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Membrane-Bound Matrix Metalloproteinase-8 on Activated Polymorphonuclear Cells Is a Potent, Tissue Inhibitor of Metalloproteinase-Resistant Collagenase and Serpinase[1]

Caroline A. Owen,[2]* Zhuma Hu,† Carlos Lopez-Otin,‡ and Steven D. Shapiro*

Little is known about the cell biology or the biologic roles of polymorphonuclear cell (PMN)-derived matrix metalloproteinase-8 (MMP-8). When activated with proinflammatory mediators, human PMN release only ~15–20% of their content of MMP-8 (~60 ng/10^6 cells) exclusively as latent pro-MMP-8. However, activated PMN incubated on type I collagen are associated with pericellular collagenase activity even when bathed in serum. PMN pericellular collagenase activity is attributable to membrane-bound MMP-8 because: 1) MMP-8 is expressed in an inducible manner in both pro- and active forms on the surface of human PMN; 2) studies of activated PMN from mice genetically deficient in MMP-8 (MMP-$8^{-/-}$) vs wild-type (WT) mice show that membrane-bound MMP-8 accounts for 92% of the MMP-mediated, PMN surface type I collagenase activity; and 3) human membrane-bound MMP-8 on PMN cleaves types I and II collagens, and α_1-proteinase inhibitor, but is substantially resistant to inhibition by tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2. Binding of MMP-8 to the PMN surface promotes its stability because soluble MMP-8 has $t_{1/2}$ = 7.5 h at 37°C, but membrane-bound MMP-8 retains >80% of its activity after incubation at 37°C for 18 h. Studies of MMP-$8^{-/-}$ vs WT mice given intratracheal LPS demonstrate that 24 h after intratracheal LPS, MMP-$8^{-/-}$ mice have 2-fold greater accumulation of PMN in the alveolar space than WT mice. Thus, MMP-8 has an unexpected, anti-inflammatory role during acute lung injury in mice. TIMP-resistant, active MMP-8 expressed on the surface of activated PMN is likely to be an important form of MMP-8, regulating lung inflammation and collagen turnover in vivo. ***The Journal of Immunology,*** **2004, 172: 7791–7803.**

Matrix metalloproteinase-8 (MMP-8;[3] collagenase-2, EC 3.4.24.34) along with MMP-1 and MMP-13 are the major members of the interstitial collagenase subgroup of the MMP family of zinc-dependent, neutral proteinases. Interstitial collagens (types I-III) are major structural components of the extracellular matrix (ECM). They are composed of three polypeptide chains arranged in a rigid triple helix conformation, rendering them resistant to degradation by proteinases other than the interstitial collagenases. Interstitial collagenases mediate the initial and rate-limiting step in interstitial collagen degradation by cleaving the three collagen polypeptide chains at a single locus three-fourths of the distance of the collagen molecule from its N-terminal end (1). The three-fourth and one-fourth fragments generated denature spontaneously, and the denatured collagen fragments (gelatins) are susceptible to further cleavage by gelatinases (MMP-2 and MMP-9) and to a lesser extent by other MMPs (including MMP-8) and serine proteinases (2). Although MMP-8 cleaves all three interstitial collagens, it differs from MMP-1 in that it cleaves type I collagen at a higher rate than type III collagen. MMP-8 also degrades type II collagen, the predominant collagen of cartilage, at a higher rate than MMP-1 (3). MMP-8 can also cleave nonmatrix proteins such as serpins, bradykinin, angiotensin I, and substance P (4, 5).

The biologic roles of MMP-8 in vivo are currently uncertain. As a result of its known catalytic activities, MMP-8 is believed to be involved in wound healing and tissue remodeling during inflammation. In addition, MMP-8 has been implicated in the pathogenesis of several chronic inflammatory diseases characterized by excessive influx and activation of polymorphonuclear cell (PMN), including cystic fibrosis (6), rheumatoid arthritis (7), periodontal disease (8), and chronic skin wounds (9). Recent data from Balbin et al. (10) demonstrate that MMP-8 has an unexpected role in vivo in protecting male mice from the development of skin tumors in a chemical carcinogenesis model. This study showed that mice deficient in MMP-8 (MMP-$8^{-/-}$ mice) have important abnormalities in their inflammatory response to chemical carcinogens. The lack of MMP-8 hampers the early PMN recruitment in response to chemical carcinogens, due in part to the loss of MMP-8-mediated cleavage and activation of LPS-induced CXC chemokine. However, once inflammation is established in MMP-$8^{-/-}$ mice, it is abnormally sustained (by unknown mechanisms), creating a more favorable environment for tumor initiation. Moreover, bone marrow transplantation experiments confirmed that PMN were the source of MMP-8 promoting protection against tumor development in this model.

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[3] Abbreviations used in this paper: MMP, matrix metalloproteinase; α_1-PI, α_1-proteinase inhibitor; APMA, 4-aminophenylmercuric acetate; BAL, bronchoalveolar lavage; ECM, extracellular matrix; HLE, human leukocyte elastase; IT, intratracheal; McaPLGLDpaAR, (7-methoxycoumarin-4-yl)-acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L2,3-diamino-propionyl)-Ala-Arg-NH_2; MPO, myeloperoxidase; PAF, platelet-activating factor; PMN, polymorphonuclear cell; TIMP, tissue inhibitor of metalloproteinase; WBC, white blood cell; WT, wild type.

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PMN are the main source of MMP-8 in humans. However, MMP-8 is also expressed at lower levels by chondrocytes (11), rheumatoid synovial fibroblasts (12), activated macrophages, smooth muscle cells, and endothelial cells (13). PMN-derived MMP-8 differs from interstitial collagenases expressed by other cells in that it is not synthesized de novo by mature PMN. Rather, MMP-8 is expressed during the myelocyte stage of development of PMN precursors in the bone marrow (14), and it is stored as a latent enzyme (pro-MMP-8, M_r 85 kDa) within the specific granules of PMN (15). Pro-MMP-8 is rapidly released from activated PMN undergoing degranulation (16, 17), and is then activated via the cysteine switch mechanism to yield the active form of the enzyme (M_r ~65 kDa (18)). Activation can be achieved in vitro by organomercurials (19), serine proteinases (20, 21), MMP-3 (22), and reactive oxygen species (18). However, it is not clear how pro-MMP-8 is activated or how it retains its activity in vivo, because tissues contain highly effective inhibitors of MMP-8, including tissue inhibitors of MMP (TIMPs).

We have shown that activated PMN are associated with striking pericellular type collagenase activity even when incubated in the presence of TIMPs. In addition, MMP-8 is expressed on the cell surface of activated PMN, and this form of the proteinase can contribute to potent, TIMP-resistant pericellular collagenase and serpinase activity. The binding of MMP-8 to the PMN surface promotes its stability and preserves its activity in the extracellular space in the presence of TIMPs. Surprisingly, our studies of MMP-$8^{-/-}$ mice in a model of acute lung injury demonstrate that MMP-8 has an anti-inflammatory role in the lung. Our data indicate that the TIMP-resistant form of MMP-8 activity expressed on the surface of human PMN is likely to contribute in important ways to its anti-inflammatory and interstitial collagen-degrading activities in the lung and other tissues in vivo.

Materials and Methods

Materials

Goat anti-rabbit F(ab′)$_2$-Alexa Fluor 546 and 488, goat anti-murine F(ab′)$_2$-Alexa Fluor 546, and phalloidin-Alexa 546 were obtained from Molecular Probes (Eugene, OR). Human pro-MMP-8, human TIMP-1, human TIMP-2, polyclonal rabbit anti-human MMP-8 IgG (AB8115), murine anti-human MMP-9, and the kit for measuring type II collagenase activity were purchased from Chemicon International (Temecula, CA). FITC-conjugated type I collagen was obtained from Elastin Products (Owensville, MO). Purified myeloperoxidase (MPO) was obtained from Athens Research (Athens, GA). Quenched FITC-conjugated gelatin and type I collagen were obtained from Molecular Probes. The (7-methoxycoumarin-4-yl)-acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L2,3-diaminopropionyl)-Ala-Arg-NH_2 (McaPLGLDpaAR) was purchased from Calbiochem Novabiochem (San Diego, CA). RS113456 and RS104210 were generously provided by Roche Bioscience (Palo Alto, CA). MMP-8 ELISA kits were purchased from R&D Systems (Minneapolis, MN). BD Biocoat Matrigel Invasion Chambers were obtained from BD Labware (Bedford, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Quantitation of MMP-8 in PMN lysates and culture supernatants

Extracts of human PMN were prepared at 5×10^6/ml in PBS containing 0.1% (v/v) Triton X-100. Cell extracts and cell-free supernatants from unstimulated and activated PMN were assayed for total human MMP-8 using a commercially available immunoassay (R&D Systems, Minneapolis, MN). This ELISA kit measures both pro- and active MMP-8, but does not detect purified human MMP-9 or human leukocyte elastase.

Human PMN isolation and activation

Human PMN (>95% pure) were isolated from peripheral blood of healthy donors (23). Human PMN were suspended at 10^7/ml in HBSS containing 1 mM Ca^{2+}, 1 mM Mg^{2+}, and 10 mM HEPES (pH 7.4), then incubated for 30 min at 37°C with or without platelet-activating factor (PAF) (10^{-6} to 10^{-10} M), TNF-α (1–1000 U/ml), LPS from *Escherichia coli* 0111:B4 (1–1000 ng/ml), fMLP (10^{-6} to 10^{-12} M), or PMA (100 ng/ml). PMN were also incubated at 37°C for 15 min with optimal concentrations of LPS (100 ng/ml), PAF (10^{-8} M), TNF-α (100 U/ml), or cytochalasin B (5 μg/ml), then optimally activated with fMLP (10^{-8} M for 30 min). To terminate assays, cells and supernatant samples were separated following centrifugation ($300 \times g$ for 3 min). For immunostaining experiments, PMN were fixed for 3 min at 4°C in PBS (pH 7.4) containing 3% paraformaldehyde and 0.5% glutaraldehyde (24).

Type I collagenase activity associated with activated PMN

LabTec chamber slides (Nunc, Naperville, IL) were coated with FITC-conjugated type I collagen (1 mg/ml) for 2 h at 37°C. HBSS, autologous serum, or autologous serum containing 20 μM RS104210, or 20 μM 4-(2-aminoethyl)benzylsulfonyl fluoride, or 20 μM leupeptin was added to the wells (300 μl/well), followed by human PMN (2×10^5 cells in 20 μl of HBSS). The cells were activated with PAF (10^{-8} M) for 5 min; then 10^{-8} M fMLP was added and the chambers were incubated at 37°C for 3 h in a humidified atmosphere of 5% CO_2. Cells adherent to the slides were fixed for 3 min at 4°C using PBS containing 3% (w/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde. The chamber slides were dismantled, and the slides were mounted in PBS containing 25% (v/v) glycerol and 250 μg/ml *p*-phenylenediamine, and then examined by bright field and incident light epifluorescence microscopy. Images of the cells were captured using a chilled charge-coupled device camera and MetaMorph software (Universal Imaging, West Chester, PA).

Isolation of PMN undergoing directed migration

We used BD Biocoat Matrigel Invasion Chambers (a modified Boyden chamber assay, with upper and lower chambers separated by an 8-μm microporous, polyethylene terephthalate membrane coated with a uniform layer of Matrigel Matrix, a prototype basement membrane (25)). The lower chambers were filled with medium alone (RPMI 1640 containing 10 mM HEPES and 1% human serum albumin, pH 7.4), or medium containing 10^{-7} M fMLP. Human PMN (8×10^6 in 2 ml of medium) were placed in the upper chambers, and the chambers were incubated for 3 h at 37°C in a humidified atmosphere of 5% CO_2. PMN that migrated through the membrane were immunostained for cell surface-bound MMP-8.

Immunofluorescence staining of human PMN for MMP-8, quantitative image analysis, and confocal microscopy

PMN were incubated at 4°C with polyclonal rabbit anti-human MMP-8 (AB8115) or rabbit IgG, as a control (both at 1 μg/10^6 cells), followed by goat anti-rabbit IgG conjugated to Alexa 546. AB8115 was raised against a peptide sequence in the hinge region of MMP-8. Western blot analysis confirmed that AB8115 recognizes both pro-MMP-8 and active MMP-8, but has no cross-reactivity for purified MMPs-9, -12, -1, or -2; human leukocyte elastase; cathepsin G; or proteinase 3. PMN were then washed twice in HBSS, incubated for 2 h at 4°C with goat anti-rabbit F(ab′)$_2$ Alexa Fluor 546, and then washed twice in HBSS. The PMN were examined by incident light fluorescence microscopy. Cell surface immunofluorescence was quantified using MetaMorph image analysis software, and the data were corrected for nonspecific staining, as described previously (24). Briefly, MetaMorph software was used to quantify the integrated fluorescence of 150–200 cells in each group in arbitrary fluorescence units. To correct for nonspecific staining, for each condition, the mean fluorescence intensity of cells stained with the nonimmune control Ab was subtracted from each of the integrated fluorescence values for cells incubated with the anti-MMP-8 Ab. The results for cell surface MMP-8 expressed by activated PMN were expressed as a percentage of cell surface MMP-8 expressed by unstimulated PMN.

Cell surface localization of MMP-8 was confirmed by analyzing cells double immunostained with: 1) rabbit anti-MMP-8 (AB8115) and goat anti-rabbit IgG conjugated to Alexa 488; 2) murine anti-human MMP-9 and goat anti-murine IgG conjugated to Alexa 546. Cells were analyzed with a Leica TCSNT confocal laser-scanning microscope (Leica, Exton, PA) fitted with air-cooled argon and krypton lasers. Fluorescent confocal micrographs were recorded under dual fluorescent imaging mode in which cells were simultaneously exposed to 488 and 568 nm light attenuated by an acusto tunable optical filter. A band pass (530 ± 30 nm) filter was used to select light emitted from the Alexa 488-labeled MMP-8, and a long-pass 590-nm filter was used to detect the Alexa 546-labeled MMP-9.

Activated human PMN were also immunostained with Alexa 488 for surface MMP-8 using AB8115 as the primary Ab, then fixed, as described above. Cells were then permeabilized using 250 μg/ml lysophosphatidyl choline in PBS for 30 min at 4°C. The cells were then immunostained for intracellular F-actin using phalloidin conjugated to Alexa 546, and them

examined by incident light fluorescence microscopy, as described previously (27).

Immunoblot analysis of MMP-8 in PMN plasma membranes

Peripheral blood PMN (6×10^8) were optimally activated (primed for 15 min with 10^{-8} M PAF, then activated for 30 min with 10^{-8} M fMLP). Viable cells were subjected to nitrogen cavitation and Percoll gradient centrifugation to separate the granule-free plasma membrane fraction (γ band), the azurophil granule faction (α band), and the specific granule fraction (β bands), and then the purity and total protein content of the plasma membrane fraction were quantified, as described previously (28). Aliquots of the plasma membrane fraction and the specific granule faction were solubilized by incubation with 0.05% Brij35 for 1 h at 4°C, followed by centrifugation at $50,000 \times g$ for 15 min. Plasma membrane samples (25 μg of total protein), along with purified, 4-aminophenylmercuric acetate (APMA)-activated MMP-8, solubilized specific granules, and cell-free supernatant samples from activated PMN, were subjected to 12% SDS-PAGE. The proteins were transferred to Immuno-Blot polyvinylidene difluoride membrane (100 V for 90 min in 12.5 mM Tris buffer (pH 8.3) containing 96 mM glycine and 20% (v/v) methanol), and the membranes were blocked with 3% nonfat milk in PBS containing 0.1% Tween 20, then probed with polyclonal rabbit anti-human MMP-8 (AB8115). Binding of Ab was detected with HRP-coupled goat anti-rabbit secondary Ab and an Opti-4CN chromogenic detection system (Bio-Rad, Hercules, CA). The intensities of the bands generated were quantified using Scion Image β 4.02 (Scion, Frederick, MD).

Pro-MMP-8 activation

Purified human pro-MMP-8 (1 μM) was incubated at 37°C for 2–4 h with 2 mM APMA. Complete activation of pro-MMP-8 was confirmed by analysis of samples on 15% SDS-PAGE. Activated MMP-8 was active site titrated using TIMP-2 and McaPLGLDpaAR as the substrate.

Murine PMN isolation and activation

Mice genetically deficient in MMP-8 (MMP-$8^{-/-}$) were generated by replacing exons 2, 3, and 4 with a phosphoglycerate kinase-neomycin fusion gene and homologous recombination (10). PMN (>85% pure) were isolated from the bone marrow of MMP-$8^{-/-}$ or wild-type (WT) mice in the same genetic background (mixed 129/SvEv × C57BL/6) by positive selection for Ly-6-G using immunomagnetic beads (29). Extracts of murine PMN were prepared in PBS containing 0.1% (v/v) Triton X-100, and analyzed for MMP-8 × 12% SDS-PAGE, followed by Western blot analysis using AB8115 as the primary Ab. Murine PMN were also activated at 37°C for 15 min with PAF (10^{-7} M), followed by fMLP (10^{-7} M for 30 min), then fixed, and immunostained for cell surface MMP-8 using AB8115 or rabbit IgG as the primary Abs, followed by goat anti-rabbit conjugated to Alexa 488. Cell surface MMP-8 expression on murine PMN was quantified using MetaMorph, as described above.

Catalytic activity of MMP-8 on the surface of murine PMN

PMN from MMP-$8^{-/-}$ and WT mice were activated and fixed (as described above), washed, and resuspended in Tris assay buffer (0.05 M Tris containing 0.15 M NaCl and 0.02 M $CaCl_2$, pH 7.4). Cells from both genotypes (10^6 cells/assay) were incubated in triplicate with and without 1,10-phenanthroline (1 mM, a synthetic inhibitor of MMPs) at 37°C for varying times in the presence of 1.8 μM McaPLGLDpaAR. Total cell surface-bound MMP activity was quantified as the 1,10-phenanthroline-inhibitable, McaPLGLDpaAR-hydrolyzing activity in cell-free supernatant samples by fluorometry (F2500 fluorescence spectrophotometer; Hitachi, Tokyo, Japan; λ_{ex} 328 nm, λ_{em} 393 nm). To quantify PMN cell surface gelatinase and type I collagenase activities, activated PMN from MMP-$8^{-/-}$ and WT mice were preincubated in triplicate for 30 min at 37°C with 1 mM PMSF (to inactivate cell surface serine proteinases having gelatinase activity (2)) with and without 1 mM 1,10-phenanthroline. Cells were incubated at 37°C for 3 h with 50 μg/ml quenched FITC-conjugated gelatin (3×10^6 cells/assay) or for 18 h with 50 μg/ml quenched FITC-conjugated type I collagen (4×10^6 cells/assay), and MMP-mediated gelatinase and type I collagenase activities were quantified as the 1,10-phenanthroline-inhibitable cleavage of substrates in supernatant fluids by fluorometry (λ_{ex} 490 nm, λ_{em} 520 nm).

Cell surface type I collagenase activity on activated human PMN

PMN were activated for 15 min with 10^{-8} M PAF for 15 min, then for 30 min with 10^{-8} M fMLP, and then fixed to prevent release of intracellular proteinases (29, 30). PMN were then incubated with 20 μg of bovine type I collagen with or without 2×10^7 PMN at 37°C for up to 20 h, and then reduced cell-free supernatant samples were analyzed using 12% SDS-PAGE and Coomassie brilliant blue G250 staining.

Catalytic activity of human membrane-bound MMP-8 on PMN

Activated human PMN express other proteinases on their surface that can cleave MMP-8 substrates other than type I collagen (24, 29, 31, 32). To study the activity of human membrane-bound MMP-8 against MMP-8 substrates other than interstitial collagens, we studied exogenous human MMP-8 bound to the surface of unstimulated PMN. This strategy permits us to load MMP-8 onto the PMN surface, and to quantify the amount bound for studies of its catalytic activity and efficiency. Proteinases that are endogenously expressed at low levels on the surface of unstimulated PMN were first inactivated (29, 31). PMN were then incubated at 4°C with activated, soluble MMP-8 (1 μg/10^6 cells), then fixed (using PBS containing 3% paraformaldehyde and 0.5% glutaraldehyde (pH 7.4) for 3 min at 4°C) to prevent release of proteinases from the cells during subsequent activity assays (29, 31). Cell surface-bound MMP-8 was quantified by incubating the following in Tris assay buffer at 37°C for 30 min with 1.8 μM McaPLGLDpaAR: 1) MMP-8 bound to PMN (2×10^6 cells/assay); 2) control PMN (which had no MMP-8 bound to their surface; 2×10^6 cells/assay); and 3) assay standards of soluble, active site-titrated, APMA-activated MMP-8 (3–400 ng/assay). Cleavage of the substrate was quantified in cell-free supernatant fluids by fluorometry. The amount of MMP-8 bound to the surface of PMN was calculated by interpolation from the standard curve for soluble, active MMP-8, and expressed as ng MMP-8 activity bound per 10^6 cells.

k_{cat}/K_M determinations

Hydrolysis of 1.5 μM McaPLGLDpaAR by soluble MMP-8 or exogenous MMP-8 bound to PMN (both at 31–500 pM in 250 μl of Tris assay buffer) was monitored over 6 min at 25°C as the increase in fluorescence (λ_{ex} 328 nm, λ_{em} 393 nm (33)). A substrate concentration of 1.5 μM fulfills the conditions of $[S] \ll K_M$ to permit the direct determination of k_{cat}/K_M under first order conditions.

Cleavage of interstitial collagens and serpins by membrane-bound MMP-8

Bovine type I collagen (20 μg) was incubated at 37°C for 4–20 h with or without: 1) human MMP-8 bound to PMN (2.5×10^7 cells); 2) human MMP-8 bound to PMN (2.5×10^7 cells) that had been preincubated with 10 μM RS113456 (a general, hydroxamate inhibitor of MMPs); or 3) 500 ng of soluble, APMA-activated MMP-8. Cell-free supernatant samples were reduced and analyzed by 12% SDS-PAGE and Coomassie brilliant blue G250 staining. A commercial kit was used to assess whether human membrane-bound MMP-8 has type II collagenase activity. Human MMP-8 bound to PMN (2×10^6 cells), control PMN (2×10^6 cells), and assay standards of soluble, active site-titrated MMP-8 (25–800 ng) were incubated for 3 h at 42°C with FITC-conjugated type II collagen (100 μg) in Tris buffer. Cell-free supernatant samples were assayed for type II collagenase activity by fluorometry (λ_{ex} 490 nm, λ_{em} 520 nm), as recommended by the kit manufacturer. The type II collagenase activity expressed by membrane-bound MMP-8 and control PMN was converted from arbitrary fluorescence units to ng of soluble MMP-8 activity per 10^6 PMN by interpolation from the standard curve generated for soluble, active MMP-8.

MMP-8 bound to PMN (8×10^6 cells), control PMN (8×10^6 PMN), or soluble MMP-8 (125 ng) were also incubated for 8 h at 37°C with 6 μg of purified α_1-proteinase inhibitor (α_1-PI) in Tris assay buffer. Reduced cell-free supernatant samples were analyzed by 15% SDS-PAGE and Coomassie brilliant blue G250 staining. To assess whether the catalytic activity associated with MMP-8 bound to PMN was due to release or leakage of proteinases from PMN, PMN that bound MMP-8 to their surface were also incubated in Tris buffer alone, and MMP activity was assayed in cell-free supernatant samples using McaPLGLDpaAR.

Effect of fixatives on the catalytic activity of membrane-bound MMP-8

PMN were fixed for 3 min at 4°C using PBS containing 3% paraformaldehyde and 0.5% glutaraldehyde (pH 7.4). Cells were then washed three times in 0.05 M Tris containing 0.15 M NaCl and 0.02 M $CaCl_2$ (pH 7.4). To assess the effects of fixatives on the catalytic activity of membrane-bound MMP-8, we incubated PMN at 4°C for 2 h with activated, soluble MMP-8 (1 μg/10^6 cells), washed the cells twice, and then divided the cells into two equal aliquots. One aliquot was fixed, as described above; the other was incubated without fixatives. Both groups were washed three

times in PBS, and then incubated in Tris assay buffer at 37°C for 30 min with 1.8 μM McaPLGLDpaAR. Cleavage of the substrate was quantified in cell-free supernatants in arbitrary fluorescence units by fluorometry, as described above. The results for fixed membrane-bound MMP-8 were expressed as a percentage of the activity associated with nonfixed, membrane-bound MMP-8.

Stability of soluble vs membrane-bound MMP-8

Pro-MMP-8 was activated with APMA; then an aliquot of active MMP-8 was bound to the surface of unstimulated PMN, and the amount bound to an aliquot of the cells was quantified, using McaPLGLDpaAR. Aliquots (50 ng) of the remaining soluble MMP-8 and membrane-bound MMP-8 were then incubated in duplicate for 30 min at 37°C in Tris assay buffer containing 1.8 μM McaPLGLDpaAR, and initial reaction velocities were quantified by fluorometry. Additional 50-ng aliquots of each form of the proteinases were incubated at 37°C in duplicate in Tris assay buffer without substrate for 2, 4, 6, and 18 h; then McaPLGLDpaAR was added, and reaction velocities were measured, as described above. The results were expressed as a percentage of the initial reaction velocity for each form of the proteinase.

Susceptibility of membrane-bound MMP-8 to inhibition

Soluble MMP-8 (10 nM) or MMP-8 bound to PMN (10 nM) were incubated at 37°C for 30 min in Tris buffer with and without: 1) 1 mM 1,10-phenanthroline; 2) 0.1 nM-1 μM RS113456; 3) 15.6 nM-1 μM TIMP-1; 4) 15.6 nM-1 μM TIMP-2; or 5) 1 mM PMSF. Membrane-bound MMP-8 was also preincubated for 30 min with 1 mM PMSF, then incubated for 30 min with 200 nM TIMP-1. Residual MMP-8 activity was quantified in cell-free supernatant samples using 1.8 μM McaPLGLDpaAR. IC_{50} values were determined by nonlinear regression analysis using SigmaStat (SPSS, Chicago, IL).

Studies of LPS-mediated acute lung injury in mice

Male MMP-8$^{-/-}$ and WT mice aged 10–16 wk, both in mixed SvEv129 × C57BL/6 genetic background, were anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg), then given 10 μg of LPS from *E. coli* 0111:B4 in 30 μl of endotoxin-free PBS, or 30 μl of PBS alone by the intratracheal (IT) route as a control. After 24 h, mice were euthanized by CO_2 narcosis, and then bronchoalveolar lavage (BAL) was performed using 8 × 0.5 ml of sterile PBS. The BAL cell and supernatant fractions were separated by centrifugation (500 × *g* for 3 min), then total and differential white blood cell (WBC) counts were performed on the BAL cell fraction. Extracts of the BAL cell fraction were prepared using PBS containing 0.1% Triton X-100. MPO activity was quantified in triplicate in BAL cell extracts, as described previously (34), using assay standards of purified MPO.

Statistics

Data are expressed as mean ± SEM or mean ± SD. The results for paired and unpaired data were compared using Student's *t* test for parametric data and the Mann-Whitney rank sum test for nonparametric data; *p* values <0.05 were considered significant.

Results

Quantitation of MMP-8 contained within and released by human PMN

Freshly isolated human peripheral blood PMN contain ~60 ng of MMP-8/10^6 cells (Table I). As expected, PMN freely release minimal amounts of MMP-8 when incubated without agonists. However, when PMN are optimally activated with proinflammatory stimuli, they freely release only 15–20% of their cellular content of MMP-8 as a soluble proteinase (Table I). When cells are activated with pharmacologic mediators (cytochalasin B and PMA), 50–70% of the PMN content of MMP-8 is freely released from PMN as a soluble proteinase. Previously, we detected no release of active forms of MMPs from PMN activated with biologic mediators using McaPLGLDpaAR, a sensitive, general MMP substrate (29). All of the MMP-8 released from PMN activated with biologic mediators is in the latent pro-MMP-8 form because Western blot analysis of supernatant samples from PAF- and fMLP-activated PMN detected a single band with M_r ~85 kDa (Fig. 4, *lane 4*). However, activated human PMN migrating upon fluorescent type I collagen are associated with striking pericellular collagenase activity (the dark areas on the fluorescent background indicated by the open arrows), even though autologous serum that contains μM concentrations of TIMPs (35) was used as the bathing medium (Fig. 1*B*). In the absence of cells, there was no degradation of the fluorescent collagen substrate (Fig. 1*A*). The type I collagenase activity associated with PMN was substantially abrogated by adding a hydroxamate MMP inhibitor to the serum (Fig. 1*C*), but addition of PMSF or leupeptin (inhibitors of serine and cysteine proteinases) to the serum had no effect (data not shown). Together, these data indicate that although activated PMN release minimal quantities of MMP-8 exclusively in the latent, proenzyme form, they express potent pericellular collagenase activity even in the presence of micromolar concentrations of TIMPs present in serum. Although this pericellular collagenase activity is TIMP resistant, it can be substantially inhibited by a low M_r MMP inhibitor.

Table I. *Quantitation of the cellular content and release of MMP-8 by human PMN*

Condition	MMP-8 (ng/10^6 PMN)
PMN total cell content[a]	59.9 ± 14.4[c]
No agonists[b]	3.0 ± 1.2
PAF + fMLP[b]	9.6 ± 1.3
TNF-α + fMLP[b]	11.2 ± 0.9
Cytochalasin B + fMLP[b]	32.1 ± 6.9
PMA[b]	42.6 ± 10.1

[a] Total cellular MMP-8 was quantified in cell extracts of freshly isolated PMN from healthy donors by ELISA ($n = 6$).

[b] PMN from the same donors were incubated at 37°C with and without agonists, as described in *Materials and Methods*, and MMP-8 was quantified in cell-free supernatant samples by ELISA.

[c] Data are mean ± SD.

Inducible expression of MMP-8 on the cell surface of PMN

We tested the hypothesis that PMN pericellular collagenase activity shown in Fig. 1*B* is mediated by MMP-8, which expressed on the PMN cell surface. To test this hypothesis, we immunostained nonpermeabilized PMN for surface-bound MMP-8. Epifluorescence microscopy of immunostained cells showed that unstimulated human PMN express minimal amounts of MMP-8 on their cell surface (Fig. 2*A*). Activated PMN have intense, focal cell surface staining for MMP-8 (Fig. 2*B*). Cells immunostained with a control primary Ab have no cell surface staining (Fig. 2*C*). Examination of the cells by confocal microscopy not only confirmed cell surface localization of MMP-8, but also demonstrated that MMP-8 is strikingly colocalized with MMP-9 (Fig. 2*D*), another enzyme that is expressed on the surface of activated PMN (29). In addition, MMP-8 and MMP-9 are often both localized to the leading edge of polarized PMN (arrows, Fig. 2*D*).

To assess the relationship between MMP-8 localized to the cell surface and intracellular events occurring during PMN activation, we immunostained activated PMN for membrane-bound MMP-8, then permeabilized the cells and stained them for intracellular F-actin (polymerized actin). MMP-8 was frequently localized to areas of the PMN plasma membrane that were adjacent to areas of cytoplasm containing F-actin (data not shown). The lack of complete colocalization of surface-bound MMP-8 and intracellular F-actin is likely to be due to the different time courses for actin polymerization and degranulation in PMN; actin polymerization is rapid, reversible, and precedes PMN degranulation (36).

We also quantified the effects of various agonists on PMN surface expression of MMP-8 using immunofluorescence staining and

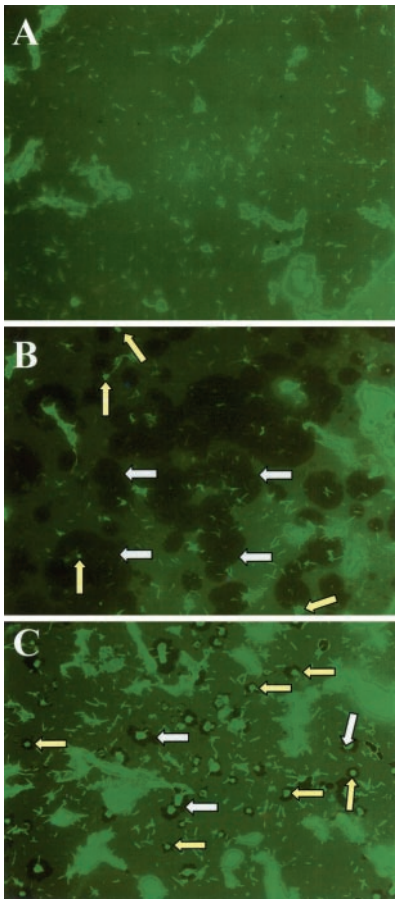

FIGURE 1. PMN are associated with pericellular collagenase activity even in the presence of serum. Chamber slides were coated with FITC-conjugated type I collagen, and either autologous serum (*A* and *B*) or autologous serum containing 20 μM RS104210, a low m.w., synthetic, general MMP inhibitor (*C*), was added as the bathing medium. The same number of PMN was added to *B* and *C*, but not to *A*. The cells were activated for 5 min with PAF (10^{-7} M); then 10^{-7} M fMLP was added and the chamber slides were incubated at 37°C for 3 h. The cells were then examined by incident light fluorescence microscopy. Areas of pericellular collagen degradation generated by cells as they migrate upon the collagen substrate are the dark areas on the background fluorescent collagen substrate (open arrows). The yellow arrows indicate cells remaining adherent to the substrate that are visible because they autofluoresce and/or ingest fluorescent substrate. Note in *B*, that as the substrate undergoes extensive pericellular degradation, cells detach from the chamber slides, and fewer cells remain adherent at the end of the assay than in *C*, in which proteolysis of type I collagen is markedly reduced by the addition of RS104210 to the serum. Magnification: ×200.

quantitative image analysis, as described in *Materials and Methods*. Activation of PMN with LPS, TNF-α, PAF, and fMLP induced concentration-dependent increases in cell surface expression of MMP-8, which were modest (2- to 4-fold), but statistically significant (Fig. 3, *A–D*). These agonists also induced additive (up to 6-fold) increases in cell surface MMP-8 expression on PMN when compared with cells incubated with agonist alone (Fig. 3*E*). The effects of agonists were also time dependent (data not shown). The optimal incubation time for activation of the cells with fMLP was 30 min, which resulted in 3- to 4-fold increases in cell surface MMP-8 expression (Fig. 3*D*). After longer incubation times with fMLP, cell surface MMP-8 expression decreased (data not shown), but was still modestly (but statistically significantly) greater than that on unstimulated PMN 3 h after the addition of fMLP (Fig. 3*F*, □).

To assess whether MMP-8 is expressed on the PMN cell surface during physiologic processes, we quantified MMP-8 expression on the cell surface of PMN undergoing directed migration. PMN migrating through Matrigel Invasion Chambers for 3 h in response to a gradient of fMLP expressed ~5-fold more cell surface MMP-8 than cells undergoing random migration in the absence of fMLP (Fig. 3*F*, ■), and >3-fold more cell surface MMP-8 than PMN incubated for 3 h in suspension with the same concentration of fMLP (Fig. 3*F*, □). These data indicate that cell surface expression of MMP-8 is up-regulated in a persistent manner on PMN undergoing directed migration in vitro.

Forms of MMP-8 expressed in plasma membranes of activated PMN

To compare intracellular and the two extracellular forms of PMN-derived MMP-8, we performed Western blot analysis for MMP-8 in cell-free supernatants from stimulated PMN, and in PMN plasma membranes and PMN-specific granules isolated by subcellular fractionation of PMN (Fig. 4). In PMN-specific granules, pro-MMP-8 is a major form of MMP-8 (*lane 2*). The specific granules of PMN contain additional forms of MMP-8 having M_r ~110, ~40, and ~30 kDa, as reported previously (15, 16). MMP-8, which is freely released from stimulated PMN as a soluble proteinase, is exclusively in the 85-kDa proenzyme form (*lane 4*). PMN plasma membranes contain three major forms of MMP-8 that differ in M_r (*lane 3*), including forms having the same M_r as pro-MMP-8 (85 kDa) and active MMP-8 (65 kDa), and a 30-kDa form, each representing 23, 24, and 30% of the total amount of MMP-8 present in PMN plasma membranes, respectively. Three additional minor forms of MMP-8 are present in PMN plasma membranes having M_r 110, 80, and 46 kDa and representing 8, 10, and 5% of the total membrane-associated MMP-8, respectively. The 46- and 30-kDa forms are likely to be proteolytically processed, inactive forms of MMP-8 because soluble MMP-8 undergoes proteolytic processing, generating forms with lower M_r that have lost catalytic activity (37).

Activated human PMN express cell surface collagenase activity

To assess whether membrane-bound MMP-8 on PMN is catalytically active, we tested whether human PMN that are optimally activated to induce surface localization of MMP-8 express surface-bound type I collagenase activity, because MMP-8 is the only PMN proteinase that degrades type I collagen (2). Activated PMN express surface-bound proteinase activity that generates the three-fourth (Fig. 5*A*, *lane 3*) and one-fourth (Fig. 5*B*, *lane 3*) fragments typical of interstitial collagenases within 12 h. After 20 h, there was complete degradation of intact type I collagen, as well as substantial degradation of the gelatins generated (Fig. 5*A*, *lane 4*). Thus, activated human PMN express interstitial collagenase (and gelatinase) activity on their cell surface.

Catalytic activity of membrane-bound MMP-8 on PMN

Our goal was to determine whether MMP-8 localized on the surface of PMN is catalytically active, and to assess whether it has similar substrate specificity and catalytic efficiency as soluble MMP-8. However, it is not possible to study MMP-8, which is endogenously expressed on the surface of human PMN to assess its catalytic activity and efficiency against substrates other than interstitial collagens because: 1) activated PMN express other proteinases on their surface that cleave MMP-8 substrates other than interstitial collagen (24, 29, 31, 32); and 2) currently, there are no inhibitors available that are specific for MMP-8. We therefore used two complementary strategies to assess the catalytic activity and

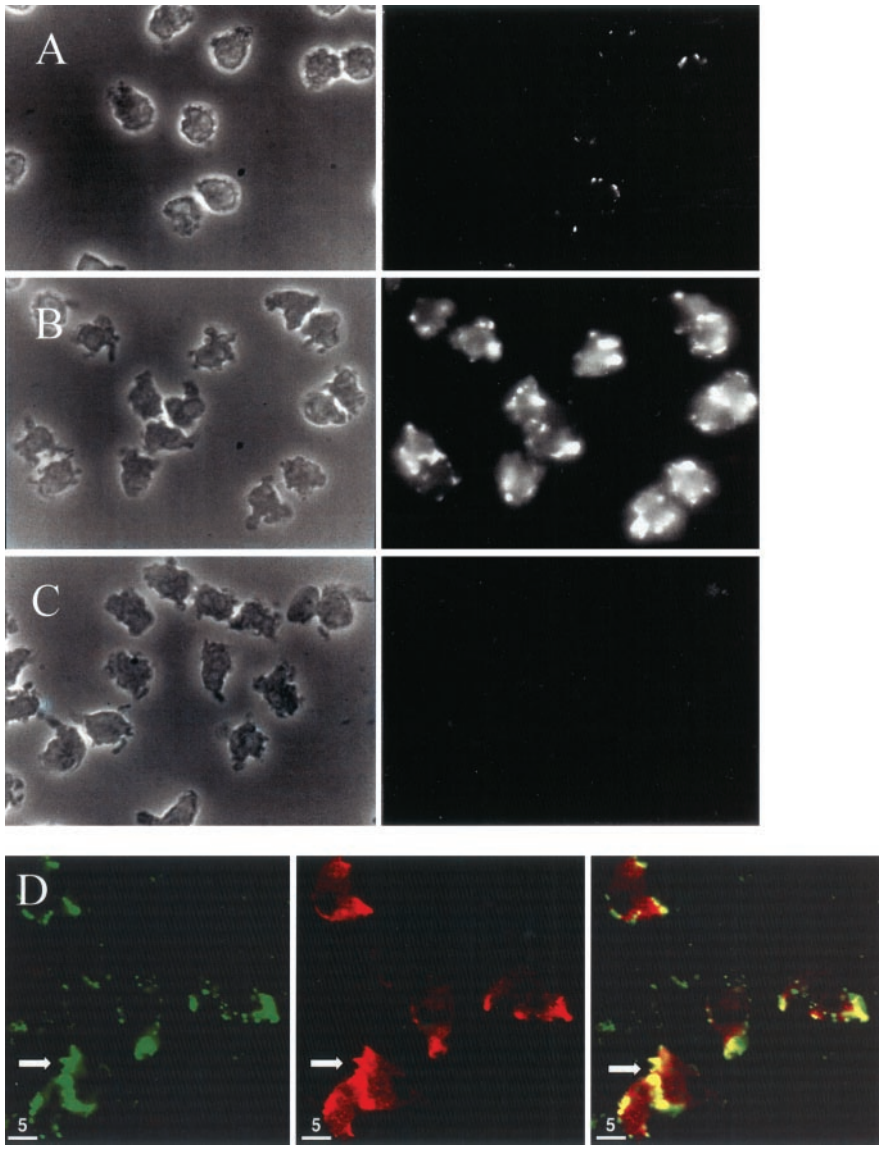

FIGURE 2. Cell surface expression of MMP-8 on unstimulated and activated PMN. PMN were incubated in the absence of agonists (*A*), or primed for 15 min with 10^{-8} M PAF, then activated for 30 min with 10^{-8} M fMLP (*B–D*). Cells were fixed, then immunostained with anti-MMP-8 (*A*, *B*, and *D*), or with rabbit IgG (*C*), as a control. Representative microscopic fields were examined both by phase-contrast microscopy (*A–C*, *left panels*) and by incident light fluorescence microscopy (*A–C*, *right panels*). *D*, To confirm cell surface localization of MMP-8, nonpermeabilized, activated PMN were also double immunostained with Alexa 488 for surface-bound MMP-8 (*D*, *left panel*) and with Alexa 546 for surface-bound MMP-9 (*D*, *middle panel*), and then examined by confocal microscopy (*D*). Dual excitation of the fluorophores (*D*, *right panel*) demonstrates substantial colocalization of both enzymes on the PMN cell surface (yellow overlay). Arrow indicates MMP-8 and MMP-9 colocalized on the leading edge of a polarized PMN. Magnification: ×1000.

efficiency of membrane-bound MMP-8 on PMN: 1) a loss-of-function strategy in which we compared the cell surface proteolytic activities expressed by activated PMN from MMP-$8^{-/-}$ vs WT mice against potential MMP-8 substrates; and 2) a model cell system in which we studied the activities of exogenous human MMP-8 bound to the surface of unstimulated human PMN.

Loss-of-function studies

We first confirmed that PMN from MMP-$8^{-/-}$ mice do not contain MMP-8, by Western blot analysis (Fig. 6*A*), and that activated PMN from WT mice express MMP-8 in an inducible manner on their cell surface (Fig. 6*B*). We then compared the capacity of cells from both genotypes to hydrolyze MMP-8 substrates. PMN from MMP-$8^{-/-}$ mice expressed ~58, 100, and 8% of the surface-bound, MMP-mediated activity against McaPLGLDpaAR (a general, synthetic MMP substrate), gelatin, and type I collagen, respectively (Fig. 6*C*). These data indicate that MMP-8 is present on the surface of activated murine PMN in a catalytically active form. In addition, membrane-bound MMP-8 on murine PMN accounts for ~40% of the total cell surface MMP activity, ~90% of the cell surface type I collagenase activity on activated murine PMN, but none of the PMN surface gelatinase activity.

Catalytic activity, efficiency, and stability of exogenous human membrane-bound MMP-8

To assess the catalytic activity, efficiency, and stability of human membrane-bound MMP-8 in isolation and in a quantitative manner, we studied exogenous, active MMP-8 bound to the surface of unstimulated PMN (which have had no detectable proteinase activity on their cell surface). We first quantified the amount of MMP-8 that bound to the surface of PMN using McaPLGLDpaAR and assay standards of soluble, active site-titrated MMP-8. PMN bound 60.2 (12.7) ng of MMP-8 activity per 10^6 PMN, whereas control cells (which had no MMP-8 bound to their surface) had no detectable activity ($n = 7$). When soluble MMP-8 and membrane-bound MMP-8 were incubated with McaPLGLDpaAR, the two forms of the proteinase produced similar, progressive cleavage of the substrate over time (Fig. 7*A*). This activity was not due to release, leakage, or detachment of proteinases from the cells, because cell-free supernatant fluids from cells incubated in buffer alone had no detectable activity against McaPLGLDpaAR ($n = 4$).

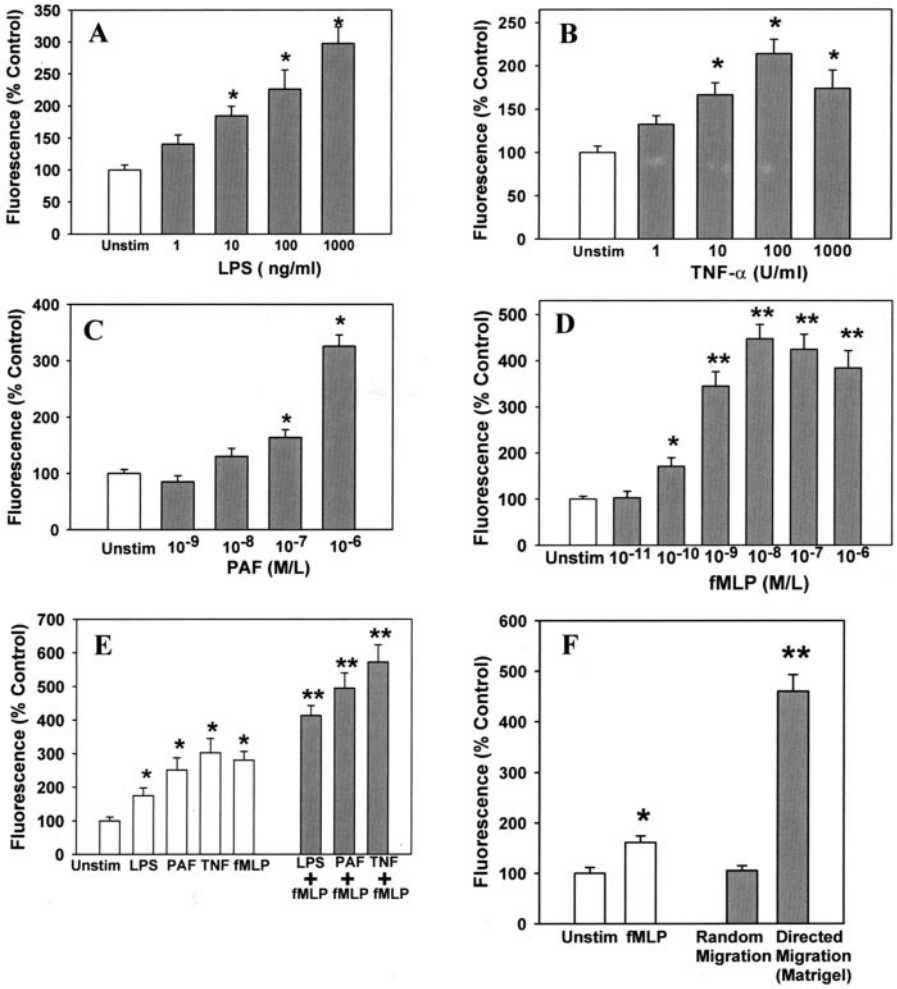

FIGURE 3. Proinflammatory mediators up-regulate cell surface expression of MMP-8 on PMN. *A–D*, PMN were incubated at 37°C for 30 min with or without varying concentrations of LPS (*A*), TNF-α (*B*), PAF (*C*), and fMLP (*D*). *E*, □, PMN incubated for 30 min at 37°C with or without 100 ng/ml LPS, 10^{-8} M PAF, 100 U/ml TNF-α, or 10^{-8} M fMLP. ■, PMN, which were primed for 15 min with the same concentrations of LPS, PAF, or TNF-α, then activated for 30 min with 10^{-8} M fMLP. Cell surface MMP-8 expression was quantified by immunofluorescence staining and image analysis. The data were corrected for nonspecific staining and expressed as a percentage of the mean integrated fluorescence of the unstimulated PMN. Data are mean values ± SEM (n = 100–150 cells). *A–C*, *, $p \leq 0.001$ compared with unstimulated cells. *D*, *, $p = 0.018$; **, $p < 0.001$ compared with unstimulated cells. *E*, *, $p < 0.001$ compared with unstimulated cells; **, $p < 0.001$ compared with cells incubated with agonists alone. *F*, ■, PMN undergoing random migration over 3 h in response to buffer alone in the lower wells (random), or directed migration in response to 10^{-7} M fMLP in the lower wells of Matrigel Invasion Chambers. □, PMN incubated in suspension for 3 h at 37°C with or without 10^{-7} M fMLP. Cell surface MMP-8 was quantified, as outlined above. The data (mean ± SEM) are expressed as a percentage of the mean integrated fluorescence of unstimulated PMN incubated in suspension (n = 150–200 cells). *, $p < 0.001$ when compared with unstimulated PMN in suspension; **, $p < 0.001$ compared with all other conditions.

Membrane-bound MMP-8 on PMN also cleaves type I collagen initially at a single locus, generating three-fourth (~98-kDa) and one-fourth length (34-kDa) collagen fragments typical of soluble interstitial collagenases (Fig. 7*B*, *lane 4*). Cleavage of type I collagen by membrane-bound MMP-8 on PMN (*lane 4*) was as efficient as that by soluble MMP-8 (*lane 2*) because both forms of MMP-8 produced almost complete disappearance of the intact type I polypeptide chains. RS113456 (a synthetic, hydroxamate MMP inhibitor) completely inhibited the type I collagenase activity associated with membrane-bound MMP-8 (Fig. 7*B*, *lane 5*). This activity was all cell associated because supernatant samples from stimulated PMN contain no type I collagenase activity (Fig. 7*B*, *lane 6*). Membrane-bound MMP-8 also cleaves type II collagen. MMP-8 bound to the surface of 10^6 PMN expressed type II collagenase activity equivalent to ~100 ng of soluble, active MMP-8 (Fig. 7*C*). As reported previously for soluble MMP-8 (38), membrane-bound MMP-8 also cleaves α_1-PI at a single locus within its active site loop, generating two cleavage products having M_r ~50 kDa (Fig. 7*D*), and ~4 kDa (data not shown). Both forms of MMP-8 produced substantial cleavage of α_1-PI. Control PMN did not cleave any of the biologic substrates tested (Fig. 7, *A–D*).

Catalytic efficiency of membrane-bound MMP-8

We compared the saturation kinetic constants (k_{cat}/K_M) for soluble and membrane-bound MMP-8. Each form of the proteinase was incubated with McaPLGLDpaAR under first order conditions, and initial reaction velocities were measured over 6 min. The k_{cat}/K_M values for soluble and membrane-bound MMP-8 were very similar (14,800 $M^{-1}s^{-1}$, n = 5; 10,100 $M^{-1}s^{-1}$, n = 3, respectively), indicating that soluble and membrane-bound MMP-8 cleave this substrate with similar catalytic efficiency.

In the above experiments, we studied exogenous membrane-bound MMP-8 that had been fixed onto the surface of PMN to prevent detachment of the enzyme from the cell surface. To assess whether our fixation process has any effect on the catalytic activity of membrane-bound MMP-8, we compared initial reaction velocities of equimolar amounts of exogenous membrane-bound MMP-8 that had been either fixed or not fixed onto the PMN surface. Fixed membrane-bound MMP-8 expressed 86.1% of the activity of nonfixed proteinase (n = 6 experiments), indicating that our fixation process has little effect on the catalytic activity of surface-bound MMP-8.

Stability of soluble vs membrane-bound MMP-8

We measured reaction velocities of both forms of the enzyme before and after incubation at 37°C for varying times, using McaPLGLDpaAR as the substrate (Fig. 7*E*). Soluble MMP-8 rapidly lost activity over time ($t_{1/2}$ at 37°C = 7.5 h). In marked contrast, membrane-bound MMP-8 retained close to 100% of its activity after 6 h, and >80% of its activity after 18 h.

Together, these data indicate that soluble MMP-8 and membrane-bound MMP-8 on PMN have a similar spectrum of catalytic activity, and similar catalytic efficiency against McaPLGLDpaAR. However, membrane-bound MMP-8 is more catalytically stable than soluble MMP-8.

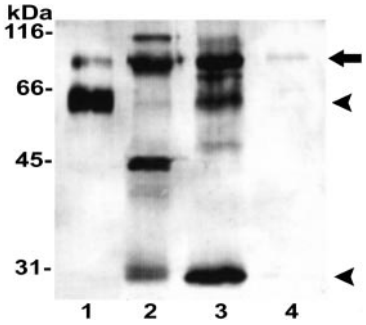

FIGURE 4. Western blot analysis of plasma membranes from activated PMN. Subcellular fractions of activated human PMN were prepared, as described in *Materials and Methods*. The following were subjected to Western blot analysis for MMP-8: *lane 1*, pro-MMP-8 partially activated with APMA (0.4 μg); *lane 2*, solubilized, purified specific granules (25 μg of total protein); *lane 3*, solubilized, purified plasma membranes (25 μg of total protein); *lane 4*, cell-free supernatant samples from PAF- and fMLP-activated PMN (10^7 PMN/ml). The arrow indicates pro-MMP-8 (~85 kDa), which represents 23% of the total amount of MMP-8 present in PMN plasma membranes, and the arrowheads indicate two additional, major forms of MMP-8 in PMN plasma membranes with M_r ~65 and 30 kDa accounting for 24 and 30% of the total amount of MMP-8, respectively. Three additional, three minor forms of MMP-8 are also present in PMN plasma membranes (M_r ~110 kDa, 80, and 45 kDa accounting for ~8, 10, and 5% of the total plasma membrane-associated MMP-8, respectively).

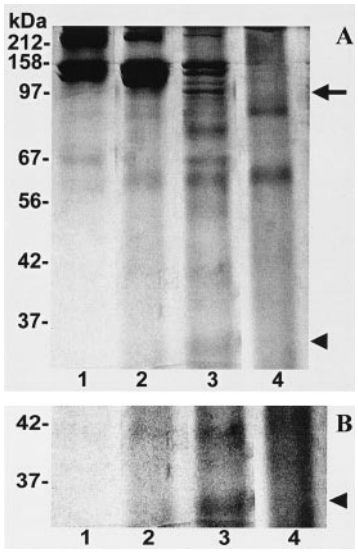

FIGURE 5. Activated human PMN express surface-bound type I collagenase activity. *A*, Bovine type I collagen (20 μg) was incubated with 2×10^7 fixed, activated human PMN for 4 h (*lane 2*), 12 h (*lane 3*), 20 h (*lane 4*), or in the absence of PMN (*lane 1*). Reduced cell-free supernatant samples were analyzed by 12% SDS-PAGE. The arrow and arrowhead indicate the three-fourth and one-fourth initial degradation products in *lane 3*, respectively. *B*, Lower one-fourth section of the gel shown in *A*, with the contrast adjusted to better demonstrate the one-fourth collagen fragments generated by activated human PMN. Note the disappearance of the intact type I collagen polypeptide bands in the presence of cells (*lanes 3* and *4*) with the appearance of the three-fourth (arrow) and one-fourth (arrowhead) cleavage products typical of interstitial collagenases in *lane 3*.

Membrane-bound MMP-8 on PMN is resistant to inhibition by TIMPs

We compared the effectiveness of inhibitors that varied in their M_r against soluble and membrane-bound MMP-8. As expected, all of the MMP inhibitors were fully effective against soluble MMP-8 (Fig. 8*A*). The low M_r MMP inhibitors (1,10, phenanthroline (M_r 194 Da) and RS113456 (M_r 426 Da)) were also fully effective against membrane-bound MMP-8. However, in marked contrast to soluble MMP-8, TIMP-1 (M_r 28 kDa) and TIMP-2 (M_r 21 kDa) only partially inhibited membrane-bound MMP-8 (~60% inhibition), even though they were tested at a 100-fold molar excess over enzyme. As expected, an inhibitor of serine proteinases (PMSF, M_r 194 Da) was ineffective against both forms of MMP-8. Preincubation of membrane-bound MMP-8 with PMSF before addition of TIMP-1 did not increase the effectiveness of TIMP-1 against this form of MMP-8 (43.4 (4.2)% vs 43.1 (1.7)% inhibition of membrane-bound MMP-8 $\times$ 200 nM TIMP-1 alone vs 1 mM PMSF and 200 nM TIMP-1, respectively). This indicates that the lack of effectiveness of TIMPs against membrane-bound MMP-8 was not due to serine proteinase-mediated cleavage of TIMPs.

Inhibitor kinetics

We quantified inhibition of membrane-bound MMP-8 by TIMP-1 as a function of time. Incubation of 10 nM membrane-bound MMP-8 with 10 nM TIMP-1 at 37°C for 18 h resulted in only 5.5 (6)% inhibition of its activity. Due to the lack of complete inhibition of membrane-bound MMP-8 by TIMP-1, it was not possible to determine the second order association rate constant for membrane-bound MMP-8 and TIMP-1. However, we compared inhibition of membrane-bound MMP-8 and soluble MMP-8 by TIMPs as a function of inhibitor concentration. The IC_{50} values for inhibition of soluble MMP-8 and membrane-bound MMP-8 by RS113456 were both low (2 and 16 nM, respectively; Fig. 8*B*), indicating that RS113456 is an effective inhibitor of both forms of MMP-8. However, the IC_{50} values for inhibition of membrane-bound MMP-8 by TIMP-1 and TIMP-2 were substantially higher (203 and 534 nM, respectively) than those for inhibition of soluble MMP-8 (IC_{50} = 22 and 7 nM, respectively; Fig. 8, *C* and *D*). In addition, 100-fold molar excess of TIMPs did not completely inhibit membrane-bound MMP-8.

To provide assurance that our method for fixing PMN had no effect on the capacity of TIMPs to bind to and/or inhibit membrane-bound MMP-8, we also tested the effectiveness of TIMPs against exogenous MMP-8 bound to PMN that had not been fixed. Incubation of 10 nM nonfixed membrane-bound MMP-8 with 125 and 250 nM TIMP-1 or TIMP-2 produced little inhibition of this form of MMP-8 (10.4 (11.3)% and 22.4 (1.0)% inhibition by 125 and 250 nM TIMP-1 and 33.7 (1.4)% and 44.3 (3.0)% inhibition by 125 and 250 nM TIMP-2, respectively). Thus, TIMPs were similarly ineffective against unfixed and fixed membrane-bound MMP-8 (Fig. 8, *C* and *D*). These data indicate that in contrast to MMP-8 that is freely released from activated PMN, MMP-8 localized on the cell surface of activated PMN during the inflammatory response is likely to retain its activity in vivo, despite the presence of TIMPs in plasma and interstitial fluids.

MMP-8 plays an unexpected, anti-inflammatory role during LPS-mediated acute lung injury in mice

To begin to assess the roles of PMN-derived MMP-8 in lung inflammatory responses, we gave MMP-$8^{-/-}$ and WT mice LPS by the IT route, then quantified influx of PMN into the alveolar space. The results show that 24 h after IT LPS, MMP-$8^{-/-}$ mice have ~2-fold greater accumulation of PMN in the alveolar space as assessed by BAL WBC total and differential counts (Fig. 9*A* and Table II, respectively). We also quantified MPO activity (a marker for PMN) in BAL cell lysates from the two genotypes. BAL cell lysates from MMP-$8^{-/-}$ mice had ~3-fold greater total MPO activity than BAL cell lysates from WT mice 24 h after IT LPS (Fig.

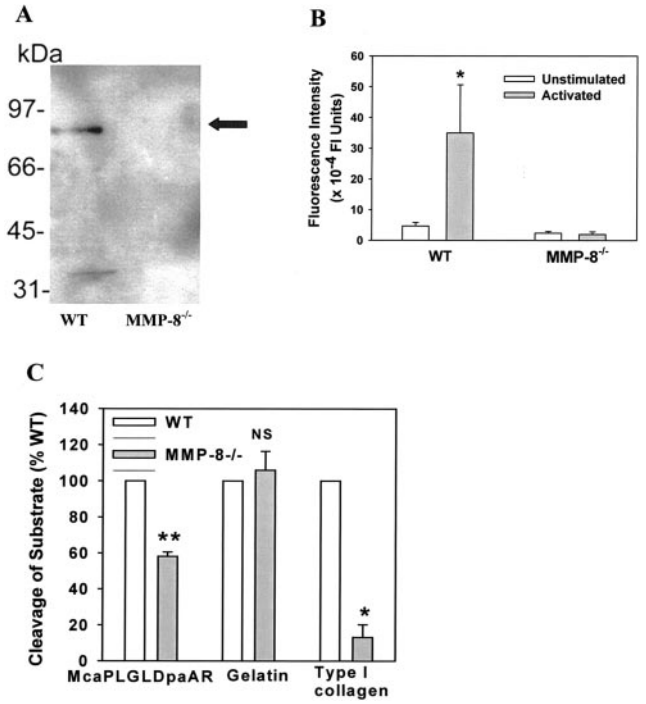

FIGURE 6. Cell surface MMP activities of activated PMN from WT and MMP-8$^{-/-}$ mice. *A*, Western blot analysis of PMN lysates from WT and MMP-8$^{-/-}$ mice. Arrow indicates pro-MMP-8 (M_r ~85 kDa). *B*, Unstimulated and PAF- and fMLP-activated PMN from WT and MMP-8$^{-/-}$ mice were immunostained for cell surface-bound MMP-8; cell surface fluorescence was quantified in arbitrary fluorescence units, and the data were corrected for nonspecific staining, as outlined in *Materials and Methods*. Data are mean values ± SEM (n = 100–150 cells). *, $p < 0.001$ compared with unstimulated PMN from WT mice. *C*, The activity associated with the cell surface of equal numbers of fixed, PAF-, and fMLP-activated PMN from pairs of WT and MMP-8$^{-/-}$ mice was quantified at 37°C against three fluorogenic substrates: 1) McaPLGLDpaAR, a general, synthetic MMP substrate (McaPLGLDpaAR; 10^6 PMN/assay; incubation time = 60 min; n = 5 pairs of mice); 2) gelatin FITC (gelatin; 3×10^6 PMN/assay; incubation time = 3 h; n = 5 pairs of mice); or 3) type I collagen FITC (type I collagen; 4×10^6 PMN/assay; incubation time = 18 h; n = 6 pairs of mice). In all assays, cells were incubated in triplicate with and without the general MMP inhibitor, 1,10-phenanthroline (1 mM). Total cell surface MMP-mediated activity against each substrate was quantified in cell-free supernatant samples using fluorometry, as the 1,10-phenanthroline-inhibitable activity. The results for cell surface MMP activity against each substrate expressed by PMN from MMP-8$^{-/-}$ mice were expressed as a percentage of the cell surface activity associated with PMN from WT mice. Data are mean ± SEM. *, $p = 0.002$; **, $p < 0.001$; and NS, not significantly different compared with cells from WT mice incubated with the same substrate.

9*B*), confirming that MMP-8$^{-/-}$ mice have a greater PMN burden in the alveolar space in response to IT LPS than WT mice. These data indicate that MMP-8 plays an unexpected, anti-inflammatory role during LPS-mediated acute lung injury in mice.

Discussion

Little is known about the cell biology of PMN-derived MMP-8, or the mechanisms by which MMP-8 retains its activity in the extracellular space following its release from PMN. In this study, we show that human PMN contain and release relatively low quantities of MMP-8, exclusively in the latent proenzyme form, yet activated PMN are associated with potent, TIMP-resistant, pericellular type I collagenase activity when they migrate on type I collagen. This activity is likely to be mediated, at least in part, by TIMP-resistant MMP-8 activity, which is expressed on the surface of activated PMN. Additional studies of MMP-8$^{-/-}$ mice in a murine model of acute lung injury show that MMP-8 plays an unexpected anti-inflammatory role in the lung. Most likely, cell surface-bound MMP-8 on activated PMN contributes in important ways to the anti-inflammatory, interstitial collagen degrading, and other activities of the enzyme in the lung and other tissues.

Cell biology of MMP-8

Surprisingly, PMN contain ~2 orders of magnitude less MMP-8 (~60 ng/10^6 cells) than the other neutral proteinases stored within their granules (PMN contain ~1–3 μg/10^6 cells of leukocyte elastase, cathepsin G, proteinase 3, and MMP-9 (31, 39)). When activated with biologic signals, PMN freely release only ~15–20% of their cellular content of MMP-8 as a soluble proteinase, which is exclusively in the latent proenzyme form. However, potent, TIMP-resistant, interstitial collagenase activity is associated with activated PMN migrating upon type I collagen, even when the cells are bathed in serum that contains micromolar concentrations of TIMPs (35). It is likely that MMP-8 localized on the surface of PMN contributes substantially to this activity because: 1) activated human PMN endogenously express MMP-8 and type I collagenase activity on their surface; 2) loss-of-function studies of PMN from MMP-8$^{-/-}$ and WT mice confirm that most of the type I collagenase activity associated with the surface of activated PMN is mediated by MMP-8; and 3) exogenous human membrane-bound MMP-8 on PMN cleaves type I collagen, but it is substantially resistant to inhibition by TIMPs. Thus, membrane-bound MMP-8 on PMN is a second form of extracellular MMP-8 that can contribute in important ways to the biologic activities of the proteinase.

Regulation of expression of membrane-bound MMP-8

Proinflammatory mediators induce additive concentration- and time-dependent increases in the expression of MMP-8 on the surface of human PMN. It is likely that these mediators up-regulate surface localization of MMP-8 by inducing PMN degranulation, followed by rapid binding of MMP-8 to the external surface of the PMN plasma membrane. This notion is supported by the following findings: 1) all of the mediators that up-regulate PMN surface expression of MMP-8 also stimulate the release of MMP-8 from PMN; 2) there is a direct relationship between the potencies with which they stimulate PMN degranulation (40) and up-regulate cell surface MMP-8 expression (LPS and fMLP > fMLP alone > LPS alone); and 3) exogenous MMP-8 binds to the external surface of the plasma membrane of PMN.

Catalytic activity of membrane-bound MMP-8 on PMN

MMP-8 localized on the surface of activated human PMN is, at least in part, catalytically active because activated human PMN endogenously express MMP-8 and type I collagenase activity on their surface, and MMP-8 is the only PMN proteinase that can degrade type I collagen (2). In addition, our loss-of-function studies of PMN from MMP-8$^{-/-}$ vs WT mice showed that membrane-bound MMP-8 accounts for ~40% of the total cell surface MMP activity (assessed using McaPLGLDpaAR) and ~90% of the PMN cell surface type I collagenase activity. Over time, activated human PMN also further degraded the denatured collagen fragments (gelatins) initially generated. Although soluble MMP-8 is reported to have weak gelatinase activity (2), the gelatinase activity associated with the surface of activated human PMN is unlikely to be mediated by membrane-bound MMP-8 because our loss-of-function studies of PMN from WT and MMP-8$^{-/-}$ mice showed that membrane-bound MMP-8 has no gelatinase activity. Most likely, this

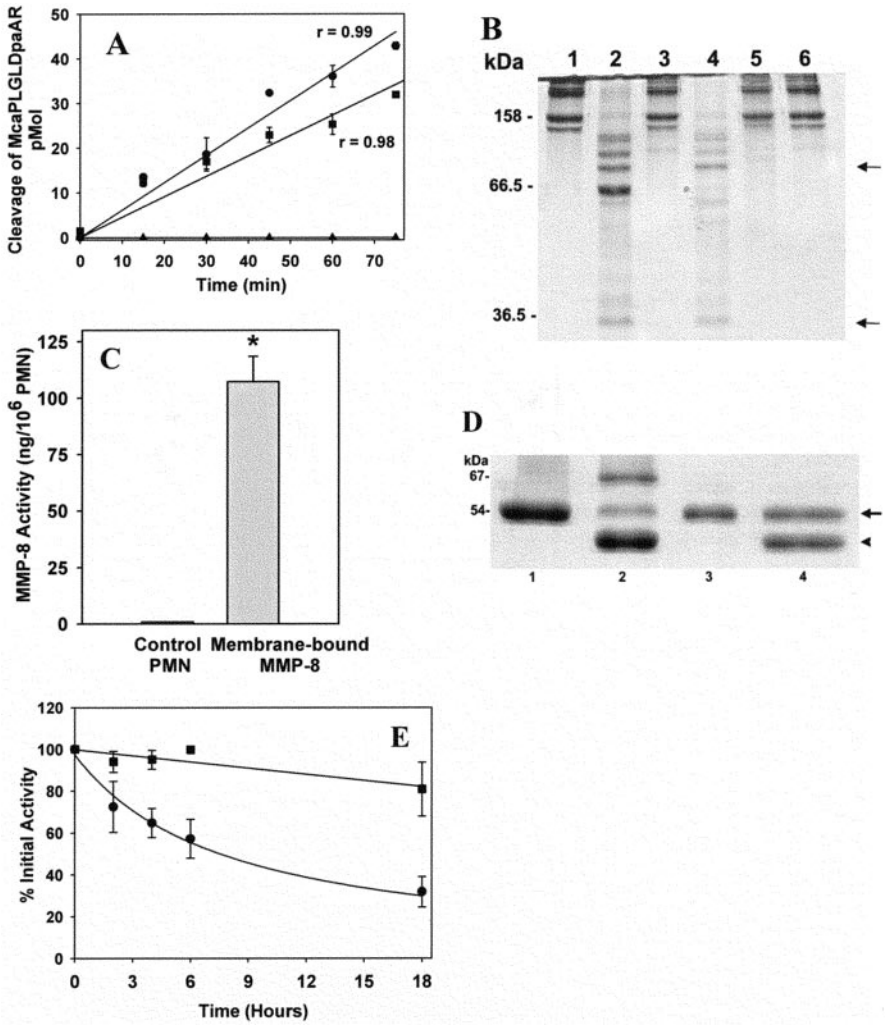

FIGURE 7. Human exogenous MMP-8 bound to PMN can degrade synthetic and biologic substrates. *A*, McaPLGLDpaAR. Cleavage of McaPLGLDpaAR by soluble MMP-8 (50 ng, squares), membrane-bound MMP-8 on PMN (50 ng, circles), or control cells incubated without MMP-8 (triangles) was quantified by fluorometry. Data are mean ± SD. *B*, Type I collagen. Bovine type I collagen was incubated for 18 h at 37°C either alone (*lane 1*) or with the following: 1) soluble, active MMP-8 (*lane 2*); 2) control PMN (*lane 3*); 3) MMP-8 bound to PMN (*lane 4*); 4) MMP-8 bound to PMN with 10 μM RS113456 (*lane 5*); and 5) cell-free supernatants from PMN that bound MMP-8 (*lane 6*). Reduced cell-free supernatant samples were analyzed by 12% SDS-PAGE. ∗, The band corresponding to soluble, active MMP-8 that is present only in *lane 2*. Arrows indicate the three-fourth and one-fourth initial cleavage products of the polypeptide chains of type I collagen in *lanes 2* and *4*. *C*, Type II collagen. We incubated PMN that bound MMP-8 to their surface or control PMN (10^6 cells/assay), along with assay standards of soluble, active site-titrated MMP-8 (25–800 ng) with FITC-conjugated type II collagen for 4 h. Cleavage of the substrate by soluble MMP-8 and membrane-bound MMP-8 was quantified in cell-free supernatant samples in arbitrary fluorescence units using fluorometry. The results for cell-associated activity against type II collagen were converted to ng equivalents of soluble MMP-8 activity by interpolation from the standard curve for soluble MMP-8 activity (*y*-axis). Data are mean values ± SD ($n = 5$ donors). ∗, $p < 0.001$ compared with control cells. *D*, α_1-PI. Purified human α_1-PI was incubated at 37°C alone (*lane 1*) or with soluble MMP-8 (*lane 2*), control PMN (*lane 3*), or MMP-8 bound to PMN (*lane 4*). Reduced cell-free supernatant samples were analyzed by 20% SDS-PAGE. The arrow indicates intact α_1-PI, and the arrowhead indicates the 50-kDa cleavage product. The 4-kDa cleavage product is not visible on the gel. *E*, Stability of soluble and membrane-bound MMP-8. Soluble MMP-8 (circles) and membrane-bound MMP-8 (squares) were incubated at 37°C in buffer for varying times (*x*-axis), and then initial reaction velocities were measured over 30 min after addition of McaPLGLDpaAR. The results were expressed as percentage of initial reaction velocities, and the $t_{1/2}$ for soluble MMP-8 (7.5 h) was calculated by nonlinear regression analysis. Data are mean ± SEM; $n = 4$.

gelatinase activity is mediated by the activities of other proteinases having gelatinase activity expressed on the surface of activated PMN, including MMP-9 (29) and human leukocyte elastase (HLE) (24).

To further assess the catalytic activity, catalytic efficiency, and susceptibility to inhibition of membrane-bound MMP-8 by TIMPs in isolation and in a quantitative manner, we studied exogenous MMP-8 bound to the cell surface of unstimulated PMN. Membrane-bound MMP-8 has similar substrate specificity as the soluble form of MMP-8 cleaving McaPLGLDpaAR, types I and II interstitial collagens, and a serpin (α_1-PI). Like soluble MMP-8, membrane-bound MMP-8 generates one-fourth and three-fourth fragments of the constituent polypeptide chains of type I collagen, and cleaves α_1-PI at a single locus within its reactive site loop, which inactivates the inhibitor (38). MMP-8 localized on the surface of PMN also has catalytic efficiency similar to that of soluble MMP-8, as determined by the similar k_{cat}/K_M values for the two forms of MMP-8 when tested against McaPLGLDpaAR. All of the activities associated with the cells were due to MMP-8 bound to the PMN surface because: 1) control PMN (which had no MMP-8 bound to their surface) had no significant activity against any of the substrates tested; and 2) no proteinase activity was detected in cell-free supernatant fluids from cells that bound MMP-8.

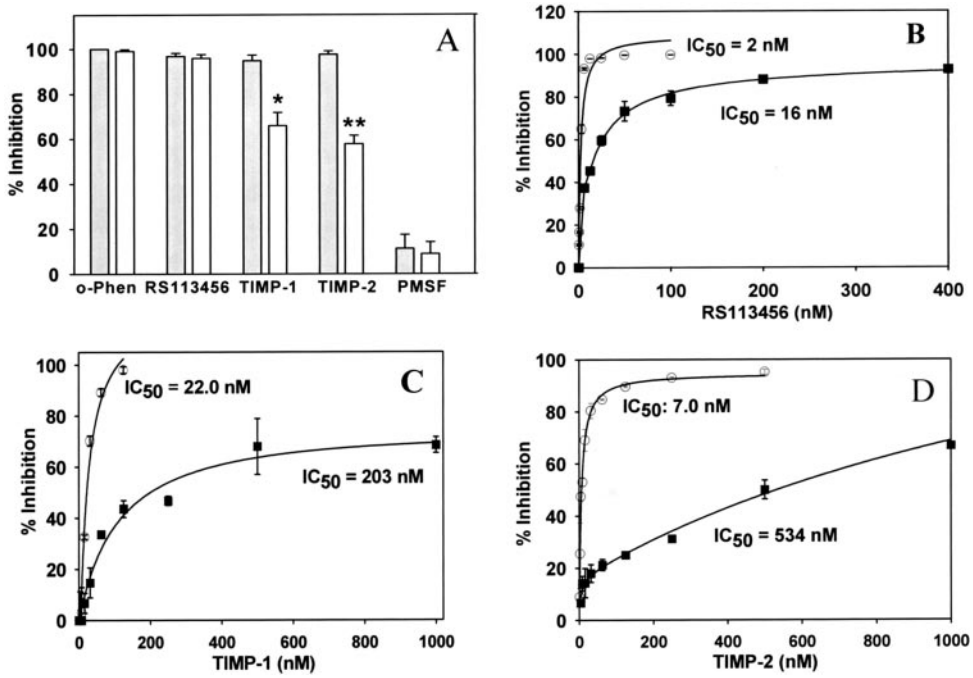

FIGURE 8. Membrane-bound MMP-8 on PMN is substantially resistant to inhibition by TIMPs. A total of 10 nM soluble MMP-8 (■) or 10 nM membrane-bound MMP-8 on PMN (□) was incubated for 30 min with and without: 1) 1,10-phenanthroline (o-Phen,1 mM); 2) RS113456 (1 μM); 3) TIMP-1 (1 μM); 4) TIMP-2 (1 μM); or 5) PMSF (1 mM). Residual MMP-8 activity was quantified in cell-free supernatant samples using McaPLGLDpaAR. Data are mean values ± SEM ($n = 5$). *, $p = 0.004$; **, $p < 0.001$ compared with soluble MMP-8 incubated with the same inhibitor. *B–D*, Concentration dependence of inhibition of soluble vs membrane-bound MMP-8. A total of 10 nM soluble MMP-8 (open symbols) or 10 nM membrane-bound MMP-8 (filled symbols) was incubated for 30 min at 37°C with or without varying concentrations of RS113456 (*B*), TIMP-1 (*C*), or TIMP-2 (*D*); then residual MMP-8 activity was quantified in cell-free supernatant samples using McaPLGLDpaAR. IC_{50} values were calculated by nonlinear regression analysis. Data are mean values ± SD.

Soluble MMP-8 and membrane-bound MMP-8 differ markedly in their stability at 37°C. Although soluble MMP-8 has a short $t_{1/2}$ at 37°C due to autoproteolysis (37), MMP-8 localized on the surface of PMN retains >80% of its activity even after incubation at 37°C for 18 h. The mechanism by which binding of MMP-8 to the PMN plasma membrane prevents loss of its activity is unclear. However, the binding of pro-MMP-9 to insoluble substrates induces a conformational change in the enzyme leading to proenzyme activation (41), and it is possible that the binding of MMP-8 to the PMN cell surface induces a conformational change that limits its autoproteolysis.

Susceptibility of membrane-bound MMP-8 to inhibition

The pericellular collagenase activity endogenously expressed by activated human PMN migrating upon type I collagen is resistant to inhibition by TIMPs present in serum, but is substantially abrogated by a low M_r MMP inhibitor. Our studies of individual MMP inhibitors tested against human membrane-bound MMP-8 showed that there is an indirect relationship between inhibitor size and its effectiveness against this form of MMP-8. In particular, the IC_{50} values for inhibition of membrane-bound MMP-8 by TIMP-1 and -2 were high (200–500 nM), and there was incomplete inhibition of membrane-bound MMP-8 × 100-fold molar excess of TIMPs. This further supports the notion that membrane-bound MMP-8 contributes to the pericellular collagenase activity associated with activated PMN. In addition, the data suggest that steric hindrance is most likely the mechanism by which membrane-bound MMP-8 on PMN evades inhibition by physiologic inhibitors. Large, globular TIMPs may form complexes less readily with MMP-8 that is sterically confined on the surface of PMN than with soluble MMP-8. This possibility is the focus of ongoing studies in our laboratory.

Although MMP-8 localized on the PMN surface can contribute to the pericellular collagenase activity associated with PMN migrating upon type I collagen, it is also possible that the generation of sequestered microenvironments by cells adherent to type I collagen contributes to this activity. Tight adherence of the PMN to the substrate could create a compartment into which diffusion of TIMPs is impaired, because α_1-PI diffuses poorly into compartments generated when PMN adhere to fibronectin (42). However, degradation of type I collagen in this protected microenvironment is likely to be mediated by membrane-bound MMP-8, rather than MMP-8 freely released by PMN into this compartment. This concept is supported by our Western blot analysis of cell-free supernatants from stimulated PMN showing that MMP-8 is released from PMN exclusively in its latent proenzyme form. In addition, we have shown previously that soluble MMP activity is not detectable in culture supernatants from activated PMN even when cells are incubated in inhibitor-free buffers (29). Our data indicate that while TIMPs can effectively control the activity of MMP-8 that is freely released from stimulated PMN, they are ineffective inhibitors of MMP-8 localized on the PMN surface. Membrane-bound MMP-8 on PMN is thus likely to substantially retain its activity in the extracellular space at sites of inflammation.

Mechanisms of activation of membrane-bound MMP-8

MMP-8 is expressed on the surface of PMN, at least in part, in a catalytically active form because: 1) activated human and murine PMN have substantial type I collagenase activity associated with

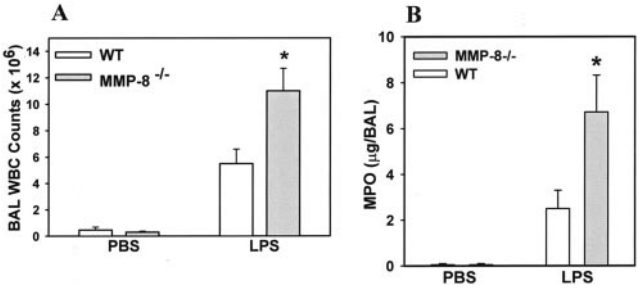

FIGURE 9. MMP-8 plays an anti-inflammatory role during LPS-mediated acute lung injury in mice. MMP-8$^{-/-}$ and WT control mice were given 10 μg of LPS (n = 10 in each genotype) by the IT route, or IT endotoxin-free PBS as a control (n = 4 in each genotype). *A*, After 24 h, the mice were euthanized, BAL was performed, and total WBC counts were performed. *, p = 0.017 compared with WT mice given IT LPS. *B*, MPO activity was also quantified in cell extracts of BAL cells, as a marker for PMN. Data are mean (SD). *, p = 0.029 compared with WT mice given IT LPS.

their cell surface; and 2) Western blot analysis of plasma membranes from activated PMN contains forms of MMP-8 that have the same M_r as active MMP-8 (~65 kDa). Proteinases (such as cathepsin G, MMP-3, and tryptase (20, 21)) and reactive oxygen species including oxidants generated by the MPO system (43) can activate pro-MMP-8 in vitro. Thus, it is possible that soluble proteinases and oxidants released by PMN and other cells during the inflammatory response activate pro-MMP-8 after its binding to the PMN surface in vivo. It is noteworthy in this respect that cathepsin G and MPO are expressed on the surface of activated PMN (30, 44, 45). Thus, membrane-bound cathepsin G and MPO could activate membrane-bound pro-MMP-8 in a juxtacrine manner. In addition, MT6-MMP is also constitutively present on the surface PMN, and is shed from the cell surface as a soluble proteinase when PMN are activated (32). MT6-MMP has been shown to activate pro-MMP-9 and -2 in vitro (32), but it is not known whether it can activate soluble or membrane-bound pro-MMP-8. The mechanisms by which pro-MMP-8 binds to and is activated on the surface of PMN will be a focus of future studies in our laboratory.

Biologic roles of membrane-bound MMP-8

Our in vitro studies suggest that MMP-8 localized on the surface of activated PMN contributes to the pericellular ECM-degrading activities of activated PMN in vivo. PMN express other ECM-degrading proteinases on their cell surface, including serine proteinases (24, 31, 46), MMP-9 (29), and MT6-MMP (32). Among these proteinases, only MMP-8 and HLE have interstitial collagenase activity, and HLE can only degrade type III collagen at a rate of only ~1% of that of the interstitial collagenases (47). Thus, the expression of MMP-8 on the surface of activated PMN adds potent interstitial collagenase (and serpinase) activity to the pericellular, proteolytic armamentarium of the activated PMN. Surface-bound MMP-8 on PMN may locally degrade interstitial collagens during inflammation and wound healing. Another biologic role of cell surface-bound MMP-8 on PMN may be to degrade serpins in the local environment of PMN because membrane-bound MMP-8 can inactivate α_1-PI. It is noteworthy that agonists that up-regulate MMP-8 localization on the surface of PMN also induce cell surface serine proteinase expression (30, 31, 44). Pericellular serpinase activity mediated by membrane-bound MMP-8 on PMN may potentiate the activities of serine proteinases coordinately expressed on the surface of activated PMN during inflammation. Finally, our studies of MMP-8$^{-/-}$ vs WT mice indicate that MMP-8 has an unexpected, anti-inflammatory role in the lung during LPS-mediated acute lung injury in mice by down-regulating the alveolar PMN burden. It is noteworthy in this respect that recent studies of MMP-8$^{-/-}$ vs WT mice in a chemical carcinogenesis model in the skin have shown that MMP-8 up-regulates early PMN accumulation in the skin (via cleavage and activation of LPS-induced CXC chemokine). At later time points in this model, MMP-8 has an anti-inflammatory activity in the skin associated with a less favorable environment for tumor initiation, but the mechanism for this is not known (10). Our future goals are to investigate whether PMN-derived MMP-8 cleaves and inactivates PMN chemokine(s), up-regulates anti-inflammatory mediators in the lung, and/or promotes clearance of PMN from the alveolar space. Thus, studies of MMP-8$^{-/-}$ mice have identified unexpected roles for MMP-8 in regulating inflammation in different organs. Moreover, TIMP-resistant MMP-8, which is expressed on the surface of activated PMN during inflammatory responses, is a bioactive form of the enzyme that is likely to contribute in important ways to its anti-inflammatory and other activities in vivo because it is a catalytically efficient and stable, but TIMP-resistant form of the proteinase.

Table II. *BAL differential WBC counts after IT LPS or IT PBS*

Genotype, Treatment[a]	% PMN Mean (SD)	% MACS Mean (SD)
WT, IT PBS	0 (0)	100 (0)
MMP-8$^{-/-}$, IT PBS	5 (4)	95 (4)
WT, IT LPS	93 (1)	7 (1)
MMP-8$^{-/-}$, IT LPS	92 (2)	8 (2)

[a] WT or MMP-8$^{-/-}$ mice were given 10 μg of LPS (n = 10) or endotoxin-free PBS (n = 4) by the IT route. After 24 h, BAL was performed, and differential counts were performed on the WBC fraction.

Conclusions

MMP-8 localized on the surface of PMN is likely to be an important bioactive form of the proteinase in vivo. During the inflammatory response, proinflammatory mediators can rapidly induce the expression of MMP-8 on the surface of PMN as they migrate through tissues. Binding of MMP-8 to the PMN surface not only leads to activation of pro-MMP-8, but also focuses, stabilizes, and preserves the collagenase and serpinase activities of the enzyme in the pericellular environment of PMN, even in the presence of TIMPs. The plasma membrane of PMN regulates the temporal and spatial localization of MMP-8 and other neutral proteinases of PMN, and thereby coordinates their activities in the pericellular environment of PMN. Although membrane-bound MMP-8 can contribute to physiologic processes of PMN, it also has the capacity to mediate tissue injury if its expression is excessive, prolonged, or inappropriate. Thus, MMP-8-mediated proteolytic events occurring at the PMN surface are likely to be critically important in physiologic and pathologic processes.

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