Overexpression of Functionally Coupled Cyclooxygenase-2 and Prostaglandin E Synthase in Symptomatic Atherosclerotic Plaques as a Basis of Prostaglandin E2-Dependent Plaque Instability

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Background—Studies have implicated a role for prostaglandin (PG) E2-dependent matrix metalloproteinase (MMP) biosynthesis in the rupture of atherosclerotic plaque. Cyclooxygenase-2 (COX-2) and PGE synthase (PGES) are coregulated in nucleated cells by inflammatory stimuli. The aim of this study was to characterize the expression of COX-2 and PGES in carotid plaques and to correlate it with the extent of inflammatory infiltration and MMP activity and with clinical features of patients' presentation.

Methods and Results—Plaques were obtained from 50 patients undergoing carotid endarterectomy and divided into 2 groups (symptomatic and asymptomatic) according to clinical evidence of recent transient ischemic attack or stroke. Plaques were analyzed for COX-2, PGES, MMP-2, and MMP-9 by immunocytochemistry and Western blot, whereas zymography was used to detect MMP activity. Immunocytochemistry was used to identify CD68+ macrophages, CD3+ T lymphocytes, and HLA-DR+ cells. The percentage of macrophage-rich areas was larger (P<0.0001) in symptomatic plaques. COX-2, PGES, and MMPs were detected in all specimens; enzyme concentration, however, was significantly higher in symptomatic plaques. COX-2, PGES, and MMPs were especially noted in shoulders of symptomatic plaques, colocalizing with HLA-DR+ macrophages. All symptomatic plaques contained activated forms of MMPs. Finally, inhibition of COX-2 by NS-398 was accompanied by decreased production of MMPs that was reversed by PGE2.

Conclusions—This study demonstrates the colocalization of COX-2 and PGES in symptomatic lesions and provides evidence that synthesis of COX-2 and PGES by activated macrophages is associated with acute ischemic syndromes, possibly through metalloproteinase-induced plaque rupture. (Circulation. 2001;104:921-927.)

Key Words: atherosclerosis ■ plaque ■ prostaglandins ■ metalloproteinases ■ inflammation

There is increasing evidence that inflammation plays a central role in the cascade of events that eventually results in plaque erosion and fissuring.1 In fact, studies examining markers of inflammation demonstrate a relation between inflammation and risk of cardiovascular disease.2 Furthermore, several studies have shown that inflammation is more common in symptomatic plaques, with greater numbers of macrophages and T cells detected in the cap of symptomatic plaques,3 and plaque rupture has been shown to be related to increased inflammation within the plaque rather than plaque morphology or degree of vessel stenosis.4

Macrophages synthesize proteolytic enzymes capable of degrading plaque constituents. One such family of enzymes, the matrix metalloproteinases (MMPs), is capable of degrading all macromolecular constituents of the extracellular matrix.4 Increased expression of active MMP-2 and MMP-9 has been reported in vulnerable regions of human carotid plaques in association with macrophages.5 Moreover, Loftus et al6 demonstrated that MMP-9 activity is significantly higher in unstable carotid plaques. Thus, localized increase in MMP has the potential to cause the acute plaque disruption that precedes the onset of symptoms in both the coronary and cerebral circulations.

Production of MMP-2 and MMP-9 by macrophages has been shown to occur through a prostaglandin (PG) E2/cAMP–dependent pathway.7 Signaling through this pathway involves the modulation of cyclooxygenase (COX).8 Two isoforms of COX have been identified, referred to as COX-1 and COX-
2.6 COX-1 is constitutively expressed and is responsible for the biosynthesis of prostaglandins involved in vascular homeostasis. In contrast, COX-2 is induced in response to growth factors, cytokines, and phorbol esters, suggesting that this enzyme is involved in the generation of prostaglandins in inflammatory diseases. Consistent with the hypothesis of COX-2 contributing to the clinical instability of coronary artery disease, incomplete suppression of thromboxane metabolite excretion has been detected in patients with unstable angina despite >95% suppression of platelet COX-1 by aspirin. Moreover, the induction of COX-2 in monocytes and the resulting production of PGE₂ have been shown to be involved in MMP production by these cells.

Thus, the identification of pathways that may regulate MMPs is critical to the formulation of strategies that may stabilize plaques. The possibility that the simultaneous induction of COX-2 and PGE synthase (PGES) by inflammatory stimuli might represent a mechanism of plaque disruption led us to investigate whether it would modulate the production of MMP by macrophages into atherosclerotic plaques. Here, we report enhanced MMP production by macrophages in symptomatic carotid plaques, most likely due to the enhancement in PGE₂ synthesis as a result of the induction of the functionally coupled inducible COX/PGES.

Methods

Patients
We studied 50 of 66 consecutive surgical inpatients (27 male, 23 female; 72±2 years old) undergoing carotid endarterectomy for extracranial high-grade internal carotid artery stenosis (≥70% lumen narrowing). Recruitment was completed when 2 predetermined equal groups of 25 patients according to clinical evidence of plaque instability were achieved. The first group included 25 patients who presented with symptoms of cerebral ischemic attack. Endarterectomy was performed 10 to 40 days after the onset of symptoms in these patients. The second group included 25 patients who had an asymptomatic carotid stenosis. Percentages of carotid diameter reduction, procedural methods, concomitant therapy, and risk factors did not differ between the 2 groups (Table). By the time of surgery, all patients were taking long-term aspirin therapy (100 mg/d). The study was approved by local ethics review committees. Informed consent was obtained from all patients.

Immunohistochemistry
Serial sections were analyzed as described by Schönbeck et al. Specific antibodies anti-CD68, anti-CD3, anti-HLA-DR, anti-α-smooth muscle actin, and anti-CD31 (Dako); anti-COX-2 and anti-PGES (Cayman Chemical); and anti-MMP-2 and anti-MMP-9 (Calbiochem-Novabiochem) were used. In addition, 4 sections from each plaque were examined for the presence of plaque ulceration and intraplaque hemorrhage. The specimens were analyzed by an expert pathologist (intraobserver variability 6%) blinded to the patient’s diagnosis. Quantitative Analysis was performed with a computer-based image analysis system (AlphaEase 5.02, Alpha Innotech Corp).

Reverse Transcription–Polymerase Chain Reaction
COX-2 mRNA expression was evaluated by reverse transcription–polymerase chain reaction (RT-PCR). RNA was reverse transcribed, and first-strand cDNA was used as a template in PCR. cDNA aliquots were amplified with primers specific for COX-2 and housekeeping gene GAPDH in a Perkin-Elmer GeneAmp 2400 cycler.

Zymography
Zymography was performed as described by Herron et al. Conditioned medium of human fibrosarcoma cell line HT1080 was used as a positive control with known gelatinolytic activity.

Statistical Analysis
Clinical and histological variables were compared by χ² test. Differences in enzyme expression and inflammatory infiltrate were analyzed by Student’s t test. Statistical significance was indicated by a value of P<0.05. All calculations were performed with the computer program SPSS 8.0.

Results

Histological Analysis
Plaque ulceration was significantly more common in the symptomatic plaques (14 of 25 [53%] versus 7 of 25 [26%]; P<0.05). In contrast, no differences (13 of 25 [52%]) were observed with regard to intraplaque hemorrhage (Table).

Inflammatory Infiltration
Immunocytochemistry revealed inflammatory infiltration in all specimens examined, more evident in symptomatic plaques.
Overall, macrophage and T-lymphocyte infiltration occurred coincidentally and was most prominent in the shoulder of the lesions and in the immediate vicinity of the atheromatous core of the lesions. Plaque area occupied by macrophages and T cells was significantly greater \( (P < 0.0001) \) in symptomatic than in asymptomatic plaque (Table).

### Macrophage Activation in Symptomatic Plaques

The site of inflammatory infiltration in the shoulder of symptomatic plaques was always characterized by strong expression of HLA-DR antigens, which contrasted markedly with the low expression of HLA-DR in the asymptomatic plaques (Figure 1). HLA-DR expression was most abundant on macrophages and lymphocytes, but HLA-DR+ smooth muscle cells (SMCs) also occurred, although limited to sites adjacent to inflammatory infiltrates.

### COX-2 Is Expressed in Macrophages of Symptomatic Plaques

Atherosclerotic lesions contained immunostainable COX-2 (Figure 2). Interestingly, COX-2 was more abundant in symptomatic lesions, as confirmed by quantitative analysis \( (21.6 \pm 4.1\% \text{ versus } 5.6 \pm 2.6\%, \text{n} = 25, \text{mean} \pm \text{SD}; P < 0.0001) \). COX-2 accumulated in the shoulder region and in the periphery of the lipid core. COX-2 staining pattern indicated its localization in the activated macrophages and SMCs. Finally, the endothelium and medial SMCs of plaque vasa vasorum also showed COX-2 staining.

### COX-2 Is Expressed in Higher Amounts in Symptomatic Plaques

Western blot and RT-PCR analyses revealed COX-2 expression in plaques (Figure 3, A and B), markedly higher in symptomatic than in asymptomatic plaques \( (6045 \pm 146 \text{ versus } 1793 \pm 801 \text{ densitometric units [DU] for protein expression, } \text{n} = 25, \text{mean} \pm \text{SD}; P < 0.0001) \).

### Regional Overexpression of PGES in Symptomatic Plaques

Immunohistochemistry revealed strong PGES immunoreactivity in all of the symptomatic plaques analyzed, but only very weak staining in the asymptomatic plaques (Figure 2). By quantitative image analysis, levels of PGES in symptomatic plaques significantly exceeded those in asymptomatic plaques \( (18.9 \pm 3.6\% \text{ versus } 4.1 \pm 1.6\%, \text{n} = 25, \text{mean} \pm \text{SD}; P < 0.0001) \), corresponding to the content of macrophages (Table). PGES localized prominently in the shoulder region of the plaque and in the periphery of the lipid core, areas characterized as macrophage-rich.

### PGES Is Expressed in Higher Amounts in Symptomatic Plaques

Only weak PGES expression was observed in asymptomatic plaques by Western blot (Figure 3A). In contrast, a 6-fold higher signal was demonstrated in symptomatic plaques \( (5482 \pm 136 \text{ versus } 1028 \pm 542 \text{ DU}, \text{n} = 25, \text{mean} \pm \text{SD}; P < 0.0001) \).

### Atherosclerotic Plaques Contain Immunoreactive MMP-2 and MMP-9

Plaques stained for both MMPs tested. Staining was significantly more abundant in the symptomatic lesions than in those of asymptomatic patients (Figure 2). By quantitative analysis, levels \( (\text{n} = 25, \text{mean} \pm \text{SD}) \) of MMP-2 as well as MMP-9 in symptomatic plaques \( (23.7 \pm 5.6\% \text{ and } 25.2 \pm 6.1\%, \text{respectively}) \) significantly exceeded \( (P < 0.0001) \) those in asymptomatic plaques \( (8.3 \pm 2.5\% \text{ and } 8.8 \pm 3.3\%, \text{respectively}) \), corresponding to the content of macrophages (Table). Immunoreactivity localized especially in the shoulder region of the plaque and in the periphery of the lipid core, areas characterized as macrophage-rich.
Colocalization of COX-2, PGES, and MMPs in Macrophages in Symptomatic Plaques

In the first experiment, serial sections of symptomatic plaques were incubated with the primary antibodies anti-CD68, anti-COX-2, anti-PGES, anti-MMP-2, and anti-MMP-9 (Figure 5). Within the lesion, all enzymes accumulated in the shoulder as well as in the periphery of the lipid core. In the second experiment, immunofluorescence double-labeling associated the expression of PGES with COX-2 and MMPs in CD68+ macrophages (Figure 6). Thus, these analyses confirmed the concomitant presence of COX-2, PGES, and MMPs in macrophages at the vulnerable region of symptomatic plaque.

**PGE2-Dependent Production of MMPs in Monocytes In Vitro**

To determine whether monocyte MMP production is regulated through a PGE2-dependent pathway involving the concomitant induction of functionally coupled COX-2 and PGES, we initially examined the effect of NS-398 on MMP production (Figure 7). LPS caused a strong enhancement in COX-2, PGES, MMP-2 and MMP-9 levels over those detected in control monocytes. COX-2 and MMP induction by LPS was significantly inhibited by NS-398; the inhibition of MMPs was reversed, however, by the addition of PGE2. Similar results were also observed when interleukin-1β was used as stimulus. Thus, MMP production appears to be secondary to the induction of functionally coupled COX-2 and PGES and the subsequent generation of PGE2.

**Discussion**

In the present report, we provide evidence for the functionally coupled involvement of COX-2 and PGES in MMP overexpression in symptomatic human atherosclerotic plaques. In particular, the present findings are the first, to the best of our knowledge, to (1) identify evidence for PGES in human atherosclerotic lesions and (2) relate the pattern of the inducible COX/PGES pathway to an acute ischemic event often precipitated by rupture of atherosclerotic plaque.

Concomitantly higher expression of COX-2, PGES, MMP-2, and MMP-9 was found in specimens obtained from the “culprit” carotid lesions of patients with recent transient ischemic attack (TIA) or stroke compared with specimens obtained from asymptomatic patients. In fact, only 4 of 25 (16%) of the asymptomatic plaques exhibited an intensity of enzyme expression comparable to those observed in symptomatic plaques, whereas the remaining specimens demonstrated only weak positivity.

There are several possible explanations for why ≈16% of asymptomatic plaques showed an enzyme expression comparable to those observed in plaques from patients with recent TIA or stroke. Postmortem studies suggest that plaque rupture is sometimes asymptomatic. Thus, silent plaque rupture resulting in nonocclusive thrombosis can occur in patients who do not develop the clinical hallmarks of any of the syndromes associated with plaque rupture. Accordingly, 2 of 4 asymptomatic patients in this study who nevertheless demonstrated high expression for COX-2, PGES, and MMPs have been in this category. Alternatively, the other patients, had they not undergone angioplasty, might have soon progressed to frank plaque rupture and one of the cerebral ischemic events.

In this study, macrophages were significantly more abundant in complicated plaques, always outnumbered the lymphocytes, and represented the major source of COX-2/PGES, MMP-2, and MMP-9. The site of inflammatory infiltration was always characterized by strong expression of HLA-DR antigens on inflammatory cells, which contrasted markedly with the low expression of HLA-DR 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 COX-2/PGES and MMP staining pattern, the histological milieu of the lesions appears to be different with regard to cellularity, presence of foam cells, and cholesterol clefts but not in the degree of vessel stenosis, suggesting that asymptomatic and symptomatic lesions are different only with regard to inflammatory burden and that differences in plaque behavior stem from differences in the presence of as yet undetermined stimuli for specific expression of proteins capable of disrupting plaque stability.

Previous studies have reported COX-2 expression in atherosclerotic lesions. These studies, however, did not provide any evidence about the real involvement of COX-2 in the pathophysiology of atherosclerotic plaque rupture. In fact, COX-2 is only an intermediate enzyme in the metabolic pathway of arachidonic acid, and the COX by-product PGH2 is further metabolized by other isomerases to various prostanoids (PGE2, PGD2, PGF2α, PGI2, thromboxane A2). Thus, the relative abundance of one specific prostanoid rather than another is the result of the expression and activity of its specific isomerase, and only the concomitant expression of functionally coupled COX-2 and PGES may lead to increased biosynthesis of PGE2-dependent MMPs in the setting of atherosclerotic plaque.

Interestingly, macrophages of the shoulder region contain most of the COX-2 protein within the lesion. This finding may have functional importance, because different cell types can regulate the production of different eicosanoids. Endothelium releases predominantly PGI2, an inhibitor of platelet activation and cholesterol accumulation, and Belton et al recently reported that COX-2 is responsible for the increase in PGI2 seen in patients with atherosclerosis. In contrast, macrophages, not present in normal arterial tissue, produce an array of prostanoids, including PGE2, considered one of the most atherogenic eicosanoids. The finding that COX-2 localizes predominantly with lesional macrophages agrees with observations in abdominal aortic aneurysms, in which macrophages also represent the majority of COX-expressing cells. Increased COX-2 expression within the plaque further agrees with reports describing COX-2 expression in atheroma-associated cells, including macrophages, on stimulation with proinflammatory cytokines such as IL-1, tumor necrosis factor-α, and CD40L, mediators found within human atherosclerotic lesions.

Recently, the intriguing and novel proatherogenic mechanism of the functionally coupled inducible COX/PGES has been supported by the demonstration that membrane-bound COX expression is markedly induced by proinflammatory stimuli in various tissues and cells and is downregulated by dexamethasone, accompanied by changes in COX-2 expression and delayed PGE2 generation. Moreover, Jakobsson et al showed that COX-2 and PGES are coregulated in nucleated cells by inflammatory stimuli and that PGE2 biosynthesis may depend on the presence of both of these enzymes. In accordance with this, an inducible PGES activity has been described in LPS-stimulated rat peritoneal macrophages, which coincides with COX-2 expression and changes the product formation in favor of PGE2. Our study thus agrees with published data demonstrating a severalfold increase in PGE2 biosynthesis and COX-2 protein in A549 cells in response to IL-1β and is the first to suggest that overexpression of the functionally coupled COX-2/PGES may realize a predominant pathway of arachidonate metabolism leading to increased biosynthesis of PGE2-dependent MMPs in the setting of human atherosclerotic plaque.

Prostanoids have potent actions on vascular SMCs, regulating contractility, cholesterol metabolism, and proliferation. Increased expression of COX might thus contribute to the accumulation of lipids in lesional SMCs (and macrophages), favoring formation of SMC- and macrophage-de-
PGE2-dependent MMP production in monocytes. PGE2, a predominant eicosanoid of macrophages and antimigratory actions of COX products on SMCs derived foam cells within atheroma. Conversely, antiproliferative and antimigratory actions of COX products on SMCs suggest potential contributions of the enzymes to the evolution of a lesion toward an SMC-depleted and macrophage-enriched, and thus more vulnerable, plaque. Furthermore, COX-2 can modulate angiogenesis by synthesis of angiogenic factors and neovessel formation. Consequently, COX-2 expression within the lesion contributes to the formation of new blood vessels, thus allowing the plaque to expand. More importantly, PGE2, a predominant eicosanoid of macrophages, induces the expression of MMP-2 and MMP-9, enzymes considered crucial in the degradation of plaque stability. Our description of these metalloproteinasises in plaque regions that are COX-2/PGES-positive and found to be macrophage-enriched suggests that such regulation of MMP expression by COX products may operate in vivo. Furthermore, we found that asymptomatic plaques expressed substantially less COX-2/PGES and MMP than symptomatic lesions. Our results are in agreement with those of Loftus et al., demonstrating higher MMP-9 activity in unstable plaques. In contrast, because no previous works have established an association between MMP-2 and vulnerable plaques, further studies will be necessary to define the precise role of MMP-2 in the setting of plaque instability.

In conclusion, this study addresses the missing link between COX-2 overexpression and plaque instability by demonstrating the high prevalence of the functionally coupled COX-2/PGES in human atherosclerotic lesions and providing evidence that synthesis of the inducible COX/PGES by activated macrophages is associated with TIA and stroke, possibly by MMP-induced matrix degradation promoting plaque rupture. These findings are potentially important from a fundamental standpoint, because they indicate a pathogenic role for the inducible COX/PGES in the evolution of atherosclerotic lesions. From a practical standpoint, these findings raise the possibility that the selective COX-2 inhibitors now currently available for clinical use or future PGES inhibitors might provide a novel form of therapy for plaque stabilization of patients with atherosclerotic disease and prevention of acute ischemic syndromes.

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