Association Between Prostaglandin E Receptor Subtype EP4 Overexpression and Unstable Phenotype in Atherosclerotic Plaques in Human

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Objective—We recently demonstrated that inducible cyclooxygenase/PGE synthase-1 (COX-2/mPGES-1) are overexpressed in symptomatic plaques in association with PGE2-dependent metalloproteinase (matrix metalloproteinase [MMP]) biosynthesis and plaque rupture. However, it is not known which of the 4 PGE2 receptors (EP1–4) mediates macrophage metalloproteinase generation. The aim of this study was to characterize EP1–4 expression in plaques from symptomatic and asymptomatic patients undergoing carotid endarterectomy and correlate it with the extent of inflammatory infiltration, COX-2/mPGES-1 and MMP expression and clinical features of patients’ presentation.

Methods and Results—Plaques were analyzed for COX-2, mPGES-1, EP1–4, MMP-2, and MMP-9 by immunohistochemistry, reverse-transcription polymerase chain reaction and Western blot; zymography was used to detect MMP activity. We observed strong EP4 immunoreactivity, only very weak staining for EP2, and no expression of EP1 and EP3 in atherosclerotic plaques. EP4 was more abundant in MMP-rich symptomatic lesions, whereas EP2 was no different between symptomatic and asymptomatic plaques. Finally, MMP induction by PGE2 in vitro was inhibited by the EP4 antagonist L-161 982, but not by its inactive analog L-161 983 or by the EP2 antagonist AH6809.

Conclusions—This study shows that EP4 overexpression is associated with enhanced inflammatory reaction in atherosclerotic plaques. This effect might contribute to plaque destabilization by inducing culprit metalloproteinase expression. (Arterioscler Thromb Vasc Biol. 2005;25:1925-1931.)

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

There is increasing evidence that inflammation plays a central role in the cascade of events that result in plaque erosion and fissuring.1,2 Lesional macrophages synthesize matrix metalloproteinases (MMPs), proteolytic enzymes capable of degrading plaque constituents.3 Interestingly, it has been shown that secretion of 72-kDa (MMP-2) and 92-kDa gelatinase (MMP-9) by macrophages in human atherosclerotic plaques occurs through a prostanlindin (PG) PGE2-dependent mechanism.4 PGE2 signaling involves the modulation of inducible cyclooxygenase-2 (COX-2) and type 1 microsomal PGE synthase (mPGES-1).5 Consistent with the hypothesis of COX-2 and mPGES-1 contributing to the clinical instability of atherosclerotic plaques, we recently reported enhanced MMP-2 and MMP-9 production by macrophages in symptomatic plaques caused by the enhancement in PGE2 synthesis as a result of the induction of the functionally coupled COX-2/mPGES-1.4 However, the specific cellular receptor(s) by which PGE2 may influence MMP generation in plaque macrophages is still unknown.

PGE2 can act through at least 4 different receptors (EPs), termed EP1–4. Recently, 2 major studies have suggested that EP4 could be the main receptor involved in the pathophysiology of inflammatory human diseases. In fact, Takayama et al6 identified EP4 receptor as the predominant PGE2 receptor isoform present in human macrophages and demonstrated EP4 receptor involvement in the inhibition of macrophage-derived chemokine production in vitro. In contrast, McCoy et al7 using mice selectively lacking each of the 4 known EP receptors showed that only the EP4-/- mice have a high resistance to development of experimental rheumatoid arthritis, and that bone samples isolated from arthritis-resistant EP4-/- mice expressed significantly less MMP-2 than did EP4+/+ cells after PGE2 treatment.8 Thus, the possibility that the overexpression of 1 specific receptor for PGE2 might influence the mechanism of PGE2-
Stenosis severity, %

Patients with

<table>
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</tr>
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<td>Percentage of macrophage-rich areas</td>
<td>23±8*</td>
<td>6±3</td>
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<tr>
<td>N of T cells per mm² section area</td>
<td>75±12*</td>
<td>17±8</td>
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<tr>
<td>Percentage of SMC-rich areas</td>
<td>19±6</td>
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** Variables indicate ischemic heart disease; NSAID, nonsteroidal antiinflammatory drug; TIA, transient ischemic attack. *P<0.0001.**

Intraperoperative plaque destabilization led us to investigate whether 1 of the 4 EP receptors would specifically modulate MMP production by macrophages into atherosclerotic plaques. Here, we reported enhanced MMP production by macrophages in carotid plaques of patients with recent ischemic event, most likely caused by increment in the signaling of the inflammatory PGE₂ as a result of the selective binding of EP4.

**Methods**

**Patients**

We studied 60 of 121 consecutive, not previously examined, surgical inpatients (34 male, 26 female; 68±3 years) enrolled to undergo carotid endarterectomy for extracranial high-grade (>70%) internal carotid artery stenosis. The patients were selected/excluded by order of completion of the 2 groups, with the symptomatic patients who first filled the allotment (30 versus 27). Recruitment was completed when 2 predetermined equal groups of 30 patients according to clinical evidence of plaque instability were achieved. The first group included 30 patients (17 male,13 female; age 67±3 years) who presented with clinical symptoms and CT scan signs of atherothrombotic stroke (“symptomatic” according to North American Symptomatic Carotid Endarterectomy Trial [NASCET] classification;** symptomatic group). The second group included 30 patients (17 male,13 female; age 69±3 years) who had an asymptomatic carotid stenosis (asymptomatic group). The asymptomatic patients never had an ischemic episode in the territory of the carotid stenosis, but carotid endarterectomy has been shown to be beneficial in these patients, as shown by the Asymptomatic Carotid Atherosclerosis Study (ACAS).**

Endarterectomy was performed in all patients ~10 days after their inclusion in the study, with no differences between symptomatic patients (mean±SD, 10±2 days) and asymptomatic ones (mean±SD, 10±3 days). Percentage of carotid diameter reduction, procedural methods, risk factors, and concomitant therapy did not differ between the 2 groups (Table). In particular, by the time of surgery, all patients were taking 100 mg daily of aspirin as antithrombotic therapy. The study was approved by local ethics review committees. Written informed consent was obtained from all patients before each examination.

**Immunohistochemistry**

After the surgical procedure, samples were immediately frozen in isopentane and cooled in liquid nitrogen (LN₉). Serial sections were prepared and analyzed as previously described. Briefly, frozen tissues were sectioned using a cryostat and serial fresh sections were fixed in cold acetone (~−20°C) for 10 minutes. Endogenous peroxidase activity was reduced by preincubation with 0.3% hydrogen peroxide in PBS. Subsequently, the sections were washed 3 times in PBS-Tween 20 (PBS-T) and then incubated with PBS containing 1% bovine serum albumin (Sigma Chemical Co, St. Louis, Mo) to minimize nonspecific binding. Consecutive sections then were incubated with the following antibodies: polyclonal rabbit anti-human EP1–2 to EP3–4, polyclonal rabbit anti-human COX-2, polyclonal rabbit anti-human mPGES-1 (Cayman Chemical, Ann Arbor, Mich); and monoclonal mouse anti-human MMP-2 and monoclonal mouse anti-human MMP-9 (Oncogene, San Diego, Calif) for 60 minutes. Biotinylated goat anti-mouse and anti-rabbit IgG (Dako Corporation, Carpinteria, Calif) was used as a secondary antibody for 30 minutes at room temperature. After 3 washes with PBS-T, we treated individual sections with horseradish peroxidase-labeled streptavidin (Dako) for 30 minutes, washed the sections 3 times with PBS-T, and determined peroxidase activity with 3,3-diaminobenzidine tetrahydrochloride (Dako). Serial sections, adjacent to those used for reaction with the above described antibodies, were incubated with the monoclonal mouse anti-human CD68 (Oncogene), monoclonal mouse anti-human CD3 (Dako), monoclonal mouse anti-human human leukocyte antigen (HLA)-DR (Dako), and monoclonal mouse anti-human α-smooth actin (Calbiochem, San Diego, Calif) to quantify inflammatory infiltrate and to characterize cellular phenotypes. Omission of primary antibodies and staining with isotype-matched control immunoglobulins served as negative controls. Antigen-adsorbed controls were also included previously. Individual sections were also counterstained with Gill’s Hematoxylin (Sigma Chemical Co). All the specimens were analyzed by an expert pathologist (intra-observed variability 6%) blinded to the clinical categorization of the patient material. In particular, CD3-positive T cells were counted individually and expressed as the number of cells per mm² section area as determined by computer-aided planimetry (AlphaEase 5.02; Alpha Innotech Corp, San Leandro, Calif). This approach was not feasible in the case of CD68 macrophages, EP4-positive, COX-2/mPGES-1–positive, and MMP-positive cells that were often present in dense, nearly confluent infiltrates, making the delineation of individual cells impossible. Instead, we determined the area occupied by positive cells planimetrically and calculated the percentage of macrophage-rich, EP4-rich, COX-2/mPGES-1–rich, and MMP-rich areas.**

**Reverse-Transcription Polymerase Chain Reaction and Real-Time Polymerase Chain Reaction**

Total RNA was isolated from plaques by using Tri-Reagent solution according to the manufacturer’s protocol (Sigma Chemical Co). Total RNA was converted to cDNA by incubation at 42°C for 20 minutes and at 99°C for 5 minutes in reverse-transcriptase buffer, Moloney Murine Leukemia Virus Reverse Transcriptase, RNase inhibitor, dNTPs, and Oligo(dt) primer (Invitrogen Corporation, Carlsbad, Calif). Then, polymerase chain reactions (PCRs) for EPs were performed as previously described by Takayama et al. Further, to provide an absolute mRNA quantification, real-time PCR for EP4 and MMP-9 was performed as previously included in the study. ** Primers for EP4 were as follows: forward primer, 5′-ATCCTAC-TCACTTGGCCACCTTCCTC-3′; reverse primer, 5′-GAAATTCTTGGGCTGATATAACTGGTGA-3′. The primers for MMP-9 were as follows: forward primer, 5′-TTGCAACCACAAATCATTAC-3′; reverse primer, 5′-GCAAAGGGCTGCTGACTTAATCA-3′. Primers and probe for β-actin mRNA (GenBank accession number X00351) were chosen using a computer program, Primer Express (Applied Biosystems, Foster City, Calif), and were as follows: forward primer, 5′-TCCCTCTGGGGCATGATGGAG-3′; reverse primer, 5′-AGGGAGAACATCTTTGAGTCTT-3′; TaqMan probe 5′(FAM)-CCTGGGATCCACAGAACTACCTTC-(TAMRA)3′. Probes
were all purchased from Applied Biosystems. Results were expressed as relative levels of EP4 and MMP-9 mRNA, referred to a sample called calibrator chosen to represent 1× expression of these genes. The calibrator used was a sample of healthy vessel that was analyzed on every assay plate with the unknown samples. All of the analyzed samples express n-fold EP4 or MMP-9 mRNA relative to the calibrator.

**Western Blot**
EP1–4, COX-2, mPGES-1, MMP-2, and MMP-9 proteins were detected by Western blot as previously described. Briefly, tissue extracts (50 μg protein) were subjected to electrophoresis on 10% SDS-polyacrylamide gels. Resolved proteins were transferred to 0.45 μm nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ) in blotting buffer. The membranes were then blocked with 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.3% Tween 20 (TBS-T) containing 10% nonfat dried milk for 2 hours. The blots then were washed with TBS-T and incubated overnight with the following primary antibodies: polyclonal rabbit anti-human EP1–2 to EP3–4, monoclonal mouse anti-human COX-2, polyclonal rabbit anti-human mPGES-1 (Cayman Chemical), monoclonal mouse anti-human MMP-2, and monoclonal mouse anti-human MMP-9 (OncoGene). The blots were washed and then incubated at room temperature for 1 hour with a horseradish peroxidase–linked anti-rabbit antibody (Amersham Biosciences) diluted in TBS-T containing 10% nonfat dried milk. The washes were repeated using TBS-T and the immuno-reactive bands were detected using ECL (Amersham Biosciences) according to the manufacturer’s instructions. Immunodetection of β-actin was performed to confirm that equal amounts of protein were loaded in individual lanes. Controls with antigen-adsorbed control antisera were also included in the study. We exposed the blots to X-ray film for 1 to 15 minutes at room temperature and then quantified the immuno-reactive bands using computer-assisted densitometry (Alpha Ease 5.02). The results are expressed as arbitrary densitometric units (DU).

**Enzyme-Linked Immunosorbent Assay Determinations**
Plaque content of MMP-2 and MMP-9 were measured by validated enzyme-linked immunosorbent assay assays according to manufacturer instructions (Amersham Biosciences, Piscataway, NJ). Furthermore, plaque content of PGE2 was measured according to a previously described and validated EIA method (Cayman Chemical, Ann Arbor, Mich).12

**Zymography**
To detect gelatinolytic activity in the proteins extracted from tissue samples and cell cultures, zymographic analyses on 7.5% acrylamide gel containing 0.1% gelatin were performed. This method detects both activated and zymogen forms of MMPs. In fact, in the presence of SDS otherwise inactive forms can lyse the substrate contained in the gel because of detergent-induced conformational change. Briefly, samples for SDS-PAGE were not boiled before the electrophoresis under nonreducing conditions. After electrophoresis, the substrate gels were soaked twice for 30 minutes with Triton-X100 solution (2.5%) at room temperature to remove SDS. Gels were then incubated in 50 mmol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl, 5 mmol/L CaCl2, and 0.02% NaN3, for 24 hours at 37°C. Conditioned medium of human fibrosarcoma cell line HT1080 was used as positive control with known gelatinolytic activity. The lysis of the substrates in the gels was visualized by staining with 2.5% Coomassie brilliant blue (Sigma Chemical Co).

**Macrophages Extraction From Atherosclerotic Plaques**
Macrophages were selectively extracted from plaques by enzymatic digestion and density gradient centrifugation, as described. Briefly, fresh lesions (1 gram) were incubated with 10 mL of an enzyme mixture containing collagenase (450 U), elastase (4.7 U), and trypsin inhibitor (10 mg) in Hanks’ buffered HEPES buffer (pH 7.4) containing 0.3% bovine serum albumin. The incubations were conducted for 60 minutes at 37°C, and the resulting suspension was filtered with through a nylon sieve (180 μm) to isolate macrophage-derived foam cells. The filtrate was centrifuged at 700g for 10 minutes at 10°C. The remaining tissue was incubated with fresh enzyme for 1 hour at 37°C. The combined pellets containing foam cells were loaded on discontinuous gradients containing the following concentrations of Nycodenz: 0.5%, 1%, 5%, 10%, and 30% (wt/vol). Gradients were centrifuged at 1200g for 15 minutes at 10°C. Pure cell populations of macrophage-derived foam cells were obtained from the 5% and 10% layer of the density gradient. The cells were allowed to adhere overnight in DMEM-containing penicillin/streptomycin (100 μg/mL), l-glutamine (2 mmol/L), and 5% bovine calf serum. Then, immunocytochemistry, Western blot, and zymography were performed as illustrated.

**Isolation and Culture of Blood Monocytes**
Monocytes were prepared from blood obtained from 5 healthy donors by density sedimentation on Ficoll-Hypaque (Amersham Biosciences) and by adherence of mononuclear cells to plastic dishes for 1 hour at 37°C as previously described. Control or stimulated (IL-1β, 10 ng/mL) monocytes (20 × 10^6/mL of RPMI) were cultured for 24 hours at 37°C in the presence of aspirin (200 μmol/L) and in the presence or absence of the EP4 receptor selective antagonist L-161 982 (100 nM), the chemically related inactive analog L-161 983 (500 μmol/L) for 30 minutes (both kind gifts from Dr Young and Dr McCusker, Merck Frosst Canada & Co, Kirkland, Quebec, Canada), and the EP2 receptor antagonist AH6809 (2 μmol/L for 30 minutes; Cayman). PGE2 (10^−7 M, Sigma) was also added to some of the cultures. At the end of the incubation, MMPs expression and activity was evaluated by Western blot and zymography.

**Statistical Analysis**
For clinical data and histological examination, variables were compared by use of the χ^2 test. The significance of difference in biochemical marker expression and inflammatory cell infiltration between symptomatic and asymptomatic patients was 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**

**Inflammatory Infiltration**
Plaque area occupied by HLA-DR^+ macrophages and T cells was significantly greater (P<0.0001) in symptomatic as compared with asymptomatic plaques (Table).

**Higher COX-2/mPGES-1 Expression and Activity in Symptomatic Plaques**
COX-2 and mPGES-1 staining was more abundant in symptomatic lesions (27±4% versus 6±2%, and 27±3% versus 6±1%, respectively; P<0.0001). COX-2/mPGES-1 accumulated in the activated macrophages and smooth muscle cells of shoulder regions. Western blot confirmed the higher COX-2/mPGES-1 expression in symptomatic compared with asymptomatic plaques (8500±140 versus 2100±120, and 7900±110 versus 2000±120 DU, respectively; mean±SD, n=30; P<0.0001). Finally, we confirmed results concerning mPGES-1 expression also by enzyme immunoassay (EIA) measurements of the plaque content of its metabolite. In fact, PGE2 was present in a higher amount in symptomatic plaques (290±13 versus 52±6 ng/mg tissue; mean±SD, n=30; P<0.0001).
EP4 Is the Main EP Receptor Expressed in Macrophages of Symptomatic Plaques

Immunohistochemistry revealed strong immunoreactivity for EP4 and very weak staining for EP2 in the atherosclerotic plaques that were studied (Figure 1). In contrast, atherosclerotic lesions did not contain immunostainable EP1 and EP3. By quantitative image analysis, levels of EP4 significantly exceeded those of EP2 ($P<0.0001$). Interestingly, EP4 was more abundant in symptomatic lesions ($25 \pm 3\%$ versus $6 \pm 1\%; n=30$, mean±SD; $P<0.0001$) (Figure 1). In contrast, we did not observe any difference regarding EP2 expression (Figure 1). EP4 localized prominently in the shoulder region and in the periphery of the lipid core, areas characterized as macrophage-rich (Figure 1, box).

Figure 1. Stain (X5) for EPs in plaques. High magnification (inset; $\times 60$) indicated EP4 localization in macrophages. Plaques did not contain immunostainable EP1 and EP3. Similar regions of the plaque are showed. These results are typical of 30 symptomatic and 30 asymptomatic plaques.

EP4 Is Expressed in Higher Amounts in Symptomatic Plaques

Reverse-transcription PCR (Figure 2a) and Western blot (Figure 2b) analyses revealed EP4 expression in plaques, markedly higher in symptomatic compared with asymptomatic plaques ($7200 \pm 140$ versus $1200 \pm 160$ DU and $7800 \pm 130$ versus $1200 \pm 140$ DU, respectively, for mRNA and protein expression; mean±SD, $n=30$; $P<0.0001$). Quantification by real time reverse-transcription PCR confirmed the higher expression of EP4 in symptomatic plaques ($7.2 \pm 2.6$ versus $4.8 \pm 2.9$; mean±SD, $n=30$; $P=0.001$) (Figure I, available online at http://atvb.ahajournals.org). In contrast, only weak EP2 mRNA (Figure 2a) and protein expression (Figure 2b) were observed in both symptomatic and asymptomatic plaques. Finally, reverse-transcription PCR and Western blot did not identify EP1 and EP3 in human plaques (Figure 2).

Figure 2. Reverse-transcription PCR (a) and Western blot (b) for EPs in plaques. These results are typical of 30 symptomatic and 30 asymptomatic plaques.

Enhanced EP4 Expression Is Not a Consequence of Plaque Rupture or Previous Stroke

To exclude that higher expression of EP4 in symptomatic plaques was merely a consequence of plaque rupture, because they became activated after the event, we also studied 10 additional patients who underwent coronary stenting with distal protection within 90 minutes from onset of acute myocardial infarction. Interestingly enough, analysis of these plaque specimens demonstrated that EP4 (as well culprit metalloproteinases) was expressed in coronary plaque macrophages at intensity levels comparable with those observed in symptomatic carotid plaques, and therefore higher with respect to those observed in asymptomatic carotid plaques. Furthermore, we studied 15 additional patients who underwent a double surgical procedure for the presence of bilateral severe stenoses. The first procedure was always performed after ischemic stroke related to the culprit carotid plaque (symptomatic plaques); in contrast, the second procedure was performed several weeks later (and therefore always on patients with previous stroke), despite the absence of symptoms related to that specific plaque. Interestingly, results showed that in the same patients symptomatic plaques always had a higher inflammatory phenotype as reflected by higher inflammatory cell infiltration, higher COX-2, mPGES-1, EP4, and metalloproteinase expression when compared with...
the asymptomatic plaques, thus suggesting that the observed differences may be a mechanistic hallmark of a future stroke and not simply consequences of a previous stroke.

**EP4 Is Expressed in Higher Amounts in Macrophages Extracted From Symptomatic Plaques**

To exclude that enhanced EP4 staining observed in symptomatic plaque sections was merely a secondary effect of higher inflammatory infiltration, we selectively extracted macrophages from 5 symptomatic and 5 asymptomatic plaques. Only weak EP4 expression was observed by Western blot in macrophages extracted from asymptomatic plaques. In contrast, a 5-fold higher signal was demonstrated in macrophages isolated from symptomatic plaques (6100 11006130 DU; n = 5, mean 1 SD; P < 0.0001).

**Plaque Expression of MMPs**

Staining for MMP-2 and MMP-9 was significantly more abundant in the symptomatic rather than in the asymptomatic lesions. By quantitative analysis, levels of MMP-2 and MMP-9 in symptomatic plaques significantly exceeded those in asymptomatic plaques (29 3% versus 6 2%, and 27 4% versus 5 3%; n = 30; P < 0.0001). Finally, quantification by real time reverse-transcription PCR confirmed the higher expression of MMP-9 in symptomatic plaques (7.9 2.1 versus 3.1 1.8; mean 1 SD, n = 30; P = 0.0001) (Figure I). In addition, zymography demonstrated that extracts from symptomatic plaques contained higher amounts of the activated form of MMP-2 and MMP-9 (3700 11006110 versus 550 31, and 4000 100 versus 720 78 DU; n = 30, mean 1 SD; P < 0.0001).

**Colocalization of EP4 with COX-2, mPGES-1, 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 EP4, anti-COX-2, anti-mPGES-1, anti-MMP-2, and anti-MMP-9 (Figure II, available online at http://atvb.ahajournals.org). 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 with confocal microscopy associated the expression of EP4 with COX-2, mPGES-1, and MMPs in CD68+ macrophages, both in plaque sections and in plaque-derived macrophages (Figure 3).

**EP4-Dependent Production of MMPs in Monocytes In Vitro**

We initially examined the effect of the selective EP4 receptor antagonist L-161 982 on MMP production (Figure 4a). In IL-1β–stimulated monocytes, we observed a significant induction of COX-2/mPGES-1 over that detected in control monocytes (Figure 4a). Notably, this overexpression of COX-2/mPGES-1 caused an enhancement in MMP-2 and MMP-9 expression. Similar results for MMPs were also observed when exogenous PGE2 was added to unstimulated cells. MMP induction by PGE2 was significantly inhibited by the specific EP4 receptor antagonist L-161 982, but not by its inactive analog L-161 983 or by the specific EP2 receptor antagonist AH6809. Thus, PGE2-dependent MMP production in mononuclear cells appears secondary to the selective expression of COX-2/mPGES-1 and MMPs in CD68+ macrophages. These results are typical of 30 symptomatic and 30 asymptomatic plaques.
activation of the EP4 receptor. In addition, blockade of EP4 also caused an increase in the generation of transforming growth factor-β and a reduction in the release of the colony-stimulating factor chemotractant, thus confirming strengthening the hypothesis that EP4 may orient the plaque toward a more unstable phenotype. Furthermore, similar results were also observed in plaque-derived cultured macrophages, thus ruling out the hypothesis that EP4 may produce these effects only on circulating monocytes but not in macrophages infiltrating the atherosclerotic plaques.

**EP4 Induces Activated MMPs in Monocytes**

We used zymography to demonstrate that extracts from cultured monocytes with PGE2-stimulated EP4 receptor contained the activated form of MMP-2 and MMP-9 (Figure 4b). In contrast, only very weak positivity for activated MMPs was observed during maximal PGE2 stimulation in monocytes in which the EP4 receptor was blocked by L-161,982 (Figure 4b). Thus, the amount of active MMP-2 and MMP-9 was significantly higher (P<0.0001) in monocytes with active EP4 receptor (2800±120 versus 230±24 and 3000±100 versus 220±65 DU, respectively).

**Discussion**

In the present report, we provide some evidence supporting the functional involvement of the EP4 receptor in PGE2-dependent MMP overexpression in human symptomatic atherosclerotic plaques. In particular, the present findings are the first, to the best of our knowledge, to identify differences for EP receptors in stable versus vulnerable human atherosclerotic lesions, and to associate the presence of the EP4 receptor on human plaque macrophages to an acute ischemic event often precipitated by rupture of atherosclerotic plaque.

Concomitantly higher expression of EP4, COX-2, mPGES-1, MMP-2, and MMP-9 was found in specimens obtained from the “culprit” carotid lesions of patients with recent stroke compared with specimens obtained from asymptomatic patients. In this study, macrophages were significantly more abundant in complicated plaques, always outnumbered the lymphocytes and represented the major source of EP4. In addition, EP4-positive areas were always characterized by strong expression of the HLA-DR antigen on inflammatory cells, thus suggesting the presence of an active, EP4-positive, inflammatory reaction in symptomatic plaques.

Our previous studies4-14 reported enhanced COX-2/mPGES-1 and PGE2-dependent MMP expression in symptomatic atherosclerotic lesions. However, these studies did not provide any information about the specific EP receptor transducing enhanced PGE2 generation in atherosclerotic plaque rupture. In fact, the final effect of PGE2 is mediated by 4 different G-protein-coupled heptahelical receptors (EP1–4) differently expressed in diverse tissues.6,7 Thus, the stimulation of specific genes by COX-2/mPGES-1-derived PGE2 is the result of the expression and activity of one specific receptor rather than another, and only the concomitant expression of COX-2/mPGES-1 and EP4 may lead to increased biosynthesis of culprit MMPs in the setting of human atherosclerotic plaques.

The fact that COX-2/mPGES-1–positive macrophages of the shoulder region contain most of the EP4 receptor within the lesion is worth attention, given that this finding may have functional importance, because PGE2 can regulate the expression of different genes in different cell types. Thus, we can speculate that increased PGE2 generation in plaque macrophages may enhance the synthesis of culprit MMPs in the same cell through an autocrine mechanism, possibly representing a vicious circle implicated in plaque instability. Furthermore, because it has been shown5,13 that PGE2 produced by mPGES may amplify COX-2 expression in inflammatory cells by an EP4-mediated positive mechanism, then EP4 overexpression in plaque macrophages might establish a positive self-stimulatory autocrine and paracrine feedback loop, amplifying and sustaining the COX-2-dependent inflammatory response leading to progressive plaque destabilization.

In our study, asymptomatic plaques expressed substantially less EP4 and MMPs compared with symptomatic lesions. Interestingly, these results support the findings of the study conducted by McCoy et al15 demonstrating significantly less MMP-2 activity in bone samples isolated from EP4−/− mice7 with respect to EP4+/+ cells after PGE2 treatment.8 In contrast, our data apparently differ from the recent report of Takayama et al16 showing that PGE2 may attenuate chemokine production in lipopolysaccharide-stimulated macrophages in vitro via EP4. However, we believe that this is only an apparent difference, because chemokines and metalloproteinases are involved in different phases of atherogenesis. Thus, we can speculate that the COX-2/mPGES-1/EP4 pathway may exert antiinflammatory effects in the early phase of atherosclerosis by inhibiting the release of chemokines from circulating monocytes, whereas it may be involved in the biosynthesis of culprit MMPs from plaque macrophages in the late phase of atherosclerosis.4,14

In this light, the controversial role of COX-2 in atherogenesis is also supported by recent animal and clinical studies. In fact, an acceleration of lesion progression in apolipoprotein-E–deficient mice has been recently reported, after 3-week treatment with a highly selective COX-2 inhibitor.16 In addition, recent reports from human trials17,18 have showed that COX-2 inhibitors may increase the risk of cardiovascular events in low-risk patients. However, these data are not in opposition with our results. In fact, COX-2 is only an intermediate enzyme in the metabolic pathway of arachidonic acid, and the COX bio-product PGH2 is further metabolized by other isomerases to various prostanooids (PGE2, PGD2, PGE1, prostacyclin [PGI2], and thromboxane A2). Thus, the relative abundance of one specific prostanooid rather than another is the result of the expression and activity of its specific isomerase, and only the concomitant expression in plaque macrophages of COX-2 and PGE synthase may lead to increased biosynthesis of PGE2-dependent MMPs. In contrast, COX-2 expression in the endothelium is protective because mainly coupled with the prostacyclin synthase, and consequently its inhibition by COX-2 inhibitors may increase the risk of thrombosis. Thus, prolonged and widespread use of COX-2 inhibitors in unselected patients, most of which have stable, asymptomatic plaques with low PGE2 activity
may be detrimental and lead to progressive plaque destabilization.6

Several limitations should be considered in translating our results to an in vivo scenario of atherothrombosis. In fact, because in this study we did not use any pharmacological tool in vivo to test our hypothesis, the potential relevance of these findings should be further investigated using pharmacological tools in vivo, in which other cells such as platelets carrying the atherogenic COX-1 are acutely involved. Furthermore, the large use of aspirin during the study could have influenced the inflammatory response in the arterial wall, because aspirin can diminish the synthesis of thromboxane and PGs, acetylated COX-2, and induce the synthesis of the antiinflammatory 15R-HETE and lipoxins.20,21 However, whereas these properties of aspirin may have modified the overall inflammatory plaque phenotype, they are unlikely to have influenced the different expression of EP receptors, because aspirin therapy was uniformly distributed between the 2 groups of patients.

Despite these limitations, nevertheless, we believe that our results may be interesting from a practical standpoint, because they raise the possibility that modification of the PGE2–EP4 receptor signaling, for example, by selective EP4 receptor antagonists, might provide a potential form of therapy (safer than COX-2 inhibitors) for plaque stabilization in patients with atherosclerotic disease and prevention of acute ischemic syndromes.

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References
1. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation. 1994;89:36–44.
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Figure I

(a) EP4 mRNA (folds of control)

(b) MMP-9 mRNA (folds of control)

P = 0.001

P < 0.0001