100 U/kg) and placement of a purse-string monofilament suture (8–0 ethilon) at the proximal end of the isolated segment, the common carotid arteries were clamped proximally and distally and a 24-gauge catheter was inserted proximally through the purse string. The blood was removed by gently massaging the artery with a wet cotton swab and a gauze wick at the open end of the catheter. One hundred microliters of the adenoviral vector–containing solution (2×10⁹ plaque-forming units per milliliter) or the same volume of diluent alone (PBS with 0.5% BSA) was instilled into the vessel lumen, the catheter was removed, and the purse-string suture tied to close the defect in the arterial wall. Twenty minutes later, flow was restored by removing the clamps. The skin incisions were closed in 2 layers and the animal allowed to recover. Five or 21 days later, the animals were anesthetized and the transduced carotid arteries were excised. The sites of arterial clamping and cannulation were not harvested. Seventeen animals were used for the 5-day experiments. Twelve animals were transduced with AdMCSF on 1 side. Ten of these were transduced with AdApoE and 2 with AdβGal in the contralateral carotid artery. In 5 additional animals, AdβGal was used on 1 side and PBS with 0.5% BSA on the other. Five animals were used for the 21-day studies.

**Evaluation of Transgene Expression**

**Detection of β-Galactosidase Activity**

Vessels harvested for 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-Gal) staining were fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in PBS, pH 7.4, for 30 minutes. Vessels were immersed in X-Gal reagent (Boehringer Mannheim Corp) for 90 minutes. Samples were rinsed with PBS, photographed, placed in 10% buffered formalin, and embedded in paraffin by using standard histological techniques. Five-micron-thick sections taken at multiple levels through the block were lightly stained with eosin only and examined by light microscopy.

**Detection of MCSF Expression by RT-PCR**

Recombinant MCSF expression was analyzed by RT-PCR. In 2 animals, total cellular RNA was isolated from arterial segments with RNeasy as per the manufacturer’s instructions. After pretreatment with amplification-grade DNase I (GIBCO BRL), RNA was reverse transcribed using SuperScript II reverse transcriptase (GIBCO) and random hexamers (GIBCO). cDNA was amplified in a thermal cycler with human MCSF–specific primers (sense, 5′-GGA-CAG-TTG-GAA-GAT-CCA-CGC-3′; antisense, 5′-TGG-GAC-GCA-GGC-CTT-GTC-ATG-3′) and the following parameters: 1 minute at 95°C, 1 minute at 55°C, and 1.5 minutes at 72°C, for 40 cycles. For
RT-PCR, samples were analyzed in the presence or absence of reverse transcriptase.

**Detection of MCSF by Immunofluorescence**

From 2 animals, fresh segments of artery were snap-frozen in LN2 and OCT and stored at −70°C. Five-micron-thick sections were fixed with acetone for 10 minutes. After nonspecific antibody-binding was blocked with 5% BSA, the tissue sections were incubated with a monoclonal mouse anti-MCSF antibody (Genetics Institute HM7/7.7.10, diluted 1:100 in 1% BSA-PBS) for 60 minutes. TRITC-conjugated anti-mouse IgG (T2402, Sigma Chemical Co, diluted 1:64) was then applied for 20 minutes. Slides were mounted with PBS-glycerol (1:1, vol/vol) and examined with a fluorescence microscope (Nikon).

**Morphometric Analysis of Intimal Infiltrates**

Carotid arteries transduced with AdMCSF, AdApoE, AdβGal, or PBS-A were analyzed for the presence of an intimal infiltrate 5 days after transduction (n=5 for each). In addition, AdMCSF- and AdβGal-transduced vessels were analyzed 21 days after transduction (n=5). Common carotid arteries were perfusion fixed in situ with 2% paraformaldehyde for 5 minutes at 80 mm Hg and harvested. Segments were snap-frozen in OCT. Fifteen 5-μm-thick sections were cut at intervals of 100 μm postfixed in acetone for 10 minutes, and stained with hematoxylin-eosin. The thickness of the intima and the underlying media was measured by light microscopy and computer-assisted morphometry (DIGICEL, ASK). Intimal thickness as a percentage of medial thickness was calculated.

**Immunohistologic Analysis of Intimal Infiltrates**

Five-micron-thick sections were fixed with acetone for 10 minutes. Endogenous peroxidase was blocked with 0.1% NaN3, and 3% H2O2 for 10 minutes. Nonspecific antibody binding was blocked with 5% normal goat serum and 0.05% Tween 20 for 10 minutes. Primary antibodies were diluted (1:50, vol/vol) for 20 minutes, and 0.4% of 3-amino-9-ethylcarbazole (A6424, Sigma) substrate solution were sequentially applied. Slides were counterstained with hematoxylin, mounted with glycerol gelatin (A6424, Sigma), and examined with a light microscope.

**Statistical Analysis**

Statistical analysis was performed by Kruskal-Wallis ANOVA using the Newman-Keuls test. A value of P<0.05 was considered statistically significant.

**Results**

**Rabbits**

Rabbit weights and plasma cholesterol and MCSF values were measured in the 21-day group at 0, 5, and 21 days. The weights were as follows: 3.5±0.3, 3.3±0.6, and 3.5±0.2 kg, respectively (P=NS). In contrast, cholesterol levels were significantly lower at 21 days compared with those at 0 or 5 days: 47.7±15.0, 48.5±7.9, and 36.0±9.2 mg/dL, respectively (P<0.05). MCSF was undetectable in the plasma of AdMCSF- or AdβGal-transduced vessels at 0, 5, or 21 days.

**Detection of β-Galactosidase in AdβGal-Transduced Carotid Arteries**

To characterize the distribution of adenovirus-mediated gene expression in the rabbit carotid artery in vivo, an adenoviral vector encoding β-galactosidase was used. Efficient gene transfer limited to the endothelium was evident 5 days after transduction (Figure 2).

**Detection of MCSF Expression In Vivo by RT-PCR**

Expression of recombinant MCSF was detected by RT-PCR 5 days after transduction. Recombinant MCSF was detected in AdMCSF- but not in AdApoE-transduced vessels (Figure 3). The reaction was negative in the absence of reverse transcriptase. MCSF expression was also detected at 21 days.
there was no intimal infiltrate or only a minimal, characteristically 1-cell-layer-thick infiltrate, with intima to media percentages of 3.69±1.7, 0.4±0.3, and 0.7±0.6, respectively (Figure 6). In addition, AdMCSF- and AdβGal-transduced carotid arteries were examined 21 days after transduction. At this time, the intima to media percentage was still greater in AdMCSF-transduced vessels, although this difference was no longer statistically significant (15.89±12.4% versus 6.04±3.4%, P=NS) (Figure 6).

**Immunohistologic Analysis of Intimal Infiltrates**

Immunohistochemical staining for rabbit macrophages with RAM11 antibody demonstrated that the cellular infiltrates in AdMCSF-transduced vessels contained small numbers of rabbit macrophages (Figure 7). Immunohistochemical staining for SMCs with an α-actin antibody demonstrated that the cellular infiltrates were composed predominantly of SMCs (Figure 8). In contrast, intimal staining for α-actin was not present in control vessels.

**Discussion**

In this article, we describe for the first time the effect of adenovirus-mediated gene transfer of human MCSF to the rabbit carotid artery in vivo. In contrast to vessels exposed to AdApoE, AdβGal, or diluent alone, AdMCSF-transduced vessels contained an intimal infiltrate consisting predominantly of SMCs and smaller numbers of macrophages. Although the infiltrate was significantly greater in AdMCSF-transduced vessels at 5 days, this difference was no longer significant at 21 days.

Adenoviral vectors are capable of mediating efficient gene transfer to the vascular wall in vivo. In the current study, an adenoviral vector encoding human MCSF was generated. In vivo experiments involved luminal administration of the vector to an isolated segment of the rabbit carotid artery. Previous experiments in the rat model have demonstrated that this mode of gene delivery results in endothelium-specific gene transfer. In our study, we found recombinant protein expression in the endothelium of AdMCSF- and AdβGal-transduced vessels, suggesting that luminal administration of adenoviral vectors may be used to obtain gene transfer to the endothelium of the rabbit carotid artery.

The MCSF vector used in this study was generated by standard techniques of homologous recombination in 293 cells as previously described. In this vector, transgene expression is driven by the major late promoter. For analysis of the effect of transgene expression at 5 days, one set of control vessels was transduced by an adenoviral vector encoding the human apoE gene, which, like the MCSF vector, was also under the control of the major late promoter. Another set of control vessels was transduced by an adenoviral vector encoding β-galactosidase. In contrast to AdMCSF and AdApoE, transgene expression in AdβGal was driven by the cytomegalovirus promoter. This vector is used to identify the efficiency of gene transfer and the cellular distribution of transgene expression. As a final control, vessels were transduced by diluent alone. We thus had a series of controls for the vector itself, the promoter used, and the effect of surgery per se.
One major difficulty associated with the use of adenovirus-mediated gene transfer to study the effects of vascular wall gene expression is vector-induced cytotoxicity. Newman et al. have described prolonged vascular cell activation, inflammation, and neointimal hyperplasia as a result of adenoviral transduction in the rabbit iliofemoral artery. These findings were minimal at 3 days and extensive at 10 and 30 days after viral transduction. Thus, interpretation of the effects of adenovirus-mediated gene transfer on vessel wall morphology must be interpreted with caution. In the current experiment, an intimal infiltrate was demonstrated 5 days after transduction of the rabbit carotid artery with AdMCSF. We chose 5 days in these experiments because the vessel wall changes in the study by Newman et al were not present until 10 days after transduction. To exclude the possibility that surgery or the adenoviral vector was inducing the cellular infiltrate, vessels were harvested from animals that had undergone sham surgery or whose vessels had been transduced with AdβGal or AdApoE. A minimal infiltrate was observed in a small number of sections from vessels harvested 5 days after viral transduction. Thus, 3 sets of controls yielded minimal evidence of intimal infiltrate 5 days after transduction.

Figure 5. Light microscopy of rabbit carotid arteries harvested 5 days after transduction with AdMCSF (top 2 panels) and AdβGal (bottom 2 panels). Intimal infiltrate is present in AdMCSF- but not AdβGal-transduced vessels. Area indicated by the arrow in upper left panel is magnified in upper right panel. In both left-hand panels, bar=0.5 mm; in both right-hand panels, bar=100 μm.

The observation that the intimal infiltrate associated with MCSF overexpression was composed predominantly of SMCs was not expected. It is of interest, however, that MCSF exerts profound effects on recruitment and terminal differentiation of pluripotent cells in other systems. MCSF is known to induce proliferation of intimal but not medial SMCs. Induction of c-fms expression in vascular SMCs by a number of growth factors, including platelet-derived growth factor, epidermal growth factor, and basic fibroblast growth factor, has been demonstrated.
Our results demonstrate that MCSF expression in the endothelium leads to intimal accumulation of predominantly SMCs and suggests that MCSF expression in the vessel wall may result in infiltration of the intima by both macrophages and SMCs. The absence of a cellular infiltrate in the intima of control vessels suggests that the infiltrate observed in AdMCSF-transduced vessels was due to MCSF overexpression and was not vector induced or the result of surgery per se.

Transduced rabbit carotid arteries were also examined 21 days after transduction. In this series of experiments, vessels were transduced with AdMCSF or AdβGal. Interestingly, as with the 5-day animals, extensive intimal infiltration was not observed in the AdβGal-transduced vessels at this time. A minimal infiltrate not more than 1 cell thick was observed in some sections. In contrast to the observation at 5 days, the intima to media thickness ratio was not significantly different in the AdMCSF- and AdβGal-transduced carotid arteries at 21 days. Therefore, the extent of the intimal infiltrate did not appear to increase with time. Although MCSF expression was still detectable by RT-PCR at 21 days after transduction, it should be noted that adenovirus-mediated transgene expression has been reported to markedly diminish with time after gene transfer. Therefore, the results of the time-course experiment suggest that expression of MCSF in the rabbit carotid artery resulted in an intimal infiltrate that did not increase with time. This may be due to the fact that adenovirus-mediated gene transfer results in transient transgene expression.
In summary, we have described the generation of an adeno viral vector encoding MCSF. Vascular wall cells were successfully transduced with this vector in vivo. Adenovirus-mediated gene transfer of MCSF to the rabbit carotid artery in vivo resulted in recombinant MCSF expression and a cellular infiltrate composed of SMCs and small numbers of macro- phages. These findings suggest that adenovirus-mediated transfer of the MCSF gene to the vessel wall may result in monocyte recruitment and the induction of SMC migration and proliferation. However, the role of the current generation of vectors in studying the role of MCSF in vascular wall pathology may be limited by the inflammatory response elicited by the vector at later times. Therefore, before further studies of this issue are undertaken, improvements in adenoviral vector technology will be necessary.

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