Expansive Arterial Remodeling Is Associated With Increased Neointimal Macrophage Foam Cell Content
The Murine Model of Macrophage-Rich Carotid Artery Lesions

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Background—Recent observations associate plaque instability with expansive arterial remodeling, suggesting a common driving mechanism.

Methods and Results—To demonstrate that macrophages, a characteristic of vulnerable plaques, also assist in expansive remodeling, we compared carotid artery remodeling due to formation of experimental macrophage-rich and macrophage-poor lesions in the flow cessation model in hypercholesterolemic apolipoprotein E knockout (ApoE KO) and wild type (WT) mice. After ligation, macrophages started to rapidly accumulate in ApoE KO but not in WT carotid artery lesions. Macrophage-rich ApoE KO intimal lesions grew fast, typically occluding within 14 days, despite a tripling of the vessel area. Outward remodeling of macrophage-rich ApoE KO arteries positively correlated with macrophage area ($r^2=0.600$, $P<0.001$). To investigate potential mechanisms of macrophage-enabled expansive remodeling, we compared levels of matrix metalloproteinases in homogenates of macrophage-rich and macrophage-poor carotid arteries. Gelatinolytic activity of macrophage-rich lesions increased faster and reached maximal levels several fold higher than in the macrophage-poor WT lesions.

Conclusions—Our results suggest that macrophages facilitate expansive arterial remodeling through increased matrix degradation by matrix metalloproteinases. This initially favorable remodeling action may eventually increase the vulnerability of macrophage-rich atherosclerotic plaques. (Circulation. 2002;105:2686-2691.)

Key Words: atherosclerosis ■ hypercholesterolemia ■ metalloproteinases ■ leukocytes ■ remodeling

Recent evidence from pathological observations, biological investigation, and in vivo imaging supports the notion that obstruction of an atherosclerotic artery causing an acute cardiovascular event is due to an occluding thrombus formed after disruption of atherosclerotic plaque, rather than occlusion by plaque bulk itself.1,2 Many of the disrupted arteries had actually undergone compensatory enlargement,3 also known as expansive, positive, or outward remodeling, which allowed preservation of blood flow. Expansive remodeling is thus currently considered one of the characteristic features of vulnerable plaques, leading to recent interest in identification of the forces allowing such remodeling. Observations of expansive remodeling in the context of stable and unstable plaques suggest that the early positive effect is related to later weakening of atherosclerotic plaques,2,4,5 raising the possibility that a common mechanism drives both phenomena.

Another characteristic of vulnerable atherosclerotic plaques is a high content of macrophages that have accumulated intracellular lipid, known as foam cells.6,7 Compared with macrophages resident in atheroma,8,9 foam cells secrete increased amounts of matrix metalloproteinase (MMP) zymogens10 and activate them,11 thus possessing an increased ability to degrade the plaque’s matrix scaffold. This MMP action has already been implicated in expansive remodeling12 and in weakening human atherosclerotic plaques.7,9,13

In the present study, we investigated the potential contribution of macrophage foam cells and their MMPs to outward remodeling of atherosclerotic arteries by establishing an experimental model that allows comparison of carotid artery remodeling due to development of macrophage-rich or macrophage-poor arterial lesions initiated using the same trigger, ligation of the carotid artery. In the atherosclerosis-prone apolipoprotein E knockout (ApoE KO) mouse, a current model for human atheroma etiology, fibro-fatty lesions occur within 6 to 8 weeks on a hypercholesterolemic diet. To obtain a macrophage-poor lesion for comparison, we used carotid artery ligation in C57BL/6 mice (WT).14 Previous observa-
tions indicate that intimal thickening occurs in this model without frank endothelial injury.\textsuperscript{14,15} Using the same procedure to initiate carotid arterial remodeling in the ApoE KO mouse, we obtained macrophage foam cell-rich atheromatous lesions. We then compared carotid artery remodeling associated with the progression of macrophage-rich or macrophage-poor intimal lesions.

**Methods**

**Animal Model**

Male C57BL/6J WT and C57BL/6J ApoE KO mice (6 weeks old, 20 to 25 g; Jackson Laboratories, Bar Harbor, Maine) were anesthetized by intraperitoneal injection of xylazine (5 mg/kg, Bayer Corporation) and ketamine HCl (80 mg/kg, Abbott Laboratories). Remodeling was triggered by ligation of the left common carotid artery near its bifurcation, using 5-0 silk ligature (Ethicon) as previously described.\textsuperscript{14} The wound was sutured and the animal recovered on a warming blanket. At the time of ligation, all ApoE KO mice were started on an atherogenic diet containing 0.5\% cholic acid and 1.25\% cholesterol (Research Diets). No neurological deficit was observed in any of the ligated mice throughout the duration of the study. The Emory University Committee on Institutional Animal Care and Use approved all animal protocols used in this study.

**Processing of Tissue**

Carotid arteries were processed for morphological studies at 0, 3, 7, 14, and 28 days after intervention, as described.\textsuperscript{14} For each time point, 8 to 10 animals were euthanized and used for morphological or biochemical measurements. For morphological evaluation, mice were perfusion-fixed with 10\% phosphate-buffered formalin at physiological pressure. Left and right carotid arteries were removed en bloc, further fixed for 16 hours, and then embedded in paraffin.

**Morphometric Features: Data Collection and Analysis**

We compared arterial remodeling in macrophage-rich hypercholesterolemic ApoE KO lesions to macrophage-poor WT lesions. Reproducibility of measurements was ensured by obtaining measurements at the apex of the lesion, which was identified as previously described.\textsuperscript{14} Groups of 5 consecutive carotid artery tissue sections (5 \(\mu\)m), spaced at equal intervals (150 \(\mu\)m), were collected along the entire length of the left and right carotid arteries. The first section in each group was counterstained with hematoxylin and eosin, and lumen size was measured. Within the group selected as the apex for each carotid lesion, the remaining 4 sections were processed for immunohistochemistry. A subset of submaximal lesions was also analyzed with the progression of macrophage-rich or macrophage-poor intimal lesions. We then compared carotid artery remodeling associated with the progression of macrophage-rich or macrophage-poor intimal lesions.

**Gelatinolytic Analysis**

Fresh ligated carotid arteries were collected separately, pulverized in liquid nitrogen, and extracted using ice-cold lysis buffer (10 mmol/L Na phosphate, pH 7.2; 150 mmol/L NaCl; 1\% Triton X-100; 0.1\% SDS; 0.5\% Na deoxycholate; 0.2\% Na azide) for 1 hour at 4\(^\circ\)C. Equal amounts of tissue extract protein (20 \(\mu\)g), assayed using the DC Protein assay (BioRad), were loaded on each lane and run in parallel with prestained molecular weight markers (BioRad) in 10\% SDS-PAGE gels containing 1\% gelatin to determine gelatinolytic activity as described previously.\textsuperscript{14} The optical volume-density product of individual lytic bands was quantified using the Molecular Analyst program (BioRad). The intensity of all lytic bands was normalized by comparison to the intensity of an MMP-9 standard obtained from culture medium conditioned by activated mouse macrophages that was loaded on every gel. Plasma cholesterol levels were measured using Infinity Cholesterol Reagent (Sigma Diagnostics).

**Data Analysis**

Average values of morphological parameters were obtained from image analysis of the lesion apex in each of the 4 to 5 individual left carotid arteries harvested at each time point and the submaximal lesions of individual carotid arteries from both study groups selected for matched intimal area irrespective of time point. Biochemical data obtained for each carotid artery by densitometric analysis of gels were averaged for 4 to 5 individual carotid arteries per each time point. Comparisons were made by ANOVA followed by Student’s \(t\) test to compare the means of multiple groups. Means were considered significantly different if \(P<0.05\).

**Results**

**Lesion Macrophage Content**

Macrophage-rich lesions developed in ligated ApoE KO carotid arteries (Figure 1). A hypercholesterolemic diet effectively increased plasma cholesterol levels in these mice, from 576±203 mg/dL to 1573±830 mg/dL (n=3) after 14 days. Tissue sections of the carotid arteries of ApoE KO euthanized before ligation (day 0) showed thin vessel walls without intimal lesions or macrophages (Figure 1). At 3 days post-ligation, however, Mac-3-positive macrophages were detected lining the lumen of ApoE KO carotid arteries (Figure 1). The intimal area occupied by macrophages grew consistently in time, from 1.05\(\times\)10\(^3\) to 1.95\(\times\)10\(^3\) \(\mu\)m\(^2\) at 3 days to 28.1\(\times\)10\(^3\) \(\mu\)m\(^2\), or 35\% of the neointimal area at 14 days (Figure 1 and Figure 2). Immunostaining of sequential ApoE KO carotid artery sections showed a similar neointimal localization of macrophages and MMP-9 (Figure 1), suggesting that macrophage foam cells may be the major source of MMP-9. In contrast, staining for Mac-3 immunopositive cells of WT ligated carotid arteries suggested limited macrophage infiltration throughout the duration of the experiment (Figure 1 and Figure 2).

**Neointimal Macrophages and Carotid Artery Remodeling**

Macrophage-rich ApoE KO carotid lesions were associated with significantly greater expansive remodeling than macrophage-poor WT carotid lesions (Figure 1 and Figure 2). After ligation, the vessel area circumscribed by the EEL in ApoE

**Immunocytochemistry**

Macrophages were detected using rat anti-mouse Mac-3 antibody. MMP-9 was detected using a polyclonal rabbit anti-mouse MMP-9 antibody (supplied by Dr Robert Senior, Washington University, St. Louis, Mo). After deparaffinization, a primary antibody was applied to specimens, followed by biotinylated rabbit anti-rat (Vector Laboratories) or goat anti-rabbit (Southern Biotechnologies) antibody and streptavidin-conjugated HRP (Jackson Immunoresearch). Specimens were then developed with a DAB kit (Vector Laboratories) and counterstained with Gill’s hematoxylin (Fisher Scientific).
KO carotid arteries more than tripled (113±9×10² μm² at day 0 to 390±20×10³ μm² at day 14, P=0.001; Figure 2). In contrast, during the same time, the corresponding area in WT carotid arteries grew by only 50% (114±73×10³ μm² at day 0 to 175±37×10³ μm² at day 14, P>0.05; Figure 2). Despite impressive compensatory enlargement, the massive neointimal growth in ApoE KO carotid arteries led to an almost complete occlusion of the lumen at 14 days (Figure 1). Significantly less lumen loss and neointimal growth occurred in WT lesions in the same time period (Figure 1). The disparity in the level of expansive remodeling between ApoE KO and WT ligated carotid arteries was obvious in occluded vessels, the endpoint of the intimal lesion (Figure 1). Because expansive remodeling is a reaction to intimal growth, we also compared the expansive remodeling of matched submaximal macrophage-rich and macrophage-poor lesions to better demonstrate the role of macrophages. Because of the accelerated course of lesion development in the ApoE KO mice, it was impossible to match lesions also for time point. By including in our analysis all of the arteries that had similar intimal areas, we found that macrophage-rich ApoE KO ligated carotid arteries had undergone significantly greater expansive remodeling (vessel area=161±11×10³ μm²) than the macrophage-poor WT ligated carotid arteries (vessel area=103±6×10³ μm²; P=0.003) for lesions of similar neointimal area (ApoE KO, 45.0×10³±3.77×10³ μm², n=5; WT,
MMP Activity and Carotid Artery Remodeling

To investigate the potential mechanism that allows for the enhanced expansive remodeling of arteries developing macrophage-rich lesions, we compared MMP gelatinolytic activity in ApoE KO and WT ligated carotid artery tissue homogenates by SDS-PAGE zymography (Figure 3). After 14 days, gelatinolytic activity corresponding in apparent molecular weight to MMP-9 rose to a maximal level of 200% of standard in ApoE KO carotid artery extracts, but activity in WT carotid artery extracts remained only 16% of standard. After 28 days, MMP-9 activity in carotid artery extracts was 140% of standard in ApoE KO, compared with 10% in WT (P<0.001; Figure 3). We also found that gelatinolytic activity corresponding in apparent molecular weight to MMP-2 increased more rapidly and to a higher maximal level in ApoE KO compared with WT ligated carotid arteries. Expressed as a percentage of standard, maximal activity was 145% in ApoE KO versus 20% in WT at 14 days (Figure 3). Thus, overall gelatinolytic activity was approximately one order of magnitude higher in the macrophage-rich ApoE KO carotid arteries.

Discussion

Arterial remodeling, defined as a persistent change in vessel size, requires structural alterations of the vessel wall, including degradation and reorganization of the matrix scaffold. Such changes occur during the course of atherosclerosis and after vascular interventions. Recent experimental and clinical evidence has revealed that the lumen size of a remodeling artery is influenced both by remodeling of the arterial wall and by growth of intimal lesions. Together with intimal thickening, constrictive remodeling of the arterial wall decreases lumen size, leading to arterial stenosis. Alternatively, expansive arterial remodeling can compensate for increased intimal thickness, therefore preserving lumen size. Despite this possible benefit, expansive remodeling is currently considered as potentially related to plaque vulnerability. Hence, understanding the factors that drive expansive remodeling may reveal a way to alter the course of atherosclerotic disease. Inflammatory cells, especially monocytes/macrophages, with their arsenal of secreted biologically active factors, are likely to fuel the growth of atherosclerotic plaques and influence arterial remodeling. In addition, high macrophage foam cell content characterizes vulnerable lesions.
To investigate the recently suspected connection between expansive arterial remodeling and macrophage foam cells, we decided to compare the remodeling of carotid arteries due to formation of experimental macrophage-rich and macrophage-poor lesions. The quest to clarify the role of specific factors in the etiology of atherosclerosis suffers from limited availability of animal models that can mimic characteristic lesions. We have shown previously\textsuperscript{14} in WT mice that outward remodeling of carotid arteries can be triggered through ligation. This intervention, which most likely triggers arterial remodeling through a deficit in basal nitric oxide synthesis,\textsuperscript{17} has been used in a number of genetically engineered mice. We found that application of the same procedure in ApoE KO mice triggered formation of lesions that accumulated large numbers of macrophages, allowing for the comparative examination of remodeling and quantitative evaluation of major morphological features in macrophage-rich and macrophage-poor lesions.

The extensive intimal lesions triggered by carotid artery ligation in hypercholesterolemic ApoE KO mice have a high content of macrophages (maximum 35% of intimal area) and a complex, human atherosclerosis-like morphology. In the absence of ligation, 14 days of hypercholesterolemic diet alone did not produce any visible morphological changes of ApoE KO carotid arteries, and we did not find any neointimal lesions or Mac-3-positive macrophages. Thus, ligation accelerates and enhances formation of lesions compared with a spontaneous course in ApoE KO carotid arteries. In view of these observations, we suggest that the use of carotid artery ligation in hypercholesterolemic ApoE KO mice offers a useful animal model for studying various aspects of macrophage recruitment and participation in arterial remodeling. We used it as a tool for investigation of the complex interplay between macrophage-rich lesions and expansive arterial remodeling.

The robust growth of intimal lesions in the ApoE KO carotid arteries, with an exuberant accumulation of macrophages, was greatly accelerated compared with the macrophage-poor lesions developing in WT arteries. The distinct time courses of lesion progression prevented the analysis of samples simultaneously matched for time point and intimal area. For instance, we found that macrophages line the luminal surface of ApoE KO ligated arteries at a very early stage (3 days after the trigger). Even without a sizable neointimal lesion at this early time point, morphological analysis indicates that expansive remodeling of ApoE KO arteries had already begun, supporting the idea that macrophages play an enabling role. As the lesion progressed, the area occupied by macrophages within the lesions correlated positively ($r^2=0.615; P<0.001$) with vessel area, further supporting the idea of a macrophage contribution to expansive remodeling.

Although the time course for lesion progression differed between the ApoE KO and WT mice, when comparing the expansive remodeling in submaximal lesions matched for intimal area irrespective of time point, we found significantly greater expansive remodeling in ApoE KO compared with WT carotid arteries. The tremendous growth eventually leads to the quick occlusion of ApoE KO carotid arteries. Interestingly, no late constrictive remodeling was observed in these arteries; thus, occlusion is not secondary to constrictive changes in this model. Comparison of this endpoint of our study reveals a greater expansive remodeling of occluded arteries in ApoE KO versus WT, seemingly allowing for the growth of a significantly larger neointima in these lesions (Figure 1). Morphometric analysis of lesions reveals a strong correlation between vessel area and intimal area in macrophage-rich ApoE KO lesions that is absent in macrophage-poor WT lesions (Figure 2). These results suggest a greater contribution to expansive remodeling from macrophage content than from intimal area.

A potential mechanism that allows expansive remodeling of the arterial wall containing macrophages is the secretion of enzymes that degrade matrix components, specifically MMPs. We recently showed\textsuperscript{14} that MMPs are associated with lesion formation and arterial remodeling triggered by carotid artery ligation in WT mice. In the present study, levels of MMP-9 and MMP-2 activity were several fold higher in ligated ApoE KO compared with WT carotid arteries at the same time points. The increase in gelatinolytic activity parallels the progressive formation of a macrophage-rich neointima and vessel enlargement, although a direct statistical correlation cannot be made because of the different nature of the variables being measured. Immunohistochemical analysis of sequential paraffin sections reveals that MMP-9 production is localized to the macrophage-rich neointimal region of ApoE KO lesions, suggesting that macrophages are the likely source of main gelatinolytic activity. Maximal levels of gelatinolytic activity and enlargement of the vessel are greatly increased in ApoE KO macrophage-rich carotid arteries compared with macrophage-poor lesions in WT carotid arteries, supporting the idea that macrophage MMPs have a role in expansive arterial remodeling. As noted previously, a limitation to our analysis is that time-matched ApoE KO lesions have larger neointima than WT lesions. Therefore, the role of other neointimal components in the larger ApoE KO lesions contributing to the observed variation in gelatinolytic activity cannot be excluded. The action of MMPs, especially MMP-9, has been implicated in unstable cardiovascular conditions. Conclusive demonstration of a critical role for macrophage MMPs, however, awaits the availability of specific MMP inhibitors or the development of genetically MMP-deficient mice in a background susceptible to atherosclerosis.

Our data support the notion that macrophages play an essential role in the expansive remodeling of atherosclerotic lesions. Degradation of the matrix scaffold by macrophage MMPs may initially allow expansive remodeling of a macrophage-rich atherosclerotic artery, but could eventually weaken the arterial wall and precipitate the destabilization of atherosclerotic plaques.

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