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Matrix Metalloproteinase (MMP)-1 and MMP-3 Induce Macrophage MMP-9: Evidence for the Role of TNF-α and Cyclooxygenase-2

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Matrix metalloproteinase (MMP)-9 (gelatinase B) participates in a variety of diverse physiologic and pathologic processes. We recently characterized a cyclooxygenase-2 (COX-2)→PGE2→EP4 receptor axis that regulates macrophage MMP-9 expression. In the present studies, we determined whether MMPs, commonly found in inflamed and neoplastic tissues, regulate this prostanoid-EP receptor axis leading to enhanced MMP-9 expression. Results demonstrate that expression of murine peritoneal macrophages and RAW264.7 macrophages to MMP-1 (collagenase-1) or MMP-3 (stromelysin-1) lead to a marked increase in COX-2 expression, PGE2 secretion, and subsequent induction of MMP-9 expression. Proteinase-induced MMP-9 expression was blocked in macrophages preincubated with the selective COX-2 inhibitor celecoxib or transfected with COX-2 small interfering RNA (siRNA). Likewise, proteinase-induced MMP-9 was blocked in macrophages preincubated with the EP4 antagonist ONO-AE3-208 or transfected with EP4 siRNA. Exposure of macrophages to MMP-1 and MMP-3 triggered the rapid release of TNF-α, which was blocked by MMP inhibitors. Furthermore, both COX-2 and MMP-9 expression were inhibited in macrophages preincubated with anti-TNF-α IgG or transfected with TNF-α siRNA. Thus, proteinase-induced MMP-9 expression by macrophages is dependent on the release of TNF-α, induction of COX-2 expression, and PGE2 engagement of EP4. The ability of MMP-1 and MMP-3 to regulate macrophage secretion of PGE2 and expression of MMP-9 defines a nexus between MMPs and prostanoids that is likely to play a role in the pathogenesis of chronic inflammatory diseases and cancer. These data also suggest that this nexus is targetable utilizing anti-TNF-α therapies and/or selective EP4 antagonists. The Journal of Immunology, 2009, 183: 8119–8127.

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induction of COX-2 expression, and PGE2 engagement of EP4. Taken together, these data identify a novel regulatory mechanism whereby MMP-1 and MMP-3 contribute to the inflammatory process by liberating TNF-α and stimulating PGE2 secretion via the induction of COX-2, which leads, in turn, to increased MMP-9 expression. The ability of select MMPs to regulate macrophage secretion of PGE2 defines a nexus between MMPs and prostanooids that is likely to play a role in the pathogenesis of chronic inflammatory diseases and cancer.

Materials and Methods

Macrophages

Thioglycollate-elicited peritoneal macrophages were obtained from Swiss Webster mice by the method of Edelson and Cohn (8) as described previously (9). Mice were injected i.p. (3 ml/mouse) with 3% Brewer thioglycollate medium (Difco). Four days later, cells were harvested by lavage with cold Dulbecco’s PBS. Peritoneal cells were recovered by centrifugation and resuspended in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 4 mM glutamine, and plated into tissue culture flasks or multiwell plates. Cells were allowed to adhere for 4 h and then washed free of nonadherent cells. All tissue culture supplies were obtained from Invitrogen. The murine macrophage cell line RAW264.7 (10) was obtained from American Type Culture Collection and maintained as adherent cultures in DMEM-10% FBS. All animal studies described in this report have been reviewed and approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee.

Matrix metalloproteinases

Human recombinant pro-MMP-1, active MMP-2, an active fragment of MMP-3, and active MMP-7 were obtained from Calbiochem. Human recombinant active MMP-9 was obtained from Oncogene. Before use, pro-MMP-1 was activated by incubation (2 h at 37°C) with 1 mM 4-amino-phenylmercuric acetate (APMA) in 50 mM Tris (pH 7.5), containing 0.01% Triton X-100 and 5 mM CaCl2 (11). The conversion of pro-MMP-1 to its active form is associated with a loss of the pro-domain (~10 kDa), which was verified by SDS-PAGE and Western blot (data not shown).

Preparation of cell lysates

Cell lysates utilized in the analysis of COX-2 were prepared by adding 1X SDS sample buffer directly to washed macrophage monolayers. In experiments designed to monitor levels of phosphorylated and total MAPK\(_{\text{ERK1/2}}\), macrophages were lysed in TBS containing 2 mM EDTA, 1% Triton X-100, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 1 mM PMSF, and 10 μg/ml aprotinin. Lysates were centrifuged (14,000 × g) for 20 min at 4°C. The supernatants were recovered, normalized for protein, and mixed with SDS sample buffer with 2-ME and boiled 5 min. Equal amounts of cell lysates were applied to gels based on normalized for protein, and mixed with SDS sample buffer with 2-ME and peptide containing amino acids 584–598 of murine COX-2 (0.5 μg/ml, H9262). Expression. The ability of select MMPs to regulate macrophage induction of COX-2, which leads, in turn, to increased MMP-9 expression. The ability of select MMPs to regulate macrophage induction of COX-2, which leads, in turn, to increased MMP-9 expression. The ability of select MMPs to regulate macrophage induction of COX-2, which leads, in turn, to increased MMP-9 expression. The ability of select MMPs to regulate macrophage induction of COX-2, which leads, in turn, to increased MMP-9 expression.

Western blots

Cell lysates were electrophoresed in 4–15% polyacrylamide gels, and proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked in 5% defatted milk in TBST, washed in PBS, and incubated 1 h in blocking buffer containing rabbit IgG raised against a peptide containing amino acids 584–598 of murine COX-2 (0.2 μg/ml; Chemicon International) or incubated at 4 °C overnight in blocking buffer containing goat anti-rabbit IgG conjugated to HRP (0.3 μg/ml; Transduction Laboratories). The membranes were washed three times in TBST, and bound HRP was visualized utilizing chemiluminescence (HyGlo). In the case of membranes determined utilizing Scion Image and normalized for levels of actin mRNA, which served as a loading control.

Determination of PGE2 and TNF-α levels in macrophage conditioned media

RAW264.7 macrophages were transfected with either COX-2, TNF-α, or nonspecific siRNA (Santa Cruz Biotechnology) according to the manufacturer’s protocol, which was modified as follows. Macrophages (T-75 flask) were washed three times with Dulbecco’s PBS to remove serum, and medium was replaced with antibiotic-free DMEM. The cells were mechanically harvested, recovered by centrifugation, and resuspended in antibiotic-free DMEM supplemented with 10% FBS. The cells (2 × 10⁵/well) were aliquoted into a 6-well plate and incubated overnight. The next morning, 9.1 μl of 10 μM siRNA was added to 152 μl of transfection medium (Santa Cruz Biotechnology) and incubated 5 min at room temperature. In another tube, 9.1 μl of the transfection reagent (Santa Cruz Biotechnology) was added to the transfection medium and incubated 5 min. The two tubes were combined and incubated 20 min to form the siRNA transfection reagent complex. Immediately before transfection, macrophage medium was removed and replaced with 1.5 ml of antibiotic-free DMEM supplemented with 10% FBS. The plate was placed on a rocker plate in the laminar flow hood, and the transfection reagent complex was added dropwise. Cells were incubated 72 h at 37°C.

RT-PCR

RNA was prepared using TRIzol reagent kits (Invitrogen). RNA (2 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Roche Applied Science) and oligo(dT)16 primer. The resulting cDNA was then used for amplification. The primers and conditions for PCR analysis of murine COX-2, MMP-9, EP4, and actin have been previously described (5). Primers for murine TNF-α were obtained from R&D Systems. PCR products were electrophoresed in a 1% agarose gel with 0.5 μg/ml ethidium bromide and photographed under UV light. PCR conditions for the target genes were optimized to ensure that experimental samples, excluding exuberant positive controls, are in the linear range when scanned. Densitometric analysis of the scanned target mRNAs were determined utilizing Scion Image and normalized for levels of actin mRNA, which served as a loading control.

Cytokine Ab array

The presence of 18 cytokines released into conditioned media from macrophages incubated 1 h with 50 nM MMP-3 was monitored utilizing the Legend MAX ELISA kit for mouse TNF-α (BioLegend).

Evaluation of cellular toxicity

To rule out toxic effects of the inhibitors utilized in these studies, cellular morphology and/or protein content following incubation are regularly monitored. Additionally, we test for potential cytotoxicity by monitoring mitochondrial dehydrogenase activity utilizing a MTT assay kit (Sigma-Aldrich). Levels of mitochondrial dehydrogenase activity were not significantly reduced following exposure to any of the inhibitors over the time periods described herein.

Cytokines and chemokines

Recombinant murine TNF-α, MIG (CXCL9), MIP-α (CCL3), INF-γ, and RANTES (CCL5) were obtained from R&D Systems.

Results

MMP-1 and MMP-3 induce macrophage COX-2 expression

In previous studies, we characterized a COX-2→PGE2→EP4 receptor axis that regulates macrophage MMP-9 expression (5). In

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the present study, we have determined whether MMP family members commonly found in inflamed and neoplastic tissues regulate expression of this prostanoid-EP receptor axis. For this purpose, RAW264.7 macrophages (10) and thioglycollate-elicited macrophages were incubated overnight with 50 nM active MMP-1, MMP-2 (gelatinase A), MMP-3, MMP-7 (matrilysin), or MMP-9. Incubation with 10 ng/ml LPS, a potent inducer of COX-2 expression by macrophages, served as a positive control (12, 13). Levels of COX-2 in cell lysates were prepared and levels of COX-2 and GAPDH were determined utilizing Western blot. A. Macrophages were dispersed into a 48-well plate (2 × 10^5 cells/well). Following adherence, cells were washed three times with Dulbecco’s PBS and media were replaced with DMEM-0.1% low endotoxin (LE) BSA alone (Ctrl) or DMEM-0.1% LE-BSA containing 50 nM MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, or 10 ng/ml LPS. The next day cell lysates were prepared and levels of COX-2 and GAPDH were determined utilizing Western blot. B. Macrophages were dispersed into a 6-well plate (2 × 10^6 cells/well). Following adherence, cells were washed. Media were replaced with DMEM-0.1% LE-BSA alone (Ctrl) or DMEM-0.1% LE-BSA containing 50 nM MMP-1, MMP-3, or 10 ng/ml LPS. After overnight incubation, total RNA was isolated, and mRNA levels for COX-2 and actin were determined utilizing RT-PCR. The data are representative of three separate experiments.

To determine whether MMP-induced COX-2 expression was dependent on their enzymatic activity, we compared the abilities of pro-MMP-1- and APMA-activated MMP-1 to induce COX-2 expression in RAW264.7 macrophages. As seen in Fig. 2 (top panel), in contrast to active MMP-1, incubation with pro-MMP-1 failed to induce COX-2 expression. Next, we determined whether two unrelated MMP inhibitors could block MMP-3-induced COX-2 expression. For this purpose, RAW264.7 macrophages were incubated with either a hydroxamic acid-based broad spectrum MMP inhibitor (GM6001) or an inhibitory peptide corresponding to the pro-domain of MMP-3 (MMP-3 inhibitor 1). As seen in Fig. 2 (bottom panel), MMP-3-induced COX-2 expression was completely blocked by either GM6001 or MMP-3 inhibitor 1. Taken together, these data demonstrate that the induction of COX-2 expression by MMP-1 and MMP-3 is dependent on their enzymatic activities.

MMP-1 and MMP-3 induce macrophage MMP-9 expression

Macrophage MMP-9 expression is regulated by COX-2-dependent synthesis of PGE_2 (4, 5). Therefore, we next determined whether MMP-1 and MMP-3 induced macrophage MMP-9 expression in a COX-2-dependent manner. As seen in Fig. 3A, the levels of COX-2 and MMP-9 Arg in RAW264.7 lysates and conditioned media, respectively, were elevated following an overnight incubation with MMP-1, MMP-3, or LPS (positive control). The MMP-9 in conditioned media from MMP-1- and LPS-treated macrophages was exclusively in the pro-form (105 kDa), which is slightly larger than the human pro-form (92 kDa). In contrast, both pro (P; 105 kDa) and active (A; 95 kDa) MMP-9 were identified in cultures incubated with MMP-3. MMP-3-dependent activation of pro-MMP-9 was confirmed utilizing zymography (not shown) as previously described (4), and this is consistent with earlier reports indicating that pro-MMP-9 is activated extracellularly by MMP-3 (6, 7, 14, 15).
In a subsequent experiment, we examined the temporal relationship between MMP-3-induced COX-2, PGE\(_2\) and MMP-9 expression (Fig. 3, B and C). Levels of COX-2 Ag were increased 4–6 h following the addition of MMP-3, and they were strongly elevated at 18 and 24 h. Levels of PGE\(_2\) in media recovered from MMP-3-treated macrophages were markedly elevated at 18 and 24 h, as compared with PGE\(_2\) levels in media recovered from control cells. Following the increase in PGE\(_2\) levels, MMP-9 Ag in conditioned media of MMP-3-treated cells was strongly elevated at 24 h. In a separate experiment, levels of PGE\(_2\) release by RAW264.7 macrophages incubated 18 h with media alone or media containing 50 nM MMP-1 or MMP-3 were compared. Media derived from control cells contained 107 pg/mg cell protein (average of duplicate wells). Following incubation with MMP-1 or MMP-3, PGE\(_2\) levels increased to 661 and 905 pg/mg cell protein, respectively.

**Activation of MAPK\(_{\text{erk1/2}}\) plays a causal role in MMP-induced COX-2 expression and MMP-9 synthesis**

We previously have shown that the activation of MAPK\(_{\text{erk1/2}}\) is a determinant of ECM-induced COX-2 expression and MMP-9 synthesis (4, 5). Therefore, we determined whether exposure of cells to MMP-1 or MMP-3 led to the activation of MAPK\(_{\text{erk1/2}}\). As seen in Fig. 4A, the levels of phosphorylated and total MAPK\(_{\text{erk1/2}}\) were determined utilizing Western blot. B, RAW264.7 macrophages (48-well plate; 2.5 x 10\(^5\) cells/well) were cultured in DMEM-0.1% LE-BSA for 24 h. Cells were then incubated with media alone (Ctrl) or media containing 50 nM MMP-1, MMP-2, MMP-3, MMP-7, or MMP-9 for 30 min, and lysates were probed for phosphorylated and total MAPK\(_{\text{erk1/2}}\). C, RAW264.7 macrophages (48 well plate; 2.5 x 10\(^5\)well) were preincubated with U0126 (10 μM; MEK-1 inhibitor) or celecoxib (5 μM; COX-2 inhibitor). Following an overnight incubation with 50 nM MMP-1, MMP-3, or 10 ng/ml LPS, levels of MMP-9 in conditioned media and COX-2 and GAPDH in lysates were determined by Western blot. The entire experiment was repeated twice with similar results.

**MMP-induced MMP-9 synthesis is dependent on COX-2**

To examine the role of COX-2 in proteinase-induced expression of MMP-9, macrophages were preincubated with the selective COX-2 inhibitor celecoxib or the MEK-1 inhibitor U0126 (5 μM) for 24 h. Following incubation with MMP-1 or MMP-3, levels of MMP-9 were determined by Western blot. The entire experiment was repeated twice with similar results.
COX-2 inhibitor celecoxib. Pretreatment with 5 μM celecoxib blocked MMP-induced MMP-9 expression (Fig. 4C), which was restored with the addition of PGE2 (not shown). The observation that celecoxib also attenuates COX-2 expression is consistent with the previously reported positive feedback loop whereby PGE2 stimulates COX-2 expression by engaging the EP4 receptor and triggering a Ras-MAPK signaling cascade (5, 16).

As an alternative strategy to examine the role of COX-2 in MMP-induced expression of MMP-9, RAW264.7 macrophages were transfected with COX-2 siRNA. As observed in Fig. 5A, levels of COX-2 Ag in cells transfected with nonspecific siRNA were increased following incubation with MMP-1, MMP-3, or LPS, whereas reduced levels of COX-2 were observed in MMP- or LPS-treated macrophages transfected with COX-2 siRNA. When conditioned media were examined for MMP-9 Ag, MMP-1, MMP-3, and LPS treatment led to a large increase in MMP-9 expression, which was markedly attenuated in macrophages in which COX-2 expression was knocked down.

Finally, the diverse biologic actions of PGE2 are mediated by the EP1–4 family of prostanoid receptors (17, 18). Earlier studies have demonstrated that PGE2-dependent MMP-9 expression was mediated via engagement of EP4 (5, 19). Thus, the induction of MMP-9 expression by selected MMPs is causally linked to MAPKerk1/2-dependent stimulation in COX-2 expression and subsequent PGE2 engagement of EP4.

MMP-dependent release of TNF-α stimulating macrophage COX-2 expression

Proteinases can affect cellular function by releasing tethered growth factors (20–22), cytokines (23, 24), or fragments of extracellular matrix (25–27) or by activating protease-activated receptor family members (28, 29). To determine whether MMP-induced COX-2 expression resulted from the release or activation of a soluble factor, conditioned media from macrophages exposed to MMP- or LPS-treatment were recovered, treated with MMP-3 inhibitor 1 overnight. The next day conditioned media from MMP-3-treated macrophages were recovered and lysates were prepared. Conditioned media alone or media to which MMP-3 inhibitor 1 was added (to block residual MMP-3 activity) were added to naive macrophages and incubated overnight. Levels of COX-2 and GAPDH were monitored by Western blot. The data are representative of three separate experiments. B. Conditioned media recovered from RAW264.7 macrophages (6-well plate; 2.5 × 10⁶/well) were incubated in DMEM-0.1% LE-BSA containing 50 nM of the indicated MMPs alone or in the presence of the broad-spectrum MMP inhibitor GM6001 (50 nM) overnight. The next day conditioned media were recovered and levels of TNF-α were determined by Western blot. The data are representative of two separate experiments.

mRNA in cells transfected with EP4 siRNA was reduced ~95% when compared with cells transfected with nonspecific siRNA (Fig. 5C, left panel). When transfected cells were exposed to proteinases, MMP-1, MMP-3, and LPS (positive control) failed to significantly induce COX-2 or MMP-9 expression by macrophages transfected with EP4 siRNA (Fig. 5C, right panel). Thus, the induction of MMP-9 expression by selected MMPs is causally linked to MAPKerk1/2-dependent stimulation in COX-2 expression and subsequent PGE2 engagement of EP4.
COX-2 expression in macrophages cocünbated with MMP-3 inhibitor 1 was noticeably reduced. Similar results are seen in Fig. 2 (upper panel). Likewise, conditioned media recovered from cells following an overnight incubation with MMP-3 stimulated COX-2 expression by naive macrophages (Fig. 6A, right panel). The ability of conditioned media to up-regulate COX-2 expression in naive cells was relatively unaffected by the addition of MMP-3 inhibitor 1. These data indicate that exposure of cells to MMP-3 released or activated a soluble factor that was responsible for MMP-induced COX-2 expression.

In an effort to identify the factors released by macrophages following exposure to MMP-3, the presence of 18 cytokines was monitored utilizing Ab array. As seen in Fig. 6B, 1 h of treatment with MMP-3 resulted in a substantial increase in levels of TNF-α previously reported to be a potent stimulator of COX-2 (5, 30) and MMP-9 expression by macrophages (5, 31). To corroborate and extend the Ab array data, conditioned media were recovered from RAW264.7 macrophages treated with MMP-1 and MMP-3 for 18 h, and probed for levels of TNF-α utilizing Western blot. Recombinant murine TNF-α (25 ng/lane) was utilized as a standard. When compared with the standard TNF-α, media samples recovered from MMP-treated macrophages contained relatively high levels of TNF-α (Fig. 6C, upper panel). In contrast, TNF-α was not detected in media recovered from cells incubated overnight with MMP-1 or MMP-3 in the presence of the MMP inhibitor GM6001 (Fig. 6C, lower panel). Finally, to obtain a quantitative measure of MMP-induced TNF-α release, media were recovered from RAW264.7 macrophages (48-well plate; 2.5 × 10⁵/well) treated with 50 nM MMP-1, 50 nM MMP-3, or 10 ng/ml LPS (positive control) for 18 h and probed for levels of TNF-α utilizing ELISA. Media from control cells contained 698 ± 109 pg/ml (mean ± SEM; n = 3). Following exposure to MMP-1 or MMP-3, levels increased 25-fold (17,555 ± 6,544) and 34-fold (23,767 ± 1,574), respectively. TNF-α levels in media from cells incubated with LPS increased 160-fold (114,333 ± 1,828).

Next, we utilized a neutralizing rat monoclonal anti-murine TNF-α IgG to determine whether MMP-induced release of TNF-α was responsible for inducing COX-2 and MMP-9 expression in RAW264.7 macrophages. For this purpose, conditioned media recovered from cells incubated with MMP-1 or MMP-3 were pre-incubated 2 h with 20 μg/ml anti-TNF-α IgG or normal IgG before transferring to naive cells. As shown in Fig. 7, conditioned media derived from MMP-1-treated cells stimulated COX-2 and MMP-9 expression in naive macrophages. Preincubation of the conditioned media with anti-TNF-α IgG blocked its ability to induce COX-2 and MMP-9 expression; in contrast, normal IgG had no effect. Likewise, the addition of MMP-3 conditioned media to naive macrophages stimulated their COX-2 and MMP-9 expression. The addition of MMP-3 inhibitor I or normal IgG to the conditioned media recovered from MMP-3-treated macrophages had no effect on its ability to induce COX-2 or MMP-9 in naive cells; in contrast, preincubation with anti-TNF-α IgG suppressed its ability to stimulate COX-2 and MMP-9 by naive macrophages.

To directly test the role of TNF-α in proteinase-induced MMP-9 expression, RAW264.7 macrophages were transfected with either TNF-α siRNA or nonspecific siRNA. The level of TNF-α mRNA in cells transfected with TNF-α siRNA was reduced ~87% when compared with cells transfected with nonspecific siRNA (Fig. 8, left panel). When TNF-α siRNA-transfected cells were exposed to proteinases, MMP-1, MMP-3 failed to significantly induce COX-2 or MMP-9 expression. Importantly, COX-2 and MMP-9 expression were restored in these TNF-α siRNA-transfected cells by the addition of exogenous TNF-α (Fig. 8, right panel).

In these knockdown studies, LPS was included as a control to monitor nonspecific effects of transfection with TNF-α siRNA. Although LPS potently stimulates macrophage expression of the inflammatory cytokines TNF-α and IL-1β, LPS-induced COX-2 expression in human macrophages is independent of either inflammatory cytokine (32). Consistent with these observations, TNF-α knockdown had relatively little effect on LPS-induced COX-2 and MMP-9 expression by murine RAW264.7 macrophages (Fig. 8, right panel). Moreover, the presence of neutralizing anti-TNF-α IgG (20 μg/ml) failed to block LPS-induced COX-2 and MMP-9 expression in murine RAW264.7 macrophages (data not shown).

Finally, the Ab array in Fig. 6 revealed that in addition to TNF-α several other cytokines and chemokines were induced by stimulation with MMP-3, although to a minor degree. These data suggest that other factors may contribute to the observed proteinase induction of COX-2 and MMP-9 expression. However, an overnight incubation of RAW264.7 macrophages with 0.1–1.0 μg/ml MIG (CXCL9), 0.5–50 ng/ml MIP-α (CCL3), 0.1–10 ng/ml INF-γ, or 1–100 ng/ml RANTES (CCL5) failed to induce COX-2 or MMP-9 (data not shown). Taken together, these data indicate that MMP-1 and MMP-3 stimulate the release of TNF-α, which induces macrophage COX-2-dependent MMP-9 expression.
FIGURE 8. TNF-α silencing blocks MMP-induced MMP-9 expression. RAW264.7 macrophages transfected with nonspecific (NS) or TNF-α siRNA were suspended in DMEM-0.1% LE-BSA and aliquoted into a 24-well plate. Following adherence, cells received media alone (Ctrl) or media containing 50 nM MMP-1, MMP-3, or LPS (10 ng/ml) and were incubated overnight. Additionally, cells transfected with TNF-α siRNA were incubated with MMP-1, MMP-3, or LPS in the presence of exogenous TNF-α (50 ng/ml). Levels of MMP-9 in conditioned media and COX-2 and GAPDH in lysates were determined by Western blot. Levels of TNF-α mRNA were determined utilizing RT-PCR. The data are representative of two separate experiments.

Discussion

Genetic evidence indicates that MMP-9 participates in the recruitment of stem cells and tissue revascularization, skeletal growth plate angiogenesis and ossification, choroidal neovascularization following injury, tumor angiogenesis, dermal-epidermal separation in bullous pemphigoid, resolution of allergic inflammation, degradation of fibrin, ventricular enlargement and collagen accumulation following myocardial infarction, and the pathogenesis of occlusive and aneurysmal vascular diseases (2, 33, 34). The activity of this pleiotropic proteinase is regulated at three levels: gene expression and pro-enzyme activation and inhibition (1, 2). The proteolytic activation of pro-MMP-9 is currently understood to take place in the fluid phase and is catalyzed by MMP-3 (6, 7, 35). However, the ability of MMP-3 and/or other MMP family members to regulate MMP-9 expression has not been explored. Results of experiments reported herein define a complex mechanism by which extracellular MMP-9 and/or other MMP family members to regulate MMP-9 expression: role of TNF-α and COX-2. Our data suggest that active MMP-1 and MMP-3 trigger the release TNF-α, which induces COX-2 expression and PGE2 secretion. PGE2 binding to EP4 stimulates MAPKerk1/2-dependent increase in MMP-9 expression.

The release of TNF-α is a well-defined mechanism by which proteinases regulate cell function. Pro-TNF-α, tethered to the cell membrane by its NH2-terminal domain, is efficiently processed to its mature form and released by membrane-associated TNF-α converting enzyme (i.e., TACE) (50, 51), which is a member of the ADAMs (a disintegrin and metalloproteinase) family of metalloproteinases (i.e., ADAM17) (52). In addition to TACE/ADAM17, several members of the MMP family are reported to process pro-TNF-α, although less efficiently (23, 24). Studies reported herein confirm the observation that MMP family members can process pro-TNF-α. We demonstrate that the levels of TNF-α in media recovered from macrophages incubated with active MMP-1 or MMP-3 were elevated as compared with control cells. The observed release of TNF-α provides a mechanism whereby these proteinases induce COX-2-dependent MMP-9 expression. We and others have reported that macrophage COX-2 (5, 30) and MMP-9 expression (5, 31) are stimulated by TNF-α, and knockdown of COX-2 expression blocks TNF-α-induced MMP-9 expression (5). Moreover, as reported herein, the ability of conditioned media recovered from macrophages incubated with MMP-1 or MMP-3 to induce COX-2 and MMP-9 was blocked by neutralizing anti-TNF-α IgG. Furthermore, proteinases failed to induce either COX-2 or MMP-9 in macrophages in which TNF-α expression was knocked down utilizing siRNA. Thus, MMP-1 and MMP-3-induced COX-2 and MMP-9 expression by macrophages is dependent on the release of TNF-α (Fig. 9).

MMP family members are potent proinflammatory and immunoregulatory molecules that are implicated in the pathogenesis of chronic inflammatory diseases and cancer (2, 53, 54). Results of experiments reported here define a complex mechanism by which collagenase-1 and stromelysin-1, commonly found in both inflamed and neoplastic tissues, stimulate macrophage MMP-9 expression via the liberation of TNF-α and subsequent modulation of the COX-2→PGE2→EP4 receptor axis (Fig. 9). The ability of MMPs to induce COX-2-dependent MMP-9 expression by macrophages defines a nexus between MMPs and prostanoids that is...
likely to play a role in the pathogenesis of chronic inflammatory diseases and cancer. These data also suggest that this nexus is targetable utilizing anti-TNF-α therapies and/or selective EP4 antagonists.

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Disclosures
The authors have no financial conflicts of interest.

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