Association Between 5-Lipoxygenase Expression and Plaque Instability in Humans

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Objective—The participation of 5-lipoxygenase (5-LO) in the development of atherosclerosis has been suggested by recent studies. However, a role for 5-LO as a modulator of atherosclerotic plaque instability has not been previously reported in humans. Thus, the aims of this study was to analyze the expression of 5-LO in human carotid plaques and to investigate the mechanism by which this enzyme could lead to plaque instability and rupture.

Methods and Results—We obtained atherosclerotic plaques from 60 patients undergoing carotid endarterectomy. We divided the plaques into symptomatic and asymptomatic according to clinical evidence of plaque instability. Clinical evidence of plaque instability was provided by the assessment of recent ischemic symptoms attributable to the stenosis and by the presence of ipsilateral cerebral lesion(s) determined by computed tomography. Plaques were analyzed for CD68+ macrophages, CD3+ T cells, α-actin+ smooth muscle cells, 5-LO, cyclooxygenase 2, matrix metalloproteinase (MMP)-2, and MMP-9 by immunohistochemical, immunoblotting, and densitometric analyses. MMP activity was assessed by zymography. Leukotriene (LT) B4 and collagen were quantified by ELISA and Sirius red polarization, respectively. The percentage of macrophage-rich and T-cell–rich areas was larger in symptomatic compared with asymptomatic patients (25±6% versus 8±4%, P<0.0001, and 74±17 versus 18±4 cell/mm², P<0.003). 5-LO expression was higher in symptomatic compared with asymptomatic plaques (24±4% versus 6±3%, P<0.0001) and was associated with increased MMP-2 and MMP-9 expression (27±4% versus 7±3%, P<0.0001, and 29±5% versus 8±2%, P<0.0001) and activity and with decreased collagen content (6.9±2.4% versus 17.8±3.1%, P<0.01). Immunofluorescence showed that 5-LO and MMPs colocalize in activated macrophages. Notably, higher 5-LO in symptomatic plaques correlated with increased LTB4 production (18.15±3.56 versus 11.27±3.04 ng/g tissue, P<0.0001).

Conclusions—The expression of 5-LO is elevated in symptomatic compared with asymptomatic plaques and is associated with acute ischemic syndromes, possibly through the generation of LTB4, subsequent MMP biosynthesis, and plaque rupture. (Arterioscler Thromb Vasc Biol. 2005;25:1665-1670.)

Key Words: plaque ■ inflammation ■ lipoxygenase ■ leukotrienes ■ metalloproteinases

Atherosclerosis is a complex disease initiated by the trapping and oxidation of low-density lipoprotein in the subendothelial layer of the arterial wall, followed by the generation of biologically active species that stimulate vascular cells to produce inflammatory molecules.1 Although numerous risk factors for atherogenesis have been identified by epidemiological studies, the mechanism(s) involved in the evolution of atherosclerotic plaques toward rupture remain elusive owing, in part, to the participation of multiple genes and pathways in this process.

There is increasing evidence that inflammation plays a central role in the cascade of events that eventually result in plaque erosion and fissuring.2 First, the risk of cardiovascular disease seems to correlate with inflammation.3 Second, inflammation is more common in the cap of symptomatic plaques as reflected by higher recruitment of macrophage and T cells.4,5 Third, plaque rupture correlates with increased inflammation within the plaque, but plaque morphology and degree of vessel stenosis do not seem to be good predictors of plaque instability.2

Macrophages produce and secrete proteolytic enzymes that degrade plaque constituents. One example is the family of matrix metalloproteinases (MMPs) that degrade macromolecular constituents of the extracellular matrix.6 Macrophages present in human atherosclerotic lesions display increased expression of active 72-kDa gelatinase (MMP-2) and 92-kDa type IV basement membrane collagenase gelatinase B
Plaque ulceration, n (%) 17 (57)* 8 (27)  
Stenosis severity, % 74±3 74±5  

MMP secretion by adjacent macrophages recruited to sympathetic carotid plaques.

Methodology

Patients

We studied 60 of 83 consecutive, no previously examined, surgical inpatients enlisted to undergo carotid endarterectomy for extracranial high-grade (>70%) internal carotid artery stenosis (Table). The degree of luminal narrowing was determined by repeated Doppler echography and intra-arterial cerebral angiography using the criteria of the North American Symptomatic Carotid Endarterectomy Trial. Recruitment was completed when 2 predetermined equal groups of 30 patients according to clinical evidence of plaque instability were achieved. The first group consisted of patients (17 men; 13 women; 74.2±2 years) who presented with clinical symptoms of cerebral ischemic attack (transient ischemic attacks and ischemic stroke) and asymptomatic ones (22.7±7 years, mean±SD) and asymptomatic ones (22.7±7 years, mean±SD). The second group included 30 patients (17 men; 13 women; 73.2±2 years of age) who had an asymptomatic carotid stenosis (asymptomatic patients, group 2). Asymptomatic carotid stenoses were diagnosed by systematic clinical examination of patients with coronary or peripheral disease. The study was approved by local ethics review committees and was performed according to institutional guidelines. Written informed consent was obtained from all patients.

Reagents

Cell phenotypes were identified using monoclonal antibodies against human muscle α-actin, CD68 (for macrophages), CD3 (for T cells), and CD31 (for endothelial cells). We used the CR3/43 monoclonal antibody to identify expression of human leukocyte antigen (HLA)-DR as a marker for cellular activation. These antibodies were from Dako Corporation (Carpinteria, Calif). The anti–5-LO polyclonal antibody and monoclonal antibodies against human MMP-2 and MMP-9 were from Calbiochem-Novabiochem (San Diego, Calif). A monoclonal antibody against human cyclooxygenase (COX)-2 was purchased from Cayman Chemical (Ann Arbor, Mich).

Immunohistochemistry

After surgery, plaque samples were immediately frozen in isopentane and cooled in liquid nitrogen. Immunohistochemistry analyses were performed as previously described.5–7 In particular, consecutive tissue sections were incubated with the following antibodies: anti-CD68 (1:50), anti-CD3 (1:20), anti–HLA-DR (1:100), anti–α-actin (1:100), anti-CD31 (1:30), anti–5-LO (1:200), anti–COX-2 (1:80), anti–MMP-2 (1:20), and anti–MMP-9 (1:30). Biotinylated goat polyclink IgG (Dako) was used as a secondary antibody (1:100). Omission of primary antibodies and staining with isotype-matched control immunoglobulins served as negative controls. Individual sections were also counterstained with Gill Hematoxylin (Sigma Chemical Co). Analysis of immunohistochemistry was performed with a personal computer–based quantitative 24-bit (16.2×10⁶ unique combinations) color image analysis system. Two expert pathologists blinded to the clinical diagnoses of the patients analyzed the specimens. The intra- and interobserver variability was 5%.

Double Immunofluorescence

Immunofluorescence analysis was performed as previously described.5–7 Anti-mouse IgG fluorescein isothiocyanate conjugate (1:60) was used as secondary antibody. Labeled specimens were examined by confocal microscopy using a Zeiss LSM 510 Meta instrument (Carl Zeiss, Jena, Germany).

Sirius Red Staining to Assess Collagen Content

Sections were performed as previously described.7 After dehydration, the sections were observed under polarized light after cover-slipping. The sections were photographed with identical exposure settings for each section.

Plaque Analysis of LTB₄

To stimulate LTB₄ production, the plaque homogenate supernatant was incubated with 1.6 mmol/L CaCl₂, 10 μmol/L calcium ionophore A23187 (Sigma Aldrich), and 10 μmol/L arachidonic acid (Cayman Chemical) at 37°C for 10 minutes. After chilling and acidifying the samples by addition of HCl to pH 3.5, precipitated protein was removed by centrifugation. The supernatant was loaded onto C18 extraction column (Sep-Pak Plus; Waters Corporation, Milford, Mass). Then, the column was washed twice and eluted with ethyl acetate. The eluate was dried under nitrogen and resuspended in ELISA buffer. LTB₄ levels were determined in duplicate assays by a commercially available ELISA (Assay Designs Inc, Ann Arbor, Mich) that had been previously validated in the measurement of LTB₄ in tissue homogenates.7

Western Blot Analyses

Tissue proteins were extracted and subjected to electrophoresis as described.7 Coimmunodetection of β-actin was performed to confirm...
that equal amounts of protein were loaded in individual lanes. Immunoreactive bands were quantified by computer-assisted densitometry (Alpha Ease 5.02). The results are expressed as arbitrary densitometric units.

### SDS-PAGE Zymography

Zymography was performed according to previously validated methods.12 Conditioned medium of human fibrosarcoma cell line HT1080 was used as positive control.

### Isolation of Macrophages From Atherosclerotic Plaques

Macrophages were selectively extracted from plaques by enzymatic digestion and density gradient centrifugation, as described. To evaluate the LTB4 release, cells (1×10⁶/mL) were incubated in medium alone or with 5 μmol/L A23187 plus 10 μmol/L arachidonic acid at 37°C for 10 minutes. The supernatants were then collected and LTB4 production was determined by ELISA as described above.

### Statistical Analyses

For clinical data and histological examination, variables were compared by use of the χ² test. Significant differences in protein expression, enzymatic activity, and inflammatory cell infiltration in symptomatic and asymptomatic patients were analyzed by Student t test. Data are expressed as percentage or mean±SD. All calculations were performed using the computer program SPSS 11.0.1.

### Results

#### Histological Analyses

The microscopic appearance of atherosclerotic plaques was assessed in all cases, and the results are shown in the Table. Plaque ulceration was significantly more common in the symptomatic plaques (17 of 30 [57%] versus 8 of 30 [27%]; P<0.0001).

#### Inflammatory Infiltration

Our immunocytochemical analyses revealed inflammatory infiltration in all specimens examined, more evident in the shoulder of symptomatic plaques. Interestingly, symptomatic and asymptomatic plaques differed in both the number and state of activation of the inflammatory cells recruited. First, the area occupied by macrophages and T cells was significantly greater (P<0.0001) in symptomatic compared with asymptomatic plaques (Table). In addition, the inflammatory cells detected in the symptomatic plaques were characterized by robust expression of the HLA-DR antigen, a feature that contrasted with the low expression of HLA-DR in cells present in asymptomatic plaques.

#### Higher 5-LO Staining in Symptomatic Atherosclerotic Lesions

We found that human atherosclerotic lesions expressed 5-LO and that staining for 5-LO was more abundant in symptomatic lesions, particularly in macrophages around vasa vasorum (Figure 1). Quantitative image analyses revealed that the levels of 5-LO in symptomatic plaques significantly exceeded those in asymptomatic lesions (24±4% versus 6±3%, mean±SD, n=30, P<0.0001). These values are in close agreement with the relative content of macrophages in symptomatic versus asymptomatic lesions (24±4% versus 9±3%), suggesting that expression of 5-LO reflects the content of recruited macrophages (Table). We also found that enhanced 5-LO expression was associated with a comparable staining pattern for COX-2, thus ruling out the possibility that these 2 arachidonic acid–metabolizing enzymes are inversely regulated in human atherosclerotic plaques.

#### Enhanced LTB4 Biosynthesis in Symptomatic Plaque Macrophages

We found that LTB4 levels were lower in plaque homogenates from asymptomatic patients compared with those from symptomatic patients and that the differences were statistically significant (18.15±3.56 versus 11.27±3.04 ng/g tissue, P<0.0001) (Figure 2). Notably, 5-LO expression showed a strong correlation (R²=0.465, P<0.001) with plaque rupture defined as intimal defect >1000 μm in width. Finally, when separate analyses were performed for patients undergoing endarterectomy, 10 to 20, 20 to 30, and 30 to 35 days after the onset of symptoms, no significant differences in 5-LO expression were observed.

#### Atherosclerotic Plaques Express MMP-2 and MMP-9

Both symptomatic and asymptomatic atherosclerotic plaques expressed MMP-2 and MMP-9. However, the staining was
significantly more abundant in the symptomatic compared with the asymptomatic lesions (Figure 3). Quantitative image analyses revealed that the levels of MMP-2 and MMP-9 in symptomatic plaques (27\% and 29\% respectively, mean±SD, n=30) significantly exceeded (P<0.0001) those in asymptomatic plaques (7±3\% and 8±2\%, respectively, mean±SD, n=30). Moreover, these values closely mirrored macrophage content (Table). Increased MMP immunoreactivity was localized around the plaque core, especially in the shoulder and the fibrous cap of the lesions (Figure 3). These areas were also associated with intense inflammatory infiltration as revealed by the abundance of macrophages.

Symptomatic Plaques Contain Activated MMPs
The increased MMP-2 and MMP-9 immunoreactivity in symptomatic versus asymptomatic atherosclerotic lesions was confirmed by Western blot analyses that revealed 2.6- and 2.3-fold increases in MMP-2 and MMP-9, respectively (Figure 4). These changes, however, do not necessarily reflect altered enzymatic activity because MMPs require proteolytic activation for optimal function. Thus, we used SDS-PAGE zymography as a complement to immunohistochemical and immunoblotting studies to assess MMP gelatinolytic activity. This approach demonstrated that extracts from symptomatic plaques contained both the zymogen and the activated forms of 72-kDa gelatinase (MMP-2) and 92-kDa gelatinase (MMP-9) (Figure 4). However, the total amount of active MMP was ≈50\% higher (P<0.0001) in symptomatic compared with asymptomatic plaques (Figure 4).

Plaque Extracellular Components
Sirius red polarization showed considerably lower content of interstitial collagen in the tissue sections of symptomatic patients compared with asymptomatic patients (6.9±2.4\% versus 17.8±3.1\%, mean±SD, n=30, P=0.01) (Figure 4).

Cultured Macrophages Isolated From Symptomatic and Asymptomatic Plaques Recapitulate Differences in 5-LO and MMP Levels Observed In Vivo
The higher 5-LO and MMP levels observed in symptomatic versus asymptomatic plaques could be attributable to a higher relative abundance of macrophages in the former tissues (Table). Alternatively, increased 5-LO expression by macrophages recruited to these sites could also account for the observed differences. To differentiate between these possibilities, we isolated macrophages from 5 symptomatic and 5 asymptomatic plaques and assessed expression of 5-LO and MMP by immunocytochemical and Western blot analyses. We observed weak expression of 5-LO, MMP-2, and MMP-9 in macrophages isolated from asymptomatic plaques. In contrast, we found 7-fold (P<0.0001) higher expression levels of 5-LO, MMP-2, and MMP-9 in macrophages isolated from symptomatic plaques (Figure 5). We conclude that the phenotype of macrophages present in asymptomatic plaques differs from that of cells in symptomatic plaques and that the differences are maintained after plating, suggesting the occurrence of sustained activation signals in macrophages recruited to symptomatic plaques.

Colocalization of 5-LO and MMPs in Macrophages Recruited to Symptomatic Plaques
In the first experiment, we incubated serial sections of tissue specimens from symptomatic plaques with primary antibod-
ies against CD68, 5-LO, MMP-2, and MMP-9. Within the lesion, all enzymes accumulated in the shoulder as well as in the periphery of the lipid core. These results suggested that 5-LO and MMPs are expressed in similar loci in macrophages and, importantly, that expression of these proteins occurs in physical locations associated with high plaque instability. In addition, we also performed double immunofluorescence staining experiments on symptomatic plaque sections. Also in this case, we found coexpression of 5-LO, MMP-2, and MMP-9 in CD68^+ macrophages. Moreover, the colocalization of these proteins was recapitulated in plaque-derived macrophages. The expression of COX-2, another arachidonic acid–metabolizing enzyme, occurred in areas characterized by elevated 5-LO levels. Finally, we found that only activated (HLA-DR^+), but not quiescent, macrophages expressed this protein.

**Discussion**

In the present report, we provide the first evidence for the association between 5-LO overexpression and atherosclerotic plaque instability in humans. First, the present findings identify marked differences in the expression of 5-LO in symptomatic compared with asymptomatic human atherosclerotic lesions. Second, we detected elevated LTB4 levels in symptomatic plaques owing to high 5-LO activity in these tissues. Third, the presence of 5-LO in human plaque macrophages correlated with morphological and immunologic features associated with plaque instability. Thus, we propose that 5-LO is a marker for increased risk of acute ischemic events precipitated by MMP-dependent processes that lead to plaque rupture.

In this study, we analyzed gene expression during the transition from stable plaque to ruptured plaque in humans and found that 5-LO had a great difference in expression in symptomatic versus asymptomatic plaques. This is particularly interesting in light of our recent finding of enhanced 5-LO activity in patients with unstable angina, a clinical event closely related to plaque rupture.14

The 5-LO pathway has been linked to atherosclerosis in humans.15 In fact, histological analyses16 revealed an abundance of 5-LO in macrophages and foam cells from human atherosclerotic lesions. Furthermore, a recent study17 reported that the carotid-artery intima-media thickness, an indicator of systemic atherosclerosis, correlates with the incidence of variant 5-LO promoter genotypes in patients who lack the common human promoter allele. However, no studies conducted to date have specifically addressed the role of this enzyme in the biological events that lead to plaque rupture. Our study provides the first evidence that 5-LO is significantly overexpressed in symptomatic compared with asymptomatic plaques, suggesting that the participation of this enzyme in atherosclerosis occurs not only during the development of atherosclerotic plaques but also during the progression of atherosclerotic plaques toward instability.

Dwyer et al17 reported that subjects harboring variant 5-LO genotypes have increased incidence of atherosclerosis. The results of this study were consistent with the hypothesis that increased leukotriene production in subjects harboring promoter variants contributed to the disease phenotype. Here, we
demonstrate that progression of atherosclerotic plaque toward rupture in humans is associated with upregulation of 5-LO protein and enzymatic activity in infiltrating macrophages. Thus, the work of Dwyer et al.\textsuperscript{17} combined with the present study support the hypothesis that subjects harboring 5-LO promoter variants may be at increased risk of developing unstable atherosclerotic plaques owing to elevated levels of both 5-LO and leukotrienes.

LTB\(_4\) is one of the downstream products of the 5-LO reaction, and it is known to function as a potent chemoattractant and proinflammatory mediator in the pathogenesis of several inflammatory diseases.\textsuperscript{18} Here, our finding that symptomatic atherosclerotic plaques express elevated levels of both 5-LO and LTB\(_4\) supports the hypothesis that LTB\(_4\) may be one of the key mediators of 5-LO-dependent plaque instability.

In this study, macrophages were significantly more abundant in symptomatic plaques, always outnumbered the lymphocytes, and represented the major source of 5-LO. In addition, the site of inflammatory infiltration was also always characterized by strong expression of 5-LO antigen on activated HLA-DR\(^{+}\) macrophages, which contrasted markedly with the low expression of this protein elsewhere in the fibrous cap. Thus, these data suggest the presence of an active inflammatory reaction in symptomatic plaques. In fact, in agreement with the difference in 5-LO staining pattern, the histological milieu of the lesions appears different with regard to cellularity and presence of foam cells but not in the degree of vessel stenosis, suggesting that symptomatic and asymptomatic lesions are different only with regard to inflammatory burden and that differences in plaque behavior stem from differences in the presence of still unknown stimuli for selective expression of 5-LO, capable of disrupting plaque stability.

Finally, we consider some interest in this study the peculiar 5-LO expression in the proximity of vasa vasorum, areas designed to blood–vessel interchange, as it has been reported that increased dietary arachidonic acid significantly blunted the effect.\textsuperscript{17}

In summary, our work is consistent with a model in which the expression of 5-LO by activated macrophages in symptomatic plaques leads to LTB\(_4\) accumulation and enhanced synthesis and release of MMPs that can promote plaque rupture. These findings raise the interesting possibility that modulation of the 5-LO/LTB\(_4\) signaling pathway with selective antagonists will provide a novel therapeutic approach for plaque stabilization and prevention of acute ischemic syndromes.

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