Remodeling of Carotid Artery Is Associated With Increased Expression of Matrix Metalloproteinases in Mouse Blood Flow Cessation Model

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Background—The matrix-degrading activity of matrix metalloproteinases (MMPs), required for cell migration and general tissue reshaping, is thought essential for pathological arterial remodeling in atherosclerosis and restenosis.

Methods and Results—We triggered remodeling of the carotid artery in C57BL/6 mice by blood flow cessation to study the relationship with gelatinases MMP-9 and MMP-2. Ligated and contralateral carotid arteries from ligated and sham-operated mice were harvested fresh, for biochemical analyses, or were perfusion-fixed, for histological studies, at 0, 1, 3, 7, 14, and 28 days after ligation. An early statistically significant \( P < 0.01 \) 4- to 5-fold increase in MMP-9 expression detected by SDS-PAGE zymography and Western blotting in tissue homogenates of ligated carotid arteries 1 day after flow cessation was maintained through day 7, after which expression gradually fell. Maximal MMP-9 levels were higher than MMP-2 levels, which became significantly increased 7 days after ligation. Proliferating cells, identified by bromodeoxyuridine incorporation, were detectable at day 1 in the adventitia and subsequently throughout the wall. Neointima was visible in 3-day specimens of ligated arteries. Suggested by morphology and predicted by theoretical considerations, maximal MMP-9 expression coincided with cell migration into the neointima, supporting its enabling role. Morphological measurements also demonstrated positive lumen remodeling up to 7 days after ligation.

Conclusions—MMP-9 induction is associated with the formation of intimal hyperplasia and does not require frank mechanical injury. Our data also show that a significant increase in MMP-9 expression preceded the positive geometrical remodeling of arteries, suggesting a potentially beneficial role for this matrix-degrading enzyme. (Circulation. 2000;102:2861-2866.)

Key Words: metalloproteinases ■ carotid arteries ■ remodeling

Besides direct mechanical injury by surgical vascular interventions, pathological vascular remodeling can occur naturally in response to immunologic, biochemical, or hemodynamic stimuli. Artery reaction to vascular injury has become the object of intense research, because of potential clues for human atherosclerotic lesion etiology and because restenosis is a major cause of morbidity in humans. However, remodeling of human vessels can only be captured as snapshots. Longitudinal observations in a variety of experimental models have provided much insight into the evolution of vascular lesions. Observations made in rat and rabbit balloon injury models of arterial remodeling\(^2,3\) support the importance of the matrix-degrading action of matrix metalloproteinases (MMPs), especially the gelatinases MMP-2 and MMP-9. These are necessary for general reshaping of the extracellular matrix scaffold of the arterial wall and likely enable migration of vascular smooth muscle cells (SMCs) and adventitial fibroblasts.\(^4\) Recently, murine models of vascular remodeling have attracted great interest because of their potential for genetic manipulation. Remodeling of murine arteries has been induced by the insertion of a thin wire,\(^5\) electric burning,\(^6\) and flow cessation.\(^7\) To study gelatinases MMP-9 and MMP-2 in relation to mouse carotid artery remodeling, we chose the flow-cessation model, characterized by rapid and reproducible remodeling with the development of significant intimal thickening.

Methods

Animal Model

Male C57BL/6J mice (2 months old, 20 to 28 g, Jackson Laboratory, Bar Harbor, Me) were anesthetized by intraperitoneal injection of xylazine (Rompun, Bayer Corp) and ketamine HCl (Abbott Laboratories) (13 mg/kg and 87 mg/kg body weight, respectively). The left common carotid artery was ligated near bifurcation with the use of 5-0 silk (Ethicon).\(^7\) In sham-operated animals, the suture was passed under the exposed left carotid artery but not tightened. The wound was sutured, and the animals were allowed to recover on a warming blanket. Two groups of injured or sham-operated animals were processed for morphological and biochemical studies at 1, 3, 7, 14, and 28 days after the procedure. Before they were euthanized (at 12

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Within the group selected as the apex for each carotid, the subsequent section in each group was counterstained with hematoxylin-eosin.

Figure 1. Graphic representation of ligated and sham-operated murine carotid artery remodeling after flow cessation. Actual average values obtained from measurements of 4 different carotid arteries per each time point were used for graphic representation of lumen (hatched area), intimal (solid black), medial (white), and adventitial (gray) layer dimensions. All layers were considered to be circular. Ligated carotid artery lumen experiences significant (*P<0.01) enlargement throughout day 7 and then becomes significantly smaller.

and 4 hours), the animals from the first group received 2 subcutaneous 2.5-mg injections of the thymidine analogue bromodeoxyuridine (BrdU, Sigma). Animal survival was >95%. A total of 110 mice were used in the final analysis, with 4 or 5 animals per each time point. The Emory University Committee on Institutional Animal Care and Use approved the protocol. For morphological analysis, animals were perfused with normal saline and fixed with 10% phosphate-buffered formalin at physiological pressure for 3 minutes. Left and right carotid arteries were removed in block, further fixed in 10% phosphate-buffered formalin at physiological pressure for 3 minutes. Left and right carotid arteries were removed in block, further fixed for 16 hours, and paraffin-embedded without further dissection. Because lesion thickness varies longitudinally, the entire length of the left and right carotid arteries was sectioned and examined for identification of the apex of the lesion, which displays the smallest lumen. Groups of 5 sections (5 μm) were collected at equally spaced intervals (measured by discarding 15×10-μm-thick slices). The first section in each group was counterstained with hematoxylin-eosin. Within the group selected as the apex for each carotid, the subsequent 4 sections were processed for detection of specific histological features.

Immunostaining
MMP-9 was detected with the use of rabbit anti-mouse MMP-9 polyclonal antibody (generously provided by Drs Senior and Shipley, Washington University, St. Louis, Mo), followed by biotinylated goat anti-rabbit IgG (Fisher Biotech), streptavidin–horseradish peroxidase (Dako), and dianaminobenzidine (DAB kit, Vector Laboratories), and counterstained with Gill’s hematoxylin (Fisher Scientific) and Scott’s solution. For negative controls, primary antibody was replaced with nonimmune rabbit serum. Cellular proliferation was detected as nuclear incorporation of BrdU with use of a monoclonal antibody (Cappel), in combination with the M.O.M. kit (Vector), followed by horseradish peroxidase or Texas red–conjugated donkey anti-mouse (Jackson); counterstaining was achieved with 0.5 mg/mL Hoechst (bis-benzimide, Sigma). Images were collected by use of a Zeiss Axioscope microscope.

Morphometric Analysis
Perimeters of the lumen, internal elastic lamina (IEL), and external elastic lamina (EEL) were obtained by tracing the contours on digitized images. Intimal thickness (distance between lumen and IEL), medial thickness (distance between IEL and EEL), and adventitial thickness (distance between EEL and the outer edge between tightly packed and surrounding loose tissue) were automatically calculated by ImagePro Plus 3.0 software (Media Cybernetics) as the mean perpendicular distance between 2 bordering tracings. All the areas were back-calculated with the assumption of circular structures. Proliferation indices were calculated as a percentage of BrdU-positive cells (pink nuclei) of the total number of cells (blue-counterstained nuclei) per arterial cross section.

Biochemical Analyses
Fresh carotid arteries were collected separately, pulverized under liquid nitrogen, and extracted with the use of ice-cold lysis buffer (10 mmol/L sodium phosphate, pH 7.2, containing 150 mmol/L NaCl [PBS], and 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and 0.2% sodium azide) for 1 hour at 4°C; centrifugation was at 10 000g for 5 minutes. For detection of MMP-2 and MMP-9, equal amounts of tissue extract protein (20 μg), assayed by using the DC protein assay (Bio-Rad), were loaded on each lane and run in parallel with prestained molecular weight markers (Bio-Rad) in 10% SDS-PAGE gels containing 1% gelatin, as described in detail previously. The optical volume-density product of individual lytic bands in Coomassie blue–stained gels was quantified by use of the Molecular Analyst (Bio-Rad). A single value was derived for MMP-9 and for MMP-2 by adding the bands associated with both pro and activated forms.

For Western blotting, samples (20 μg protein) were loaded on 10% SDS-PAGE mini gels and transferred onto nitrocellulose with use of a SemiDry system (Bio-Rad). MMP-9 was detected with rabbit anti-mouse MMP-9 polyclonal antibodies, followed by enhanced chemiluminescence (ECL kit, Amersham). Signals on x-ray films were quantified by use of the Multi-Analyt (BioRad).

Data Analysis
Average values were obtained per time point from image analysis of the apex section of each of the 4 or 5 carotid arteries. Densitometric
data from Western blotting and SDS-PAGE zymography were generated through analysis of 4 or 5 individual carotid arteries per each time point. Comparisons were made by ANOVA followed by the Tukey protected test to compare the means of multiple groups. Means were considered significantly different at $P < 0.05$.

Results

General Features of Vascular Remodeling in Flow Cessation Murine Model

The ligated carotid artery underwent rapid remodeling, as previously reported. A neointima was visible at day 3 and was well developed by 7 days. By 28 days, neointima accounted for $>30\%$ of the total wall thickness. Morphometric analysis (Figure 1) showed that compared with the sham-operated artery, the ligated carotid artery lumen initially undergoes a statistically significant enlargement, followed later by a significant decrease. Measurement of individual layer and total wall thickness showed both neointima development and overall wall shrinkage. The neointimal layer steadily increased throughout 28 days (Figure 2). Medial and adventitial layer thickness increased until day 14 but decreased thereafter. Cell number over time paralleled the layer thickness, suggesting that cell dynamics, the net result of cell turnover and migration between layers, is a major determinant of vascular remodeling in this model.

Expression of Gelatinases During Mouse Carotid Artery Remodeling

MMP gelatinolytic activity in the carotid artery was increased after ligation (Figure 3). At day 1, MMP-9 activity had already increased 4- to 5-fold ($P < 0.01$) in the ligated arteries. MMP-9 activity was detected also in sham-operated arteries at day 1 (this activity was likely stimulated by the surgical injury) and then returned to baseline levels but remained significantly elevated in ligated arteries through day 7. In contrast, activity corresponding to MMP-2 apparent molecular weight became significantly increased only after 7 days and remained lower than absolute MMP-9 levels. Pro-MMP-2 activation was detectable in 14- and 28-day specimens. Immunoblotting confirmed significant MMP-9 protein upregulation in ligated arteries up to 7 days after ligation (Figure 4). By immunocytochemistry, MMP-9 was not detectable in normal mouse carotid arteries and had very low levels in sham-operated arteries at all time points. In contrast, MMP-9 expression was clearly detectable by day 3 in many adventitial and medial cells of ligated arteries. MMP-9-positive cells appeared to be engaged in migration (Figure 5, inset). Scattered MMP-9-positive cells were detected in contralateral arteries as well. By day 7, intense MMP-9 staining was visible in the well-developed neointima of ligated arteries. Neointimal cells remained highly positive for MMP-9 in the ligated arteries harvested at day 28. Adventitial MMP-9 expression was also maintained.

We analyzed the dynamics of cell accumulation and proliferation within the 3 layers of the ligated artery. BrdU-positive cells were detected throughout the wall (Figure 5). For each time point, average individual layer proliferation indices (intimal, medial, and adventitial) and the average overall arterial wall index were calculated (Figure 6). The proliferation index was maximal at day 1 in the adventitia, where it subsequently decreased sharply. Up to 14 days, proliferation slowly increased in both intimal and medial layers and then decreased in the medial layer, while being sustained in the intimal layer. Comparison of total cell counts...
(Figure 2) and proliferation index data (Figure 6) produces a picture of cell dynamics within different layers. In the intima, the total cell count continued to increase throughout day 28, consistent with the increased proliferation index. In contrast, the total medial cell number initially modestly increased and then tapered off, in spite of sustained proliferation. Similarly, the adventitial cell number initially increased and then decreased after 14 days. The decrease observed in the outer 2 layers is compatible with cells migrating into the intimal layer.

The murine carotid artery does not contain intimal SMCs under normal conditions; thus, cell migration is an absolute requirement for neointimal development. MMP-9–positive cells appeared to be engaged in migration, which is thought to require MMP activity. However, such an in situ association remains circumstantial, inasmuch as to date there are no definitive markers of cell migration. The number of neointimal cells is also influenced by the rate of cell proliferation and cell loss. To gain further insight regarding the specific contribution of migration to neointimal formation, we made some theoretical considerations. With the use of data recorded from cell proliferation analysis, for the intimal layer these data were fit to a log curve (Figure 6) with the following equation: 

$$r = -0.015 + 0.032 \ln(t)$$

where $r$ is the proliferation rate, and $t$ is days $\times$ doublings per day. The cell number, plotted with use of actual neointimal proliferation rates (Figure 6), and doubling times reported for mouse cells were compared with the actual neointimal cell numbers counted in the specimens (Figure 2). Because of the simplicity of our model, we limited calculations to early proliferation rates (up to 7 days). We confirmed that the actual cell number detected in the neointima up to day 7 was higher than what was possible solely through proliferation, indicating that additional cells had to have migrated from other wall layers. The time course obtained is similar to that of MMP-9, which is consistent with MMP-9 contribution to cell migration.

**Discussion**

On the basis of the requirement that cells have a matrix-degrading capacity to free themselves from the matrix cage to move and proliferate and to reshape the tissues, we and others have hypothesized that MMPs play a key role in tissue remodeling associated with vascular lesion progression. After activation by soluble mediators, SMCs increase their secretion of matrix-degrading enzymes, including MMP-9. Previous studies support the role of MMP-9 in SMC migration and proliferation in vitro and in situ after carotid balloon angioplasty injury in rat and rabbit models. Data available for the mouse from studies of remodeling after perivascular femoral artery electric burning, another type of frank injury, also show increased MMP-9 expression.

In the present study, we investigated the expression of gelatinases during vascular remodeling of the murine carotid artery induced by flow cessation. Low flow is thought to contribute to the development of human vascular lesions and was previously reported to upregulate MMP expression in a rabbit model of carotid artery remodeling. Like other experimental models, the murine flow cessation model is an obvious departure from...
Arterial lumen stenosis after mechanical injury was shown to resemble the evolution of the human restenotic artery. Carotid artery undergoes initially positive and then late MMP-2 activity may be associated with carotid artery remodeling. Similarly, although not examined in detail, the late increase in MMP-9 activity could be related to the regulation of adventitial MMP-9. Recently reported actions of MMP-9 include proteolytic modification of biological activity of important nonmatrix substrates, such as interleukin-1β and several inhibitors of serine proteases, which in turn modulate vascular cells, suggesting that MMP-9 may influence vascular remodeling beyond matrix degradation.

Our present study of murine carotid artery remodeling suggests that MMP-9 participates in positive remodeling, an initial beneficial phase after vascular injury. Additionally supporting this finding was the result of analysis of contralateral carotid arteries, which experience increased blood flow after ligation. We found that MMP-9 gelatinolytic activity was also doubled in contralateral arteries 1 day after ligation (not shown). After 7 days, the lumen of arteries contralateral to ligated arteries had increased on average by 30% (P<0.01), and the total wall areas increased >50% (P<0.05) compared with arteries contralateral to sham-operated carotid arteries. The potential role of MMP-9 in positive remodeling is of great interest, inasmuch as therapeutic inhibition of vascular MMPs is currently contemplated in restenosis. Currently available strategies, including the administration of chemical or biological MMP inhibitors and the overexpression of TIMPs, suffer from inescapable limitations, such as poor efficiency or tissue access, and also from nonspecific effects. A definitive confirmation of the role of MMP-9 in vascular remodeling proposed by the present and previous studies awaits the development and utilization of better experimental tools, including the recent availability of MMP-deficient animals.

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