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Human Mast Cell-Derived Gelatinase B (Matrix Metalloproteinase-9) Is Regulated by Inflammatory Cytokines: Role in Cell Migration

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Mast cells are key effectors in the pathogenesis of inflammatory and tissue destructive diseases such as rheumatoid arthritis (RA). These cells contain specialized secretory granules loaded with bioactive molecules including cytokines, growth factors, and proteases that are released upon activation. This study investigated the regulation of matrix metalloproteinase MMP-9 (gelatinase B) in human mast cells by cytokines that are known to be involved in the pathogenesis of RA. Immunohistochemical staining of synovial tissue showed abundant expression of MMP-9 by synovial tissue mast cells in patients with RA but not in normal controls. The expression, activity, and production of MMP-9 in mast cells was confirmed by RT-PCR, zymography, and Western blotting using cord blood-derived human mast cells (CB-HMC). Treatment of CB-HMC with TNF-α significantly increased the expression of MMP-9 mRNA and up-regulated the activity of MMP-9 in a time- and dose-dependent manner. By contrast, IFN-γ inhibited MMP-9 mRNA and protein expression. The cytokine-mediated regulation of MMP-9 was also apparent in the human mast cell line (HMC-1) and in mouse bone marrow-derived mast cells. Furthermore, TNF-α significantly increased the invasiveness of CB-HMC across Matrigel-coated membranes while the addition of IFN-γ, rTIMP-1, or pharmacological MMP inhibitors significantly reduced this process. These observations suggest that MMP-9 is not a stored product in mast cells but these cells are capable of producing this enzyme under inflammatory conditions that may facilitate the migration of mast cell progenitors to sites of inflammation and may also contribute to local tissue damage. The Journal of Immunology, 2006, 177: 2638–2650.

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Mast cells are derived from hemopoietic progenitor cells that home to tissue as committed progenitors (1–3). Maturation and differentiation of mast cells occurs in tissue in response to local production of stem cell factor (SCF) (4). Mast cells can also undergo significant change in number or in phenotype during allergic or nonallergic inflammation (1, 5–9). These cells play a crucial role in IgE-dependent immune responses that mediate immediate hypersensitivity reactions associated with allergic phenomena and host resistance to parasites (5, 10). They also participate in innate immunity to bacterial infection (11) and play a direct role in the pathogenesis of a mouse model of inflammatory arthritis (12). There is considerable circumstantial evidence implicating mast cells in the pathogenesis of human rheumatoid arthritis (RA). These include the production of inflammatory (13, 14) and tissue destructive (15, 16) mediators. Moreover, a significant increase in mast cell numbers has been documented in human synovial tissue derived from patients with RA (8, 9). Despite the extensive knowledge regarding the mediators that regulate mast cell phenotype and number (10), factors that regulate the homing of mast cell progenitors from the bone marrow to the tissue of residence are not well-defined.

Matrix metalloproteinases (MMPs) are a family of neutral proteolytic enzymes active against all major components of the extracellular matrix. To date, 24 members have been cloned and characterized in humans and are divided into four groups depending on structure and substrate preference (17). Broadly, they include the collagenases, gelatinases, stromelysins, and the membrane-type MMPs. Gelatinase B (92-kDa, MMP-9) and gelatinase A (72-kDa, MMP-2) are the largest members of the MMP family and are to date the only gelatinases identified. MMP docking molecules such as CD44 for MMP-9 (18) and membrane-type MMPs for MMP-2 (17) have been identified on the surface of various cells. Cells use these proteinases to promote local proteolysis and to enhance their invasive potential (18, 19). Proteolytically active and inducible levels of MMPs have been observed in synovial cells from patients with RA (20).

MMP-9 is regulated posttranscriptionally at multiple levels. For example, its activity is neutralized by binding to naturally occurring tissue inhibitors of metalloproteinase (TIMP-1 to TIMP-4) with preferential binding observed between MMP-9 and TIMP-1 (21). MMPs (including MMP-9) are generally synthesized and secreted as latent soluble enzymes that require activation in the extracellular space. Several studies have identified mast cell-derived chymase and tryptase as potent activators of MMPs (22, 23). MMPs are also regulated at the level of transcription and their expression is modulated by a variety of stimuli including cytokines (20, 24, 25), growth factors (26), and cell-to-cell and cell-to-matrix interactions (27–29). Interestingly, the expression of MMP-2...
rarely varies, and it is regarded as a housekeeping-like gene in MMP biology.

MMP-9 was first identified in neutrophils (30) but these granulocytes lack TIMP-1 (31). MMP-9 is also a product of other inflammatory leukocytes including T cells, macrophages, and eosinophils (32). Relevant to acute and chronic inflammation, MMP-9 can cleave IL-8 and potentiate its activity as a neutrophil chemoattractant by at least 10-fold (18). There is now compelling evidence that along with the arsenal of stored serine proteases, mast cells are also a major source of MMPs such as MMP-1 (33), MMP-3 (34), and MMP-9 (23, 35–37). Although there is limited evidence for the expression of MMP-9 in mast cells in rheumatoid synovium (35), its regulation in RA is poorly understood. In the current study, we confirm MMP-9 expression in rheumatoid synovial mast cells and for the first time, demonstrate its regulation by TNF-α and IFN-γ in cord blood-derived human mast cells (CB-HMC), bone marrow-derived mouse mast cells (mBMMC), and the human mast cell line-1 (HMC-1). Furthermore, we provide evidence implicating TNF-inducible MMP-9 in the migration of mast cells through matrix proteins.

Materials and Methods

Patients

Formalin-fixed paraffin-embedded archival synovial tissue from patients with RA (n = 3) and normal controls (n = 3) was obtained from a tissue collection held at the Department of Pathology, University of New South Wales (Sydney, Australia). Institutional human ethics committee approval was obtained for this study.

Immunohistochemical studies

Serial sections (2–4 μm) of formalin-fixed paraffin-embedded synovial tissue were used for immunohistochemical studies. Specific mouse IgG1 mAbs against MMP-9 (Ab-8) and mast cell tryptase (AA1) were purchased from Oncogene Research Products and DakoCytomation, respectively. An irrelevant mouse IgG1 negative control Ab was purchased from DakoCytomation. These Abs were used in a modified three-step alkaline phosphatase staining procedure (38). In brief, paraffin-embedded adjacent sections were dewaxed and equilibrated with TBS (pH 8.0) followed by a 5-min Ag retrieval by microwave in 0.01 M citrate buffer (pH 6.2). The citrate buffer was allowed to cool, sections were re-equilibrated in TBS, then blocked with 20% goat serum for 20 min at room temperature. Sections were incubated with anti-MMP-9 (4 μg/ml), anti-trypatase (5 μg/ml), or an isotype control (5 μg/ml) Ab in 2% BSA/TBS overnight at 4°C. Sections were washed extensively with TBS, and then incubated with a biotinylated goat anti-mouse IgG (Vector Laboratories) for 1 h at room temperature. Sections were rinsed in TBS, and incubated with streptavidin-alkaline phosphatase conjugate (Vector Laboratories) for 45 min at room temperature. Immunoreactivity was detected using an alkaline phosphatase substrate (Vector Red; Vector Laboratories) and the sections were briefly counterstained with hematoxylin.

Morphometry

Overlapping images (1345 × 1033 pixels), each spanning an area of ~1.5 mm², were taken from sections using an Olympus BX60 microscope with a ×10 objective linked to Spot Advanced software version 3.5.6 for Windows (Diagnostic Instruments). Multiple images taken from each section were stitched into a single continuous image using Photoshop version 8 (Adobe Systems) software and the total surface area was measured. Adjacent sections that were stained for tryptase and MMP-9 were overlapped, rotated until they were aligned, and tryptase-positive and MMP-9/tryptase double-positive cells were counted. Finally, the proportion of MMP-9-positive mast cells from each tissue specimen was calculated. An area of 1.56–2.43 mm² from two to three images was sufficient to count over 100 mast cells in RA synovium. However, it was necessary to count the entire normal synovium tissue section (5.84–7.35 mm²) to acquire ~100 mast cells.

Human mast cell cultures

Cord blood from human placentas was obtained after routine Caesarian section from the Australian Cord Blood Bank at the Royal Women’s Hospital (Sydney, Australia). Cord blood was collected in accordance with established institutional guidelines and ethics committee approval. CB-HMCs were established from the mononuclear cell fraction of the cord blood as described (39, 40). In brief, heparin-treated cord blood was sedimented with 4.5% dextran solution to remove erythrocytes. Buffy coats were layered onto Ficoll-Hyphaque (Pharmacia) and the mononuclear cell interface was obtained after centrifugation. After repeated washes with PBS containing 5 mM EDTA, mononuclear cells were suspended at 2 × 10⁷/ml in high glucose RPMI 1640 (Invitrogen Life Technologies) containing 10% FBS, 2 mM l-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml gentamicin (all from Sigma-Aldrich), and 0.2 μM 2-ME (Invitrogen Life Technologies). Cells were cultured in the presence of 100 ng/ml SCF (gift from Amgen), 50 ng/ml IL-6 (R&D Systems), and 10 ng/ml IL-10 (R&D Systems). The nonadherent cells were transferred every week for up to 10 wk into culture medium containing fresh cytokines. The purity and maturity of cells was assessed weekly by flow cytometry using a fluorochrome-conjugated mouse Ab directed against human c-kit (CD117; BD Pharmingen), by metachromatic staining of cytoplasmic preparations using toluidine blue, and by immunostaining for both tryptase (DakoCytomation) and chymase (Chemicon International) using acetone-fixed cytosin preparations. Maturity was defined by >95% CD117high cells, >95% toluidine blue positivity, and positive immunostaining for tryptase and chymase. Once cells reached maturity, no other immunocytochemical or functional differences were noted between 7- and 12-wk-old cells. Therefore, cells were used for this study when they reached >95% toluidine blue positivity rather than a specific number of weeks in culture.

The human mast cell leukemia cell line (HMC-1) was provided Dr. J. Butterfield (Division of Allergic Diseases and Internal Medicine, Mayo Clinic, Rochester, MN) and cultured in the same medium as the CB-HMC (RPMI 1640 complete medium without growth factors). Because of the functional heterogeneity of the HMC-1 bulk culture, a limiting dilution assay was performed to isolate individual HMC-1. Cells from passages 5 to 8 of the newly propagated clones were used.

Mouse mast cell cultures

mBMMC were obtained by culturing primary bone marrow cells from female 6- to 8-wk-old BALB/c mice (BRC) in 50% RPMI 1640 complete medium, 30% WEHI-3B supernatant (as a source of murine IL-3) and 20% 3T3 supernatant (as a source of murine SCF) (41). The nonadherent cells were transferred every week for up to 4 wk into fresh culture medium. The purity and maturity of cells was assessed weekly by flow cytometry using fluorochrome-conjugated rat Ab against mouse c-kit (CD117; BD Pharmingen) and by metachromatic staining of cytosin preparations using toluidine blue. Maturity was defined by >95% CD117high cells and >95% toluidine blue positivity. Cells reached >95% maturity within 3–5 wk and no differences in morphology and function were noted between 3- and 5-wk cells. Cells were used when they reached >95% toluidine blue positivity rather than a specific number of weeks in culture.

MMP-9 production by human and mouse mast cells after cytokine stimulation

CB-HMC were washed twice with medium alone and resuspended at 2 × 10⁶ cells/ml in assay medium (2.5% FBS/RPMI 1640 and SCF (100 ng/ml)). HMC-1 and mBMMC were resuspended in the same medium and at the same density, but without SCF. Human mast cells were incubated with 25 ng/ml TNF-α (R&D Systems) or 25 ng/ml IFN-γ (Endogen) in 24-well flat-bottom Falcon plates at 37°C and 5% CO₂. mBMMC were incubated with 25 ng/ml recombinant mouse TNF-α (R&D Systems), 12.5 ng/ml recombinant mouse IFN-γ (R&D Systems), or the combination of both for 24–36 h. For some experiments, RNA was extracted and supernatants harvested for gelatin-substrate zymography and Western blotting, and each preparation was stored at ~70°C.

To assess the effects of IFN-γ on TNF-α-mediated induction of MMP-9, CB-HMC were incubated with the optimal concentration of TNF-α (25 ng/ml) and increasing doses of IFN-γ (0.005–50 ng/ml). Total RNA was extracted after 4 h for RT-PCR analysis and supernatants were collected 24 h after stimulation for zymography. To determine whether production of MMP-9 from CB-HMC was due to de novo protein synthesis, cells were stimulated with 25 ng/ml TNF-α and coincubated with varying concentrations of cycloheximide (0.5–0.5 μg/ml; Sigma-Aldrich) for 24 h.

Quantitation of histamine released by CB-HMC

Degranulation of CB-HMC was induced after passive IgE sensitization and anti-IgE activation as described elsewhere (40). In brief, CB-HMC were washed twice with RPMI 1640 and resuspended at 2 × 10⁶ cells/ml in

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culture medium containing 100 ng/ml SCF. Cells were primed with semi-purified human myeloma IgE (10 μg/ml; Chemicon International) and IL-4 (10 ng/ml; Endogen) for 5 days. After washing with PBS containing 0.1% BSA (ICN Biomedicals), cells were activated with 1 μg/ml rabbit anti-human IgE Ab in 0.1% BSA/PBS for 30 min at 37°C in 5% CO2. For some experiments, cells were treated for 4–8 h with varying concentrations of A23187 (Sigma-Aldrich) or 50 ng/ml phorbol ester (PMA; Sigma-Aldrich). Supernatants were harvested and stored at -20°C before histamine determination, zymography, and Western blot analysis (see below). Some cells were collected by centrifugation, resuspended in RPMI 1640, and lysed by rapid freeze/thawing (three cycles). Histamine in the supernatant and cell pellet fractions was measured by ELISA (ICN Biomedicals). Percentage of histamine release was quantitated by the equation: histamine in supernatant/(histamine in supernatant + histamine in pellet) × 100.

Gelatin-substrate zymography

Zymography was performed as previously described (25, 33, 42, 43). Supernatants standardized for cell numbers were diluted 1/4 using serum-free culture medium and 8 μl of nonreducing sample buffer (0.25M Tris-HCl (pH 6.8), 10% SDS, 4% sucrose, 0.1% bromophenol blue) was added to 25 μl of diluted culture supernatant and loaded onto 10% SDS-PAGE gels containing 1 mg/ml gelatin (Sigma-Aldrich). After electrophoresis, gels were fixed twice for 5 min in 2.5% Triton X-100 (Sigma-Aldrich), incubated overnight at 37°C in substrate buffer (50 mM Tris-HCl (pH 7.4), 10 mM CaCl2, and 0.02% NaN3), stained with Coomassie Blue R-250 (Bio-Rad) for 1.5 h, then destained (3 × 50 min) to expose gelatinolytic bands. A low range of molecular mass protein ladder (Bio-Rad) was run in adjacent lanes. MMP identity was verified by adding proteinase inhibitors such as 10 mM EDTA, 1 mM 10-phenanthroline (Sigma-Aldrich), and 2 mM 2-(4-morpholino)ethanesulfonic acid (Sigma-Aldrich) to the substrate buffer. Gelatinolytic bands were semiquantified with the Gel Doc 2000 and the Quantity One program (Bio-Rad).

Western blotting

Western blotting was performed as previously described (25, 33, 43). Culture supernatant from control or cytokine-stimulated CB-HMC (25 μl) was electrophoretically separated on SDS-PAGE using 4% stacking and 10% resolving gels under nonreducing conditions. Proteins were transferred to PolyScreen polyvinylidene difluoride transfer membranes (PerkinElmer Life Sciences), blocked in 5% skim milk powder in TBST for 1 h, washed briefly in TBST, then incubated with 5 μg/ml primary mAb directed against human MMP-9 (Ab-8; Calbiochem-Novabiochem) diluted in 5% BSA/TBST for 1 h at room temperature. Membranes were then extensively washed in TBST (3 × 5 min) and incubated with a 1/2000 dilution of HRP-conjugated rabbit anti-mouse Ab (DakoCytomation) for 1 h at room temperature. Membranes were again washed (3 × 5 min) in TBST, then placed in chemiluminescent reagent for 1 min (Western Lightning; PerkinElmer Life Science) and exposed to Hyperfilm MP (Amersham Biosciences). A prestained low molecular mass protein ladder (Bio-Rad) was run in adjacent lanes.

RNA extraction and RT-PCR

Total RNA was extracted (RNAgent Total RNA Extraction kit; Promega) from control and cytokine-stimulated CB-HMC after 4 h. Reverse transcription was performed using the PreCipitation System for First Strand cDNA Synthesis kit (Invitrogen Life Technologies) and as previously described (25, 33, 43) using Superscript III. Aliquots (4 μl) of cDNA were amplified by PCR using 100 nM each of the forward of 5′-TTTC AAG GCT GGG GAG TAC TG-3′ and reverse 5′-TCC GGA GGG CCC CCG TCA CCT-3′ primers for MMP-9, TIMP-1, and GAPDH (43). A 2-min hot start at 95°C was performed to denature the double stranded cDNA, followed by 37 cycles of PCR (each cycle: 95°C, 30 s; 55°C, 30 s; 72°C, 30 s) and the reactions terminated with a 2-min extension at 72°C. Cycle number was predetermined so that the products generated were within the linear portion of the amplification curve. PCR products were visualized on 2% agarose gels, pretreated with ethidium bromide and semi-quantified with the Gel Doc 2000 and the Quantity One program (Bio-Rad). A 100-bp ladder (Invitrogen Life Technologies) was run in adjacent lanes.

Migration of CB-HMC through Matrigel

CB-HMC were washed twice in RPMI 1640 and resuspended at 2 × 106 cells/ml in RPMI 1640 complete medium containing 100 ng/ml SCF. Cells were preincubated for 6 h in the same medium with or without TNF-α (25 ng/ml), IFN-γ (25 ng/ml), or a combination of TNF-α and IFN-γ in 15-ml round-bottom Falcon tubes at 37°C and 5% CO2. Cells were washed once with 10 ml of binding buffer (1% BSA, 25 mM HEPES in RPMI 1640) then adjusted to 1.5 × 106/ml Polyvinylpyrrolidone-free membranes (10 μm pore, 25 × 80 mm; Osmonics) were coated with a 1/6 dilution of Matrigel (Sigma-Aldrich) in 75 mM HEPES/RPMI 1640 for 2 h at room temperature then 10 μg/ml human fibronectin (Invitrogen Life Technologies) was applied for 1 h at room temperature and allowed to dry.

Varying concentrations (10-10 to 10-8 M) of the mast cell chemotaxant C5a (R&D Systems) or binding buffer alone in a final volume of 30 μl were dispensed in triplicate into the lower well of a 48-well chemotaxis chamber (Neuro Probe). A suspension of cytokine-stimulated CB-HMC (50 μl) was dispensed into the upper wells of the chemotaxis chamber that was separated by a coated membrane. Chambers were subsequently incubated at 37°C in a humid 5% CO2 incubator for 14 h, the membranes were removed, rinsed in PBS, and the upper surface gently scraped over with a wiper blade to remove noninvading cells. Membranes were stained with Diff Quick according the manufacturer’s instruction (Lab Aids), mounted on glass slides and coverslipped. Cells that had invaded to the lower surface were counted under high power (×400) using a light microscope. Three randomly selected fields were counted from each well and each treatment consisted of triplicate wells giving nine counts per treatment. The migration index from three independent experiments was then calculated by dividing the mean cell counts for each treatment divided by counts from corresponding medium only control.

To determine whether the migration of CB-HMC through Matrigel was indeed MMP dependent, migration assays were performed in the presence of several MMP inhibitors. A broad-spectrum synthetic MMP inhibitor BB3103 (0–10 μM) was provided by Bio-Science Ltd. (Campinas, Brazil). Recombinant human (rh) TIMP-1 and TIMP-2 were purchased from Calbiochem-Novabiochem and dexamethasone was purchased from Sigma-Aldrich. Briefly, CB-HMC were preincubated in RPMI 1640 complete medium that contained 100 ng/ml SCF and TNF-α (25 ng/ml) for 6 h at 37°C in 5% CO2. Cells were then washed once with 10 ml of binding buffer and adjusted to 1.5 × 106/ml in binding buffer containing BB3103 (0–10 μM), rhTIMP-1/2 (0–5 μM), or dexamethasone (0–5 μM). Cells in binding medium alone were used as positive control. To determine toxicity, a 100-μl cell suspension containing each inhibitor at the respective concentration was dispensed into the wells of a 96-well flat-bottom plate. Chemotaxis chambers and 96-well plates were incubated for 14 h at 37°C in 5% CO2. Membranes were rinsed, stained, and migrated cells counted as described above. Supernatants from the 96-well plates were harvested for zymographic analysis and cells were used for viability assays using a fluorometric, 96-well plate LIVE/DEAD Viability assay kit (Molecular Probes).

Cell surface expression of C5a receptor and c-Kit on CB-HMC by flow cytometry

CB-HMC (2 × 106/ml) incubated in RPMI 1640 complete medium containing 100 ng/ml SCF with or without TNF-α (25 ng/ml) for 6 h at 37°C in 5% CO2 were washed with 10 ml of binding buffer and an aliquot (1 × 106 cells/ml) was sampled (6-h time point). The remaining cells (1 × 106 cells/ml) were transferred to a 96-well flat-bottom plate in binding buffer containing 5% FBS and incubated at 37°C in 5% CO2 for 14 h. Before staining, cells were washed with cold PBS containing 0.05% NaN3 and 1% BSA (PAB buffer) and resuspended in the same buffer at 2 × 106/ml. Human serum (10% final) was added and cells (50 μl) were incubated for 30 min at room temperature with 5 μg/ml unconjugated mouse IgG2a (AbD Serotec) or a combination of TNF-α (25 ng/ml), and FITC-conjugated mouse IgG1 mAb to c-Kit (CD117; BD Pharmingen), or with PE-conjugated control mouse IgG1 (BD Pharmingen). After washing in cold PAB buffer, 500 μl of 1% parar- maldehyde in PBS was added to the cell suspensions and stained with conjugated Abs before storing at 4°C in the dark. Cells stained with unconjugated Abs were incubated on ice for 45 min with 10 μg/ml (100 ng/ml) of FITC-conjugated Fab (Fab′), goat anti-mouse IgG (Fab′), specific), secondary Ab (Jackson ImmunoResearch Laboratories). Cells were washed twice with PAB buffer, fixed with 1% paraformaldehyde in PBS, and analyzed by using a FACSScan flow cytometer (BD Biosciences). A unimodal shift in fluorescence intensity of cells stained with specific Abs compared with cells stained with the isotype-matched negative control Abs was considered positive.

Statistical analysis

Significance of mast cell degranulation in response to A23187, PMA, and IgE cross-linking was determined using two-tailed t tests. Statistical significance in the activity of MMP-9 in mast cells treated with various doses...
FIGURE 1. Immunohistochemical staining for MMP-9 in rheumatoid synovium. Serial sections of synovial tissue from three patients with RA (patient 1, A and B; patient 2, C and D; patient 3, E and F) were stained with Abs to mast cell tryptase (A, C, and E) and MMP-9 (B, D, and F), respectively. Cells in adjacent sections positive for MMP-9 and tryptase are identified with arrows. Serial sections of normal synovial tissue (normal 1, G and H) were stained with Abs to mast cell tryptase (G) and MMP-9 (H). Several mast cells were identified in this tissue, but most did not express MMP-9. The arrowheads in D, G, and H identify neutrophils within blood vessels that are positive for MMP-9. Insets, A neutrophil at high power (×400) which is negative for mast cell tryptase (A) but positive for MMP-9 (B).
Results

Production of MMP-9 by human mast cells in vivo

Upon stimulation, mast cells release a battery of enzymes that are generally stored in specific granules. In addition to the well-characterized serine proteases, previous studies have identified members of the MMPs in these cells (22, 33–37, 44). Initially, it was our intention to confirm these primarily in vitro observations in vivo, using synovial tissue obtained from patients with RA by immunohistochemistry. Staining for MMP-9 (Fig. 1, B, D, and F) was observed in tryptase-positive (Fig. 1 A, C, and E) mast cells in the RA synovium whereas in normal human synovium, despite the presence of several tryptase-positive mast cells (Fig. 1 H), there was limited immunoreactivity for MMP-9 in these cells (Fig. 1F). MMP-9 was also identified in synovial lining cells, macrophages (data not shown) and intravascular neutrophils in RA tissue (Fig. 1, B, inset, D, arrowhead) and normal synovium (Fig. 1 H, arrowhead). Quantitative analysis of tissue sections demonstrated substantially more tryptase-positive mast cells in RA synovium with an average of 57.3 ± 12 cells/mm² compared with 13.0 ± 1.8 cells/mm² in normal synovium (Table I). There were also higher proportions of MMP-9-positive mast cells in RA tissue (65.4 ± 9.5%) compared with normal synovium (11.1 ± 1.1%) (Table I).

Characterization of CB-HMCs

To confirm expression of MMP-9 by human mast cells, primary mast cells were propagated from cord blood and characterized. These cells displayed typical morphological, phenotypic, and functional characteristics of mast cells including dense granular cytoplasm (Fig. 2 A), intense metachromatic staining (>95%) with acidic toluidine blue (Fig. 2 A), and abundant expression (>97%) of tryptase (Fig. 2 B) and 95–99% chymase positively (Fig. 2 C). Flow cytometric analysis demonstrated high intensity cell surface expression for c-kit/CD117 on >95% of these cells (Fig. 2 D).

Production of MMP-9 by CB-HMC

CB-HMCs were used to determine whether MMP-9 is stored in mast cell granules or whether it is newly synthesized and secreted upon mast cell activation. To test the former hypothesis,
degranulation of mast cells was induced by cross-linking of the high-affinity IgE receptor with anti-IgE for 30 min at 37°C. Although this treatment caused significant degranulation as measured by histamine release (Fig. 3C), no difference was noted in the level of MMP-9 activity either in the supernatants or lysates (Fig. 3A). To confirm this observation, experiments were performed using other agents (A23187 and PMA) that are known to cause the release of prestored mediators from mast cells. Activation of mast cells with PMA or A23187 (calcium ionophore) did not alter the relative production of MMP-9 even when cells were left in these agents for up to 8 h (Fig. 3B). However, a significant amount of histamine was detected in the supernatants obtained from the cells treated with PMA or A23187, indicative of their degranulation (Fig. 3D). Hence, degranulation of mast cells in response to PMA, A23187, and IgE-cross linking in the absence of any significant increase in MMP-9 production suggests MMP-9 is predominantly not a stored product. This is consistent with the lack of MMP-9-positive cells in normal synovial tissue (Fig. 1, G and H).

Cytokine regulation of mast cell-derived MMP-9

TNF-α plays a key role in the pathogenesis of RA and is currently a therapeutic target in this and other inflammatory diseases (45). TNF-α positively regulates expression of MMPs that

![FIGURE 3](image-url). Gelatinolytic activity and histamine release by CB-HMC. Cell supernatants and lysates were harvested after CB-HMC activation (30 min) with IgE and anti-IgE or IgE alone (control), neat supernatants, and lysates used for zymography (A). CB-HMC in RPMI 1640 medium containing 2.5% FBS and 100 ng/ml SCF were incubated without (control) or with 50 ng/ml PMA or A23187 (0.5 or 5 μM) for 4 or 8 h and neat culture supernatants were analyzed by zymography (B). Mast cell degranulation was measured by histamine release after activation of cells with IgE and anti-IgE for 30 min (C) or treatment of cells with PMA or A23187 (D). Results are representative of three independent experiments. *, p < 0.05; **, p < 0.01 when compared with corresponding controls.
are responsible for tissue destruction that characterizes RA. Therefore, we determined whether this cytokine induced the expression of MMP-9 in mast cells that are typically present in rheumatoid synovium and are believed to play a key role in the pathogenesis of the disease. Treatment of CB-HMCs with TNF-α caused significant up-regulation of MMP-9 in a time-dependent manner (peak 18 h) (Fig. 4). By contrast, treatment of these cells with IFN-γ significantly suppressed MMP-9 production (Fig. 4A) with the greatest decrease noted at the 24-h time point (Fig. 4B). This is consistent with previous studies that have demonstrated the down-regulation MMP-9 by IFN-γ in macrophages (46, 47).

Following the kinetic studies, CB-HMCs were treated for 24 h with varying concentrations of TNF-α (0–50 ng/ml) and supernatants analyzed by zymography. MMP-9 production was significantly induced by TNF-α in a dose-dependent manner, while MMP-2 remained unchanged (Fig. 5A). Maximum MMP-9 production was observed when cells were stimulated with 5–50 ng/ml TNF-α (Fig. 5C). Conversely, when CB-HMCs were treated with IFN-γ, constitutive MMP-9 levels significantly decreased (Fig. 5B), while MMP-2 was not affected. Maximum inhibition was observed with 5–50 ng/ml IFN-γ (Fig. 5C).

To confirm the identity of the 92-kDa gelatinolytic band as a metalloproteinase, zymograms were incubated in substrate buffer containing EDTA (metal chelator) or 1,10-phenanthroline (an MMP inhibitor). Both agents abolished all activity associated with MMP-2 and -9 (data not shown, Refs. 42 and 43), whereas the serine protease inhibitor ABSF had no effect on the activity of MMP-2 and -9 (data not shown). Moreover, stimulation with TNF-α not only increased expression of the 92-kDa band, but also induced the appearance of a minor 83-kDa component (Fig. 4A, 24- to 72-h arrows; Fig. 5A, arrow), a size typical of activated MMP-9. Western blotting confirmed the identity of the 92-kDa band as MMP-9 that was increased after treatment with TNF-α (Fig. 5D). An immunoreactive band at 83 kDa that corresponds to the active form of MMP-9 was observed following TNF-α stimulation (Fig. 5D). Interestingly, TIMP-1, a natural inhibitor of MMP-9 protein, was not detected in the same supernatants by Western blotting (data not shown) suggesting that activation of mast cells by TNF-α might be biased toward the process of increased tissue permeability and degradation.

To determine whether MMP-9 production in CB-HMC was due to de novo protein synthesis, cells were stimulated with 25 ng/ml TNF-α and coincubated with varying concentrations of cycloheximide (0 – 0.5 μg/ml). MMP-9 was dose-dependently inhibited by cycloheximide and this was visualized by a decrease in intensity of this proteinase (Fig. 6A). Inhibition of MMP-9 was statistically significant with 0.125– 0.5 μg/ml cycloheximide (Fig. 6B).

The effects of IFN-γ on the TNF-α-mediated induction of MMP-9 were examined by stimulating cells with 25 ng/ml TNF-α.

**FIGURE 4.** Time course production of MMP-9 in cytokine-stimulated CB-HMC. CB-HMC in RPMI 1640 containing 2.5% FBS and 100 ng/ml SCF were stimulated with either 25 ng/ml TNF-α or 25 ng/ml IFN-γ and supernatants were collected at the indicated time points for zymographic analysis (A). Small arrows in A identify active MMP-9 (83 kDa) evident at 24–72 h after TNF-α stimulation. B, The intensity of the lytic bands from three independent experiments was measured and the fold change in intensity was calculated by dividing values from the different treatments to the values from corresponding untreated control. Bars represent mean and SE of three experiments and statistical analysis for each time point performed using Kruskal-Wallis test followed by Dunn’s posttest. *, p < 0.05; **, p < 0.01 compared with untreated control.
in the presence of varying amounts (0–50 ng/ml) of IFN-γ. Treatment of cells with IFN-γ caused inhibition of TNF-α-mediated MMP-9 production in a dose-dependent manner (Fig. 7).

The effect of TNF-α and IFN-γ on the expression of MMP-9 mRNA was studied in cells treated with optimal concentrations of these cytokines for 4 h. RNA was analyzed by RT-PCR using gene-specific primers for human MMP-9. Consistent with the protein data, treatment of cells with TNF-α induced MMP-9 mRNA by 2.4-fold as compared with untreated controls (data not shown), whereas coinoculation of cells with TNF-α and IFN-γ caused a modest (1.7-fold) decrease in the TNF-α-induced expression of MMP-9 (data not shown). TIMP-1 mRNA was detected in CB-HMC but levels were not affected by TNF-α and/or IFN-γ.

Our observations in CB-HMC further corroborated HMC-1 and mBMMC. Treatment of HMC-1 or mBMMC with TNF-α induced the production of MMP-9 in these cells (Fig. 8) and IFN-γ completely abrogated TNF-α-mediated up-regulation of this enzyme in both mast cell types (Fig. 8, A and B, lane 4). MMP-9 activity was not detected in supernatants derived from control or IFN-γ-treated HMC-1 or mBMMC, suggesting that these cells produce lower constitutive levels of this protease compared with CB-HMC. Alternatively, cytokines such as SCF, IL-6, and/or IL-10 that were used to maintain CB-HMC in culture may be responsible for the higher basal expression of MMP-9 in these cells.

Invasion of CB-HMC through Matrigel is mediated by MMP-9 and not by C5aR up-regulation

Mast cells are generally found in abundance at sites of inflammation. The means by which the progenitors of these cells traverse the vascular endothelium and migrate through the surrounding connective tissue is poorly understood. However, evidence now suggests that MMPs may facilitate the extravasation, migration, and recruitment of inflammatory cells from blood vessels to the surrounding connective tissue (48, 49). MMP-9 produced by activated mast cells may play such a role. Migration of cytokine-treated CB-HMC through a Matrigel-coated membrane was investigated. Dose-dependent migration of mast cells toward C5a was observed irrespective of the cytokine treatment (Fig. 9A). However, the highest number of invading cells was observed when cells were pretreated with TNF-α. This was statistically significant at all doses of C5a (p < 0.01) when compared with cells that were preincubated in basal medium containing SCF alone. Numbers of
invading cells in samples preincubated with IFN-γ were similar to those cultured in basal medium containing SCF, indicating that IFN-γ did not alter invasion of CB-HMC through matrix components. However, the combination of IFN-γ and TNF-α significantly (p < 0.01) reduced cell invasion induced by TNF-α to control levels (Fig. 9A). The increased migration of TNF-treated cells toward C5a was not due to up-regulation of C5αR, as its constitutive expression was not altered by pretreatment of cells with TNF-α for 6 h or subsequent withdrawal of TNF-α for 14 h (Fig. 9B).

To confirm whether the increased invasion of mast cells through the reconstituted basement membrane after treatment with TNF-α was mediated by MMPs, TNF-α-primed CB-HMC were treated with MMP inhibitors throughout the duration of the invasion assay. Treatment of cells with broad spectrum MMP inhibitors, BB3103 (Fig. 10A) and dexamethasone (Fig. 10B), or specific inhibitors including rhTIMP-1 (Fig. 10E) or rhTIMP-2 (Fig. 10G) caused dose-dependent abrogation of cell invasion through the reconstituted basement membrane by up to 80% (range 20–80%). The viability of the cells following treatments with the various concentrations of inhibitors was consistently >95%, confirming that the effect of these mediators on cell migration was not due to toxicity. Zymographic analysis of supernatants obtained from the TNF-α-primed cells showed that BB3103 (Fig. 10B), rhTIMP-1 (Fig. 10F), or rhTIMP-2 (Fig. 10H), despite their strong inhibition of cell invasion, did not inhibit the production of MMP-9 by these cells. In contrast, treatment of these cells with dexamethasone caused dose-dependent down-regulation of MMP-9 production by ~2-fold as displayed by the decrease in the intensity of the gelatinolytic band (Fig. 10D, lane 4).

Discussion
In this study, we have demonstrated the induction of MMP-9 in primary human mast cells by a proinflammatory cytokine (TNF-α), a cytokine strongly linked to the pathogenesis of RA (45). The TNF-mediated up-regulation of MMP-9 was strongly abrogated by IFN-γ. A similar pattern of regulation of MMP-9 by TNF-α and IFN-γ has previously been reported in human macrophages (47), however its regulation by TNF-α and IFN-γ in primary human mast cells (CB-HMC) is novel. Although the production of MMP-9 by human mast cells has been reported (23, 33–37, 44), we provide substantial evidence to demonstrate that MMP-9 is primarily not a stored product. Other investigators have speculated that latent MMP-9 coexists with histamine and tryptase in specialized secretory granules within mast cells (37). In that particular study, the HMC-1 cell line was used and cell-to-cell contact with lymphocytes induced MMP-9. Given that MMP-9 protein was first detected at 6 h and peaked at 22 h, this suggested de novo synthesis and not the release of preformed protein as the authors of that study alluded (37). In CB-HMC, enhanced production of MMP-9 was observed at 6 h and reached maximum levels by 18–24 h following TNF-α stimulation (Fig. 4). Moreover, no additional MMP-9 was released following mast cell degranulation (Fig. 3). These differences may be attributed to modifications in culture conditions, level of mast cell maturity and/or incubation times with PMA. In a previous investigation, we observed potent induction of MMP-9 in HMC-1 and canine BR mastocytoma cells after a 48-h incubation with this agent (33). Because TNF-α is one of the key cytokines in RA, synovial tissue from patients with RA was used to investigate in vivo expression of MMP-9 in mast cells. In addition to various other cell types, MMP-9 was colocalized in a large proportion of tryptase-positive mast cells in diseased tissue (Fig. 1, A–F; Table I), whereas, MMP-9 reactivity was scant in
normal synovium despite the presence of numerous mast cells (Fig. 1, G and H; Table I). These data suggest that mast cell-derived MMP-9 may be induced during an inflammatory response. Others have shown similar in vivo results in humans (35) and mice (51). Interestingly, while MMP-9 was identified in mast cells from diseased human synovial tissue, it was also detected in mast cells found in normal human skin and lung (35). Yet other studies have not detected MMP-9 despite the presence of mast cells in normal skin (44). The above disparities in the results regarding the expression of MMP-9 in normal tissue may be attributed to differences in Abs, immunohistochemical technique, processing of the tissue and/or site from which the tissue was obtained.

Studies have shown that mast cell-derived MMP-9 can be down-regulated (36) or up-regulated (52) by SCF depending on the type of mast cells. Tanaka et al. (36) demonstrated down-regulation of MMP-9 in bone marrow-derived mouse mast cell progenitors, whereas, Fang et al. (52) demonstrated induction of MMP-9 in canine BR mastocytoma cells treated with SCF. In the current study, CB-HMCs were cultured in the presence of SCF and this may have accounted for the substantial amount of constitutive MMP-9 (Fig. 4). This is supported by the increase in production of MMP-9 in cells cultured over 6–72 h (Fig. 4). In contrast, no basal production of MMP-9 was evident in HMC-1 or mBMMC, neither of which requires rSCF for their maintenance in culture (Fig. 8). We also showed that IFN-γ inhibited constitutive (Figs. 4 and 5) and TNF-α-mediated induction of MMP-9 in CB-HMCs (Fig. 7).

The inhibitory effects of IFN-γ come as no surprise as IFN-γ suppresses the TNF-α-mediated induction MMP-9 (46, 47) and MMP-1 (53) in tumor cells, primary macrophages, and in synovial fibroblasts. Although, further work is required to determine the mechanism in CB-HMCs: this inhibitory effect may be mediated via STAT-1 or via caspase 8-independent apoptosis as described in tumor cells (46) and synovial fibroblasts (54).

In this study, we have shown that the significant increase in migration of CB-HMC through reconstituted basement membrane was associated with the TNF-α-mediated induction of MMP-9 but not C5aR in these cells (Fig. 9). Furthermore, the TNF-α-mediated increase in migration was abrogated by coinucubation with IFN-γ, which also inhibited MMP-9 production in these cells. These results suggest that MMP-9 might be important for the transmigration of human mast cell progenitors across a basal lamina or/and connective tissue matrix. To investigate whether this was indeed an MMP-related phenomenon, a selection of MMP inhibitors was included in this model. We observed that the synthetic hydroxamic acid derivative (BB3103) and the corticosteroid dexamethasone significantly reduced the TNF-α-mediated induction of MMP-9 in CB-HMCs (Fig. 7).

In contrast, dexamethasone has a direct effect on MMP-9 production in CB-HMCs. Our results suggest that TNF-α-mediated induction of MMP-9 is due to the release of MMP-9 from preexisting stores within the cell. TNF-α treatment increased MMP-9 expression from both untreated cells and cells pretreated with IFN-γ. Pretreatment of cells with TNF-α for 6 h (a and b) or removal of TNF-α thereafter for 14 h (c and d) did not change surface expression of C5aR or c-kit (regular line histogram) when compared with untreated cells (bold histogram). Histograms on the left of each diagram show the corresponding isotype-matched negative controls.
band corresponding to TIMP-1 was detected and attributed to pro-
cessing and deactivation of this inhibitor by mast cell-derived chy-
mase (56). It is possible that TIMP-1 protein is degraded by chy-
mase and tryptase that are abundant in these cells (Fig. 2, B
and C). Conversely, mast cell-derived serine proteases such as chymase
are known to convert the inactive pro-MMP-9 to active MMP-9
(56).

Along with the many known functions of MMP-9 in wound
healing, connective tissue remodeling, angiogenesis, and leuk-
cyte migration, several additional key functions are emerging.
MMP-9 can cleave the precursor form of IL-1β to an active
molecule and IL-8 is truncated at the N terminus by the same
enzyme, causing amplification of its activity as a neutrophil
chemoattractant (57). Thus, specific inhibition of MMP-9 may
result in anti-inflammatory effects, a proposal confirmed in
MMP-9 knockout mice in which deficiency in this enzyme pro-
tected mice from developing autoimmune diseases (58, 59) and
prolonged delayed-type hypersensitivity responses (60). Heis-
sig et al. (61) recently showed that MMP-9 induced in bone
marrow cells caused release of SCF thereby permitting the
transfer of endothelial and hemopoietic stem cells from a qui-
escent to a proliferative niche. This is an essential prerequisite
for the recruitment and maturation of progenitor cells from the
bone marrow to the tissue of residence (61). In MMP-9 knock-
out mice, release of SCF and motility of endothelial and hemo-
poietic stem cells are impaired. Bone marrow ablation in these
animals resulted in failure of hemopoietic recovery and in-
creased mortality (61).

Despite the extensive knowledge regarding proteins that regu-
late mast cell phenotype and numbers in tissue, factors that regu-
late the homing of mast cell progenitors from the bone marrow to
the tissue of residence are not fully understood. A critical role
for adhesion molecules such as $\alpha_4\beta_7$ in directing tissue-select-
ive homing of mast cell progenitors to the peripheral tissue has
been shown (62). However, the mechanisms on how mast cell
progenitors move within the usually dense connective tissue are

FIGURE 10. Inhibition of invasion by TNF-α-primed CB-HMC through
matrix components with MMP inhibitors. Invasion of CB-HMC toward C5a
(10^{-8} M) through Matrigel-coated membranes was performed on cells
pretreated with TNF-α and incubated with increasing amounts of synthetic
(A, BB3103), steroid (C, dexametha-
sone), or natural MMP inhibitors (E,
TIMP-1; G, TIMP-2). Cells in three
high power fields were counted for
each well and results are presented as
the mean cell count ± SD of triplicate
wells for each treatment. The effect of
each inhibitor on MMP-9 levels was
evaluated by gelatin zymography (B,
D, F, and H). Inhibition of migration
was determined using a two-tailed $t$

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. INDUCTION OF MMP-9 BY TNF-α IN HUMAN MAST CELLS

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unclear. In this study, we have demonstrated increased migration of CB-HMC through reconstituted basement membrane that was associated with increased production of MMP-9 (Figs. 9A and 10). Thus, MMP-9 produced by mast cell progenitors may enable these cells to migrate through connective tissue and release SCF by a membrane cleavage mechanism thereby generating conditions that sustain mast cell maturation and differentiation. It is possible that the significant increase in mast cells observed during allergic or nonallergic inflammation (1, 5, 6, 8, 9) might be due to TNF-α-induced MMP-9 production that facilitates migration of mast cell progenitors and causes local release of SCF. This suggestion is supported by our demonstration of increased numbers of MMP-9-positive mast cells in inflamed synovial tissue. Modulation of MMP-9 production by mast cells might therefore be a useful therapeutic strategy to minimize the inflammatory reaction in RA.

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Disclosures
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