Targeting Prostaglandin E₂ Receptors as an Alternative Strategy to Block Cyclooxygenase-2-dependent Extracellular Matrix-induced Matrix Metalloproteinase-9 Expression by Macrophages

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COX-2-dependent prostaglandin (PG) E₂ synthesis regulates macrophage MMP expression, which is thought to destabilize atherosclerotic plaques. However, the administration of selective COX-2 inhibitors paradoxically increases the frequency of adverse cardiovascular events potentially through the loss of anti-inflammatory prostanooids and/or disturbance in the balance of pro- and anti-thrombogenic prostanooids. To avoid these collateral effects of COX-2 inhibition, a strategy to identify and block specific prostanooid-receptor interactions may be required. We previously reported that macrophage engagement of vascular extracellular matrix (ECM) triggers proteinase expression through a MAPK™/ERK1/2-dependent increase in COX-2 expression and PGE₂ synthesis. Here we demonstrate that elicited macrophages express the PGE₂ receptors EP1–4. When plated on ECM, their expression of EP2 and EP4, receptors linked to PGE₂-induced activation of adenylyl cyclase, is strongly stimulated. Forskolin and dibutyl cyclic-AMP stimulate macrophage matrix metalloproteinase (MMP)-9 expression in a dose-dependent manner. However, an EP2 agonist (butaprost) has no effect on MMP-9 expression, and macrophages from EP2 null mice exhibited enhanced COX-2 and MMP-9 expression when plated on ECM. In contrast, the EP4 agonist (PGE₁-OH) stimulated macrophage MMP-9 expression, which was inhibited by the EP4 antagonist ONO-AE3-208. When compared with COX-2 silencing by small interfering RNA or inhibition by celecoxib, the EP4 antagonist was as effective in inhibiting ECM-induced proteinase expression. In addition, ECM-induced MMP-9 expression was blocked in macrophages in which EP4 was silenced by small interfering RNA. Thus, COX-2-dependent ECM-induced proteinase expression is effectively blocked by selective inhibition of EP4, a member of the PGE₂ family of receptors.

Atherosclerosis is a chronic inflammatory disease characterized by lipid accumulation, macrophage recruitment, smooth muscle proliferation, and fibrosis (1, 2). Macrophage proteinase expression compromises the structural integrity of atherosclerotic lesions by degrading components of the extracellular matrix (ECM), which contributes to lesion ulceration or rupture and subsequent sequelae of thrombosis, myocardial infarction, and stroke (3–6). A substantial body of evidence has identified cyclooxygenase (COX)-2 as a targetable component of the signaling pathway responsible for increased proteinase expression by macrophages in athero sclerotic lesions. COX metabolizes arachidonic acid to an unstable endoperoxide, which is then converted to the principal prostaglandins (PG) by specific synthases (7, 8). COX-2 expression is elevated in atherosclerotic lesions (9–14). PGE₂, an important mediator of the inflammatory response, stimulates proteinase expression by a variety of cells including macrophages (15–18). Both PGE synthase and matrix metalloproteinase (MMP) activities are elevated in regions of symptomatic plaques rich in macrophages and susceptible to rupture (13, 19). Moreover, treatment of low density lipoprotein receptor-deficient mice with a specific COX-2 inhibitor resulted in reduced aortic atherosclerosis (20), and statin-dependent plaque stabilization was associated with decreased COX-2, PGE synthase, and MMP activities (21–24). Finally, a naturally occurring polymorphism in the COX-2 promoter that is associated with reduced expression appears to protect against myocardial infarction and stroke (25).

Taken together, these data suggest that selective inhibition of COX-2 would lead to reduced PGE₂ and MMP expression, resulting in greater plaque stability. However, recent data indicate that administration of COX-2-selective inhibitors increases adverse cardiovascular events (26, 27). The explanation for this effect is not fully understood; however, inhibition of COX-2 results in the loss of all downstream PGs, some of which have anti-inflammatory functions (28). Likewise, it has been suggested that selective COX-2 inhibitors block PG₃ production by vascular endothelium, without inhibiting COX-1-dependent platelet thromboxane A2 expression, thereby supporting a pro-thrombotic state (29, 30). Finally, several cyclooxygenase-independent effects of the COX-2 inhibitors have been described (31), which may contribute to adverse cardiovascular events.

Despite the adverse cardiovascular events associated with selective COX-2 inhibition, the COX-2–PGE₂–MMP-9 axis remains an attractive target to block macrophage proteinase expression at sites of chronic
inflammation. In this regard, we previously reported that macrophage engagement of vascular smooth muscle cell (SMC)-derived ECM triggers proteinase expression through a protein kinase C-dependent activation of MAPK erk1/2, which leads to increased COX-2 expression and PGE2 synthesis (32). Selective inhibition of macrophage COX-2 activity blocked ECM-induced proteinase expression and ECM-induced PGE2 production, and MMP-9 expression was markedly reduced in COX-2−/− macrophages compared with wild type macrophages (32). In studies reported here, we have determined whether selective inhibition of the PGE2 receptor family (EP1–4) can attenuate ECM-induced MMP-9 expression by macrophages. We demonstrate that thioglycollate-elicited macrophages express EP1–4. When plated on ECM, their expression of EP2 and EP4, receptors linked to PGE2-induced activation of adenylyl cyclase (33), is strongly stimulated. Although forskolin and dibutyryl cAMP stimulate macrophage MMP-9 expression in a dose-dependent manner, the selective EP2 agonist (butaprost) has no effect on macrophage MMP-9 expression, and macrophages from EP2 null mice exhibited enhanced COX-2 and MMP-9 expression when plated on ECM. In contrast, a selective EP4 agonist (PGE2−OH) stimulated macrophage MMP-9 expression, which was inhibited by the EP4 antagonist ONO-AE3-208. Finally, preincubation of macrophages with the EP4 antagonist or inhibition of their EP4 expression by siRNA was as effective in inhibiting ECM-induced MMP-9 expression as treatment with the selective COX-2 inhibitor celecoxib or inhibition of COX-2 expression by siRNA. Thus, COX-2-dependent ECM-induced proteinase expression is markedly attenuated by selective inhibition of EP4, a member of the PGE2 family of receptors.

**MATERIALS AND METHODS**

**Isolation of Peritoneal Macrophages**—Thioglycollate-elicited peritoneal macrophages were obtained from Swiss Webster, EP2 wild type, and EP2 null mice (34) by the method of Edelson and Cohn (35) as described previously (36). Mice were injected intraperitoneally (3 ml/mouse) with 3% Brewer thioglycollate medium containing 0.3 mM thioglycollate (Difco). Four days later cells were harvested by lavage with cold DPBS. Peritoneal cells were recovered by centrifugation and resuspended in Dulbecco’s modification of Eagle’s medium (DMEM) supplemented with 10% Collec Gold fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml), and 4 mM glutamine (Invitrogen) and plated into tissue culture flasks or multiwell plates. Cells were allowed to adhere for 4 h and then were washed free of nonadherent cells. Experiments to determine the effect of matrix on macrophage proteinase expression were carried out in DMEM supplemented with 0.1% low endotoxin bovine serum albumin.

**Murine Macrophage Cell Line**—RAW264.7 macrophages (37) were obtained from American Type Culture Collection and maintained as adherent cultures in DMEM-10% FBS.

**Preparation of Extracellular Matrix-coated Dishes**—SMC-derived matrices were prepared as previously described (38) with the following modifications. Rat aortic smooth muscle cells (SMC; VEC Technologies, Inc.) were plated into 6-, 12-, or 24-well plates in DMEM supplemented with supplied growth medium. 3–4 days after reaching confluence, the cell layer was removed by sequential exposure to 0.1% Triton X-100 in DPBS (2 min at room temperature) and 0.2 mM NH4OH in DPBS (2 min at room temperature). The remaining insoluble matrices were washed 3× with DPBS and stored at 4 °C.

**RT-PCR**—RNA was prepared using RNeasy Mini kits from Qiagen. RNA (2 μg) was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Roche Applied Science) and oligo d(T)16 primer. The resulting cDNA was then used for amplification. The PCR reaction volume was 25 μl and contained 5 μl of cDNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl2, 0.4 mM dNTPs, 400 nM forward primer, 400 nM reverse primer, and 2.5 units of Taq polymerase (Applied Biosystems) in a thermocycler under the following conditions. For EP1, denature for 30 s at 95 °C, anneal for 30 s at 60 °C, and extend for 45 s at 70 °C; repeat for 35 cycles with a final extension for 10 min at 70 °C. For EP2 and EP4, denature for 30 s at 95 °C, anneal for 30 s at 62 °C, and extend for 45 s at 70 °C; repeat for 35 cycles with a final extension for 10 min at 70 °C. For EP3, denature for 30 s at 95 °C, anneal for 30 s at 60 °C, and extend for 45 s at 70 °C; repeat for 40 cycles with a final extension for 10 min at 70 °C. For MMP-9, denature for 20 s at 95 °C, anneal for 20 s at 60 °C, and extend for 45 s at 72 °C; repeat for 35 cycles with a final extension for 10 min at 72 °C. For COX-2, denature for 20 s at 94 °C, anneal for 20 s at 65 °C, and extend for 30 s at 72 °C; repeat for 35 cycles with a final extension for 10 min at 72 °C. Primers for murine EP1 were: sense, 5′-TTAACCCTGACCTGAGGATGATG-3′ (nucleotides 311–331), and antisense, 5′-CGCTGAGGCTTACATGCACTA-3′ (nucleotides 955–976); primers for murine EP2 were sense, 5′-GTCGCCCTGGCTCGGCGAAA GTC-3′, (nucleotides 446–468) and antisense, 5′-GGCAAGGAGCATGATGCAAGTTG-3′ (nucleotides 957–981); primers for murine EP3 were sense, 5′-CCCGGCACTGTTGCTGTTCAT-3′ (nucleotides 538–557), and antisense, 5′-TAGCAGCATAAACCAGG-3′ (nucleotides 956–975); primers for murine EP4 were sense, 5′-TTCCGCTGTTGTCGCTGTTTCGTTCCC-3′ (nucleotides 1074–1097), and antisense, 5′-GAGGAGGTTTGCTTGGGTTGAC-3′ (nucleotides 1539–1562). Primers for murine MMP-9 were sense, 5′-CGTCTGTGATCCACTCAGTACT-3′ (nucleotides 651–671), and antisense, 5′-AACACACAGGGTTTGCGCCTT-3′ (nucleotides 855–875). Primers for murine COX-2 were sense, 5′-GG-TCTGGTGT CCTGTTGATGTTGAC-3′ (nucleotides 935–958), and antisense, 5′-GTCCTTTCAGGAAATGGT-3′ (nucleotides 1637–1658). The primers for β-actin were forward, 5′-GGTATCCCACTGTGATCCCAT-3′, and reverse, 5′-GGATGCCCAGGACTCC-ATGC-3′. PCR products were electrophoresed in a 1% agarose gel with 0.5 μg/ml ethidium bromide and photographed under UV light. The identity of each PCR product was confirmed by DNA sequencing.

**Preparation of Cell Lysates**—Macrophages were lysed in Tris buffer, pH 7.5, containing 20 mM Tris-HCl 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 1 mM sodium vanadate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin. Lysates were centrifuged (14,000 × g) for 20 min at 4 °C. The supernatants were recovered, normalized for protein, mixed with SDS sample buffer with β-mercaptoethanol, and boiled for 5 min. Equal amounts of cell lysates were applied to gels based on protein content.

**Determination of Metalloproteinase Activity**—The presence of metalloproteinase activity in cellular-conditioned media was determined utilizing enzyme zymography as previously described (39). Conditioned media were mixed with SDS sample buffer (without mercaptoethanol) and incubated for 30 min at 37 °C. Samples and molecular weight markers were electrophoresed in a 10% polyacrylamide gel containing 0.1% gelatin. The gel was then washed (2×) in 2.5% Triton X-100 to remove SDS. The gel was incubated at 37 °C for 48 h in 200 mM NaCl containing 40 mM Tris-HCl and 10 mM CaCl2 (pH 7.5) and stained with Coomassie Blue. The presence of gelatinolytic activity was identified as clear bands on a uniform blue background after destaining.

**Western Blot Identification of MMP-9**—Macrophage-conditioned media were electrophoresed in 4–15% polyacrylamide gels, and proteins were transferred to a polyvinylidene difluoride membrane. After
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Western Blot Identification of COX-2—Cell lysates were electrophoresed in gradient gels, and proteins were transferred to a polyvinylidene difluoride membrane. After transfer, the membrane was blocked, washed in PBS (1×), and incubated for 1 h in blocking buffer containing 75 ng/ml rabbit anti-phosphospecific p44/p42 MAPK IgG or rabbit anti-phosphospecific p38 MAPK IgG (Cell Signaling Technology). The membranes were washed (2×; TTBS) and incubated for 1 h in blocking buffer containing 0.3 μg/ml goat anti-rabbit IgG conjugated to HRP. After visualization of bound HRP, membranes were stripped in 62.5 mM Tris buffer (pH 6.7) containing 100 mM β-mercaptoethanol and 2% SDS for 30 min at 50 °C, washed, and probed for total MAPKerk1/2 or p38 (Cell Signaling Technology).

RESULTS



FIGURE 1. SMC-ECM stimulates macrophage expression of EP2 and EP4 receptors. Thioglycollate-elicited macrophages were suspended in DMEM supplemented with 0.1% low endotoxin bovine serum albumin (LE-BSA) and separated into aliquots in 12-well control or ECM-coated plates (7.5 × 10⁵ cells/well). After overnight incubation, total RNA was isolated, and mRNA levels for PGE₂ receptors (EP1–4) were determined by RT-PCR as described under “Materials and Methods.” The data are representative of three separate experiments.

Mature engagement of ECM triggers protein kinase C-dependent activation of MAPKerk1/2, which leads to the stimulation of COX-2 expression and MMP-9 synthesis (32), and activation with LPS triggers the p38-dependent increase in COX-2 expression and MMP-9 synthesis (44). Thus, signaling through the MAPK cascade regulates COX-2 and MMP-9 expression by macrophages. Therefore, we determined whether the observed ECM-dependent increase in EP2 and EP4 expression (Fig. 1) was also dependent on the MAPK cascade. As observed in Fig. 2A, levels of phosphorylated MAPKerk1/2 and p38 were increased when cells were plated on SMC-ECM (Fig. 2A). Preincubation of cells with an inhibitor of protein kinase C (calphostin C) or MEK-1 (U0126) blocked ECM-induced activation of MAPKerk1/2. In contrast, ECM-induced levels of phosphorylated p38 were slightly increased in cells preincubated with calphostin C and decreased in cells incubated with
Macrophages were preincubated with U0126 (10 μM MEK-1 inhibitor U0126 or 50 nM protein kinase C inhibitor (Calphostin C (Calp)) before plating on ECM. Lysates were prepared 30 min later, and levels of phosphorylated (p) MAPKerk1/2 and p38 were assessed by Western blot as described under “Materials and Methods.”

**FIGURE 2.** The causal role of MAPKerk1/2 in ECM-induced EP2 and EP4 expression. **A,** macrophages (2 × 10⁶ cells/well) were preincubated for 30 min with 10 μM MEK-1 inhibitor U0126 or 50 nM protein kinase C inhibitor (Calp) before plating on ECM. Lysates were prepared 30 min later, and levels of phosphorylated (p) MAPKerk1/2 and p38 were assessed by Western blot as described under “Materials and Methods.” **B,** macrophages were preincubated with U0126 (10 μM) or an inhibitor of p38 kinase (10 μM; SB202190) and separated into aliquots in 6-well control plates or plates coated with SMC-ECM (2.0 × 10⁶ cells/well). Conditioned media were collected the next day and assayed for MMP-9 expression by Western blot. **C,** macrophages were preincubated with U0126 or an inhibitor of p38 kinase and separated into aliquots in 6-well control plates or plates coated with SMC-ECM (2.0 × 10⁶ cells/well). After overnight incubation, total RNA was isolated, and mRNA levels for PGE2 receptors (EP1–4) were determined by RT-PCR as described under “Materials and Methods.” Data presented in panels A and B are representative of two experiments, and panel C is representative of three experiments.

U0126. Equal protein loading was confirmed by probing for total MAPKerk1/2 and p38.

The causal relationship between MAPKerk1/2 activation and ECM-induced MMP-9 expression is shown in Fig. 2B. As previously reported, the expression of MMP-9 by macrophages cultured on SMC-ECM was markedly elevated as compared with that in cells cultured on plastic (32). Virtually all the MMP-9 secreted by murine macrophages was in the pro form (105 kDa), which is slightly larger than that expressed by human cells (92 kDa). High molecular weight multimers of MMP-9 are also visible in some preparations. Preincubation of macrophages with U0126 completely blocked ECM-induced MMP-9 expression; in contrast, proteinase expression was partially attenuated by exposure to a p38 inhibitor (SB202190; Calbiochem). We next determined the role of MAPKerk1/2 and p38 on ECM-induced EP2 and EP4 expression. RNA isolated from elicited macrophages plated on ECM in the presence of a MEK-1 or p38 inhibitor was probed for expression of EP2 and EP4 utilizing RT-PCR. ECM-induced expression of the PGE2 receptors was blocked by preincubation with the MEK-1 inhibitor, whereas inhibition of p38 had no effect (Fig. 2C). Taken together with earlier studies (32), these data demonstrate that ECM-induced activation of MAPKerk1/2 after macrophage engagement on ECM is responsible for increased COX-2 and EP2 and EP4 receptor expression.

**ECM-induced MMP-9 Expression Is EP4-dependent**—EP2 and EP4 are coupled to Gας and, when activated by PGE2, lead to an increase in intracellular cyclic AMP (33). In an effort to determine whether either of these receptors played a role in ECM-induced MMP-9 expression, macrophages were incubated with increasing concentrations of an activator of adenylyl cyclase (forskolin) or dibutyryl cAMP. As seen in Fig. 3, incubation of macrophages with either forskolin or dibutyryl cAMP led to a dose-dependent increase in MMP-9 expression, determined by both Western blot and enzyme zymography of conditioned media.

EP2- and EP4-dependent actions of PGE2 can be distinguished utilizing relatively specific receptor agonists or antagonists and genetic approaches. To determine whether ECM-induced MMP-9 is dependent on EP2 signaling, macrophages were incubated with butaprost (free acid; Cayman), a selective EP2 agonist (45). The free acid form of butaprost has a >10-fold higher affinity to EP2 than the ester form. As seen in Fig. 4A, butaprost (0.01–10 μM) had no effect on macrophage MMP-9 expression. AH 6809 (Cayman) is a receptor antagonist for EP2 (as well as EP1, EP3, and DP2) (46). Preincubation of macrophages with AH 6809 had little or no effect on ECM-induced MMP-9 and COX-2 expression (Fig. 4B). To directly test the role of EP2, we next compared the effect of ECM on macrophages isolated from wild type and EP2−/− mice (34, 47) (Fig. 4C). When plated on ECM, COX-2 and MMP-9 expression were stimulated in both wild type and EP2 null macrophages. Based on these data, the prostanoid receptor EP2 does not appear to play a central role in ECM-induced MMP-9 expression.

PGE1-OH is a selective EP4 agonist (48). Incubation of macrophages with 0.05–10 μM PGE1-OH resulted in a dose-dependent increase in MMP-9 expression, which was inhibited by the selective EP4 antagonist ONO-AE3-208 (Fig. 5). We next determined whether EP4 receptor blockade would inhibit ECM-induced protease expression and com-
phages isolated from EP2
COX-2 in lysates were determined by Western blot.
were collected, and lysates were prepared. Levels of MMP-9 in conditioned media and
plate or an SMC-ECM-coated plate. Cells were incubated overnight, conditioned media
concentrations of AH 6809 (30 min) and separated into aliquots into a control 24-well
blot.
conditioned media were collected, and levels of MMP-9 were determined by Western
the indicated concentrations of butaprost (free acid). Cells were incubated overnight,
Washed 3
wells). After 4 h of adherence, cells were
phosphate dehydrogenase.
ared for MMP-9 antigen, LPS and TNF
treatment led to increased
expression was first verified in LPS (10 ng/ml)-
and TNFα (100 ng/ml)-
treated RAW264.7 macrophages. As observed in Fig. 6 (left panel),
COX-2 antigen in mock-transfected macrophages was markedly
increased after incubation with LPS or TNFα. In contrast, neither LPS
nor TNFα stimulated COX-2 expression by macrophages previously
transfected with COX-2 siRNA. When conditioned media were exam-
ined for MMP-9 antigen, LPS and TNFα treatment led to increased
proteinase expression, which was not observed in macrophages trans-
ferred with COX-2 siRNA. Next we determined the effect of COX-2
silencing on ECM-induced MMP-9 expression. As reported earlier,
macrophase expression of COX-2 and MMP-9 is up-regulated when
plated on ECM. Inhibition of COX-2 gene expression by siRNA blocked
ECM-induced MMP-9 expression. Thus, cytokine- and ECM-induced
proteinase expression is causally linked to enhanced COX-2 expression
and effectively blocked by COX-2 silencing.
We next compared the abilities of the selective COX-2 inhibitor cele-
oxib and the EP4 antagonist to inhibit matrix-induced proteinase expression (Fig. 6, middle and right panels). ECM-induced MMP-9
expression was markedly inhibited when cells were preincubated with
celoxib (5 μM). Likewise, ECM-induced proteinase expression was
nearly blocked by preincubation with the EP4 antagonist (10 μM).
As an alternative strategy to test the role of EP4 in ECM-induced
MMP-9 expression, we utilized EP4 siRNA to knock down macrophage
expression. Levels of EP4 mRNA were markedly increased when
either control or nonspecific siRNA transfected RAW264.7 macro-
phages were plated on ECM (Fig. 7A). In contrast, EP4 expression
was not detected in macrophages transfected with EP4 siRNA. As expected,
the level of MMP-9 mRNA was increased when either control or non-
specific siRNA transfected cells were plated on ECM. When EP4 knock-
down macrophages were plated on ECM, the level of MMP-9 mRNA
was reduced to that observed in controls.
COX-2 expression is reportedly stimulated by PGE2 via a positive
feedback loop (49, 50). Our observation that celoxib and the EP4
agonist partially inhibited ECM-induced COX-2 expression (Figs. 6)
is consistent with a feedback loop and identifies EP4 as the receptor
responsible for PGE2 effect. To test this hypothesis, EP4 "knockdown"
macrophages were plated on ECM, and COX-2 expression was deter-
mixed utilizing RT-PCR (Fig. 7A). The expression of COX-2 was mark-
edly increased when either control or nonspecific siRNA-transfected
RAW264.7 macrophages were plated on ECM, whereas ECM-induced
COX-2 expression was partially inhibited in EP4 knockdown
macrophages.
We next determined the effects of EP4 knocked down on ECM-
induced MMP-9 and COX-2 expression utilizing zymography and West-
ern blotting (Fig. 7B). Levels of MMP-9 activity and antigen were mark-
edly increased when either control or nonspecific siRNA transfected
macrophages were plated on ECM. Likewise, levels of COX-2 antigen
were increased when either control or nonspecific siRNA-transfected
macrophages were plated on ECM. When EP4 knockdown macrophages
were plated on ECM, levels MMP-9 and COX-2 expression were
reduced to that observed in controls. Taken together, these data dem-
strate that targeting a single prostanoid receptor achieves the same
level of proteinase inhibition as gene silencing or pharmacological inhi-
bition of COX-2.
Finally, the ability of an EP4 agonist to restore COX-2 and MMP-9
expression by macrophages pretreated with selective COX-2 inhibitors
was determined. As seen in Fig. 8A, COX-2 and MMP-9 expression
were stimulated by exposure to LPS. Co-incubation of macrophages
with the selective COX-2 inhibitors NS398 and celoxib blocked LPS-
induced COX-2 and MMP-9 expression, which was restored by the
addition of the EP4 ligand, PGE1,OH.
To corroborate these results, we knocked down EP4 expression with
siRNA and subsequently treated macrophages with LPS (Fig. 8B). Nei-
ther COX-2 nor MMP-9 antigen levels were up-regulated by LPS in cells
transfected with EP4 siRNA. In contrast, COX-2 and MMP-9 expres-
sion was strongly up-regulated in cells transfected with nonspecific
siRNA. As predicted, exogenous PGE1,OH was unable to restore

FIGURE 4. ECM-induced MMP-9 expression by macrophages is EP2-independent. A, RAW264.7 macrophages were suspended in DMEM containing 10% FCS and separated into aliquots in 24-well plates (3 × 10⁵ cells/well). After 4 h of adherence, cells were washed 3× with DPBS, and media were replaced with DMEM, 0.1% LE-BSA containing the indicated concentrations of butaprost (free acid). Cells were incubated overnight, conditioned media were collected, and levels of MMP-9 were determined by Western blot. The data are representative of two experiments. B, cells were suspended in DMEM, 0.1% LE-BSA, preincubated with the indicated concentrations of AH 6809 (30 min) and separated into aliquots into a control 24-well plate or an SMC-ECM-coated plate. Cells were incubated overnight, conditioned media were collected, and lysates were prepared. Levels of MMP-9 in conditioned media and COX-2 in lysates were determined by Western blot. C, thioglycollate-elicited macrophages isolated from EP2+/+ or EP2−/− mice were suspended in DMEM, 0.1% LE-BSA and separated into aliquots in 12-well control or ECM-coated plates (4.0 × 10⁵ cells/well). After overnight incubation, conditioned media were collected, and cell lysates were prepared. Levels of MMP-9 and COX-2 were determined by Western blot as described under "Materials and Methods." Dose response data presented in panels A and B are representative of three experiments. Data in panel C are derived from duplicate wells and are representative of two experiments. WT, wild type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

FIGURE 5. EP4-agonist stimulates MMP-9 expression by macrophages. RAW264.7 macrophages were suspended in DMEM containing 10% FCS and separated into ali-

quots in 24-well plates (3 × 10⁵ cells/well). After 4 h of adherence, cells were washed 3×
with DPBS, and media were replaced with DMEM, 0.1% LE-BSA containing the selective
EP4 agonist PGE1,OH (0.5–10 μM) or PGE1-OH and the EP4 antagonist ONO-AE3-208.
Cells were incubated overnight, conditioned media were collected, and levels of MMP-9
were determined by Western blotting (Fig. 7A). In contrast, EP4 expression was
markedly increased when either control or nonspecific siRNA-transfected
RAW264.7 macrophages were plated on ECM, whereas ECM-induced
COX-2 expression was partially inhibited in EP4 knockdown
macrophages.

EP4-dependent Proteinase Expression by Macrophages
EP4-dependent Proteinase Expression by Macrophages

FIGURE 6. EP4 antagonist is as effective as either COX-2 silencing or celecoxib in the inhibition of ECM-induced MMP-9 expression. Left panel, RAW264.7 macrophages transfected with COX-2 siRNA or mock-transfected were suspended in DMEM, 0.1% LE-BSA and separated into aliquots in a control 24-well plate or SMC-ECM-coated plate. After adherence, the indicated wells received LPS (100 ng/ml) or TNFα (10 ng/ml). Cells were incubated overnight, conditioned media was collected, and lysates were prepared. Levels of MMP-9 in conditioned media and COX-2 in lysates were determined by Western blot. Data for each condition are derived from a single well. The entire experiment was repeated twice with similar results. Ctrl, control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Middle and right panels, cells were suspended in DMEM, 0.1% LE-BSA preincubated with celecoxib (5 μM) or EP4 antagonist (10 μM) and separated into aliquots in a control 24-well plate (plastic (Plst)) or SMC-ECM-coated plate. Cells were incubated overnight, conditioned media were collected, and lysates were prepared. Levels of MMP-9 in conditioned media and COX-2 in lysates were determined by Western blot. The data are representative of two experiments.

FIGURE 7. EP4 silencing blocks ECM-induced MMP-9 expression. A, RAW264.7 macrophages transfected with EP4 siRNA or nonspecific (NS) siRNA were separated into aliquots in plastic (Plst) or SMC-ECM-coated 6-well plates (1 × 10⁶/well) in DMEM, 0.1% LE-BSA, and the cells were incubated overnight. The next day total RNA was isolated, and mRNA levels for EP4, MMP-9, and COX-2 were determined by RT-PCR as described under "Materials and Methods." B, EP4 siRNA or nonspecific siRNA-transfected macrophages were separated into aliquots in plastic or SMC-ECM-coated 12-well plates (5 × 10⁵/well) in DMEM, 0.1% LE-BSA, and the cells were incubated overnight. The next day media were recovered, and lysates were prepared. Levels of MMP-9 expression were determined by both zymography and Western blot. Levels of COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by Western blot as described under “Materials and Methods.”

COX-2 and MMP-9 expression by EP4 knock-down cells. Thus, PGE₂-dependent COX-2 expression appears to be mediated by the EP4 receptor.

In summary, ECM-induced proteinase expression by macrophages is COX-2-dependent and blocked by a selective inhibitor of COX-2 or an EP4 receptor antagonist. Thus, targeting the EP4 prostanoid receptor appears to be an alternative strategy to block COX-2-dependent MMP-9 expression by macrophages.

DISCUSSION

Pathologic, pharmacologic, and genetic evidence indicate that COX-2-dependent PGE₂ synthesis regulates macrophage MPP expression, which is thought to destabilize atherosclerotic plaques and contribute to the formation of aneurysms (9–14, 20, 21, 25, 51). PGE₂ and the other prostanoid family members (PGF₂α, PGD₂, PGI₂, and thromboxane A₂) are synthesized from the common endoperoxide intermediate PGH₂, which is generated by the action of COX-1 and COX-2 on arachidonic acid (7, 8). COX-1 expression is constitutive, whereas COX-2 expression is induced in response to a variety of inflammatory mediators (52). Consequently, inhibition of COX-2 would appear to be a rational therapeutic target for the stabilization of vascular lesions. Paradoxically, the administration of selective COX-2 inhibitors leads to increased frequency of adverse cardiovascular events (26, 27). It has been suggested that the loss of anti-inflammatory prostanoids and/or disturbance in the balance of pro- and anti-thrombotic prostanoids is responsible for the adverse cardiovascular effects of COX-2 inhibitors (29, 30). To avoid these collateral effects of COX-2 inhibition, a strategy to block a specific prostanoid receptor(s) would be necessary. In studies reported here, we have determined whether selective inhibition of the PGE₂ receptor family (EP1–4) can block ECM-induced proteinase expression by macrophages. We demonstrate that macrophage engagement of ECM selectively stimulates expression of EP2 and EP4 prostanoid receptors, and signaling through EP4 is responsible for increased MMP-9 expression. Moreover, inhibiting EP4 expression by siRNA or blocking PGE₂ binding to EP4 inhibits ECM-induced proteinase expression as effectively as celecoxib inhibition of COX-2 activity or inhibition of COX-2 expression by siRNA.

The pleiotropic effects of prostanoids are mediated by a family of G-protein-coupled receptors (53). Each prostanoid is recognized by its own receptor or receptor subtypes. Four subtypes of the EP receptor for PGE₂ have been described (EP1–4) and cloned (54). The regulation of EP receptor expression as well as cellular functions triggered by their engagement is an area of intense investigation (55–57). In this regard, several studies have demonstrated differential expression of macrophage EP receptors in response to tissue injury and inflammatory stim-
FIGURE 8. EP4 agonist restores MMP-9 and COX-2 expression in macrophages treated with selective COX-2 inhibitors. A, RAW264.7 macrophages were separated into aliquots into a 12-well plate in DMEM, 10% FBS (5×10^5/well). After 4 h of adherence, cells were washed 3× with DPBS, and media were replaced with DMEM, 0.1% LE-BSA. Cells were preincubated (30 min) with either NS398 (5 μM) or celecoxib (5 μM) after which LPS (100 ng/ml) and PGE1-OH (10 μM) were added to the indicated wells. The next day conditioned media were collected, and lysates were prepared. Levels of MMP-9 expression in the media and COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the lysates were determined utilizing Western blot as described under "Materials and Methods." Ctrl, control; 8, EP4 siRNA or nonspecific (NS) siRNA-transfected macrophages were separated into aliquots into a 12-well plate in DMEM, 10% FBS (5×10^5/well). After adherence, cells were washed, media were replaced with DMEM, 0.1% LE-BSA, and LPS and PGE1-OH were added to the indicated wells. The next day media were recovered, and lysates were prepared. Levels of MMP-9 expression were determined by both zymography and Western blot. Levels of COX-2 and glyceraldehyde-3-phosphate dehydrogenase were determined by Western blot as described under "Materials and Methods."
