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Human Mast Cells Release Metalloproteinase-9 on Contact with Activated T Cells: Juxtacrine Regulation by TNF-α[1]

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Mast cells, essential effector cells in allergic inflammation, have been found to be activated in T cell-mediated inflammatory processes in accordance with their residence in close physical proximity to T cells. We have recently reported that mast cells release granule-associated mediators and TNF-α upon direct contact with activated T cells. This data suggested an unrecognized activation pathway, where mast cells may be activated during T cell-mediated inflammation. Herein, we show that this cell-cell contact results in the release of matrix metalloproteinase (MMP)-9 and the MMP inhibitor tissue inhibitor of metalloproteinase 1 from HMC-1 human mast cells or from mature peripheral blood-derived human mast cells. The expression and release of these mediators, as well as of β-hexosaminidase and several cytokines, were also induced when mast cells were incubated with cell membranes isolated from activated, but not resting, T cells. Subcellular fractionation revealed that the mature form of MMP-9 cofractionated with histamine and tryptase, indicating its localization within the secretory granules. MMP-9 release was first detected at 6 h and peaked at 22 h of incubation with activated T cell membranes, while TNF-α release peaked after only 6 h. Anti-TNF-α mAb inhibited the T cell membrane-induced MMP-9 release, indicating a possible autocrine regulation of MMP release by mast cell TNF-α. This cascade of events, whereby mast cells are activated by T cells to release cytokines and MMP-9, which are known to be essential for leukocyte extravasation and recruitment to affected sites, points to an important immunoregulatory function of mast cells within the context of T cell-mediated inflammatory processes. ***The Journal of Immunology*, 2001, 167: 4008–4016.**

Mast cells are central effector cells in the elicitation of the early and late phase allergic inflammatory reactions. Via triggered exocytosis of their secretory granules, these cells release a variety of mediators, including vasoactive amines, specific proteolytic enzymes, multifunctional cytokines, and chemoattractants. In addition, some cytokines and metabolites of arachidonic acid are synthesized de novo. Mast cells also contribute to host defense mechanisms against bacterial and parasite infections as potential activators of T cell-dependent immune responses (1–3).

Morphologic studies have revealed that mast cells reside in close apposition to T cells within inflamed allergic tissues and at sites of parasitic infections (4, 5). Hence, a functional relationship between these two immune cell types has been proposed (6). Consistent with this hypothesis, it has been demonstrated that the mononuclear cell infiltration in the late allergic response is mast cell dependent (7). Studies have also shown that mast cells are activated during T cell-mediated inflammatory reactions, such as cutaneous delayed hypersensitivity (8), rheumatoid arthritis (6), and hypersensitivity pneumonitis (9). It seems likely that such a relationship may provide bidirectional signals, since T cells can mediate mast cell proliferation and activation (6, 10, 11), and, on the other hand, mediators of activated mast cells may affect T cell-mediated inflammatory reactions (6, 12). Thus, intercellular communication via cell-cell interactions is likely a significant mechanism of immune cell functions at inflamed sites, where several cell types tend to accumulate (13).

We have previously reported on the effects of direct contact between mast cells and T lymphocytes on mast cell degranulation. Mast cells were found to degranulate in response to direct contact with activated T cells as well as to produce TNF-α (14). Moreover, studies using murine mast cells and PMA- or anti CD3-activated T cells attributed the degranulation induced by cell-cell contact to ICAM-1-LFA-1 interactions (15), thus providing further evidence of a putative relationship between the two cell types.

Mast cell function in potentiating multiple responses in their resident tissues, via the release of soluble mediators, may depend on interactions with extracellular matrix (ECM)[4] molecules within connective tissues. Several studies have highlighted the potential role of mast cells in mediating ECM degradation through the activation and production of matrix metalloproteinases (MMPs). Mast cell tryptase and chymase have been shown to activate the precursors of MMP-2 (gelatinase A) (16), MMP-9 (gelatinase B) (17), collagenase, and stromelysin (18). Recently, it has been demonstrated that activation of mast cells by long (48 h) incubation with PMA stimulated MMP-9 expression in vitro. This enzyme has been identified in mast cells localized in various healthy and diseased tissues (19). Reports of differential regulation by the c-*kit* ligand stem cell factor (SCF), a potent stimulator of mast cell

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[4] Abbreviations used in this paper: ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; SCF, stem cell factor.

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proliferation and differentiation, and the pleiotrophic cytokine TGF-β implicate the importance of growth factors in mediating MMP-9 secretion by mast cells from various sources (20, 21). Although previous studies have indicated a role for direct cell-cell contact in potentiating metalloproteinase expression in monocytes (13) and T lymphoma cells (22), thus far mast cell-T cell contact has not been reported to induce mast cell production of MMPs.

The current study was aimed at gaining insight into the functional role of mast cell-T cell contact in expression and release of MMPs, in the context of modification of the inflammatory environment. We now demonstrate that mast cell-T cell heterotypic adhesion up-regulates mast cell MMP-9 expression as well as release of active MMP-9 and tissue inhibitor of metalloproteinase (TIMP) 1 from granular stores. Furthermore, although mast cell expression or release of the inflammatory cytokines TNF-α, IL-4, and IL-6 was induced by direct contact with activated T cells, only TNF-α regulated induction of MMP-9 expression. These results suggest that physical interactions between mast cells and activated T lymphocytes may promote mast cell release of soluble cytokines and proteases that regulate ECM degradation during T cell-mediated inflammation.

Materials and Methods

Antibodies

The following Abs were purchased from R&D Systems (Minneapolis, MN): normal mouse IgG1 isotype control, anti-human IL-4-neutralizing mAb, anti-human IL-6-neutralizing mAb, and anti-human pro/active MMP-9 mAb. Anti-TNF-α-neutralizing mAb was purchased from BD PharMingen (San Diego, CA). Anti-human cathepsin D Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human mast cell tryptase mAb was purchased from Chemicon (Temecula, CA).

Cells

Reagents for cell culture were purchased from Biological Industries (Beit Haemek, Israel). HMC-1 cells (23), a human mast cell leukemia cell line, and the Jurkat T cell lymphoma line were each maintained in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 12.5 U/ml nystatin. Human peripheral blood T lymphocytes were isolated from healthy donors as previously described (12). Briefly, the mononuclear cells were isolated on Ficoll gradients, then washed and incubated for 2 h at 37°C in 5% CO_2 in a humidified incubator. Nonadherent cells were collected and incubated on nylon wool columns. Unbound cells were eluted by extensive washing with PBS, and the resulting cell population was >98% T lymphocytes.

To study the effects of coculture with T cells, freshly isolated human peripheral blood T lymphocytes or Jurkat T cells (1×10^6/ml) were first activated with 50 ng/ml PMA for 60 min at 37°C, followed by extensive washing (three times) with RPMI 1640. Mast cells (1×10^6/ml) were then cocultured for 20–22 h with activated or nonactivated T cells (at 1:1 ratio) in RPMI 1640 supplemented with L-glutamine, penicillin-streptomycin-nystatin, and 0.1% BSA in a humidified incubator in 96- or 24-well culture plates.

Human peripheral blood $CD34^+$ progenitor cells were isolated as described elsewhere (24), then cultured in serum-free medium (StemPro-34 SFM; Life Technologies, Grand Island, NY) supplemented with L-glutamine (200 mM), streptomycin (50 mg/ml), penicillin (100 U/ml), recombinant human IL-6 (100 ng/ml), human SCF (100 ng/ml; PeproTech, Rocky Hill, NJ), and 30 ng/ml rhIL-3 (for the first week only). Half of the culture medium was replaced with fresh medium every 7 days. Purity of mast cells was determined by metachromatic staining using acidic toluidine blue (pH 1.0). After 8–10 wk of establishing the culture, >95% of the cells were identified as mast cells. Contaminating monocytes/macrophages were removed by incubation on plastic culture dishes (35 × 10 mm) for 2 h, finally yielding a >99% mast cell population.

Preparation of T cell membranes

Two methods for isolation of Jurkat T cell membranes were used. Method 1 is a modification of a method described previously (25). Resting or PMA-activated cells (2×10^8) were washed three times with PBS (8 min, 150 × *g*) and resuspended at 10^7 cells/ml in ice-cold TKMS lysis buffer comprised of 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$, 0.25 M sucrose, 1 mM PMSF, and Complete, a mixture of protease inhibitors (Boehringer Mannheim, Mannheim, Germany). The cells were kept on ice for 20 min and lysed by five cycles of freezing and thawing in liquid nitrogen until no living cells could be observed by trypan blue exclusion. The cells were then centrifuged at 800 × *g* for 5 min at 4°C. The supernatants were collected and subjected to centrifugation for 60 min at 100,000 × *g* at 4°C. The pellets were suspended in PBS and stored at −70°C. Method 2 is a modification of a method described previously (26). Briefly, resting or PMA-activated cells (2×10^8) were washed with PBS as above and resuspended at 10^7 cells/ml in ice-cold STM lysis buffer comprised of 0.25 M sucrose, 5 mM Tris-HCl (pH 7.2), and 1 mM $MgCl_2$. PMSF and Complete protease inhibitors were added. The cells were kept on ice for 10 min and lysed by two cycles of freezing and thawing in liquid nitrogen. The cell lysates were then centrifuged at 280 × *g* for 5 min at 4°C. The supernatants were collected and centrifuged at 1500 × *g* for 10 min at 4°C. The pellets were suspended in 9 ml of 1.42 M sucrose-STM, overlaid with 2 ml of 0.25 M sucrose-STM, and centrifuged for 60 min at 82,000 × *g* (SW41 rotor). The interphases between the two sucrose layers were collected and 8 ml of 5 mM Tris-HCl (pH 7.2) were added. These suspensions were further centrifuged for 60 min at 100,000 × *g*. The final pellets were suspended in PBS and stored at −70°C.

Gelatin zymography

Supernatants of mast cells, T cells, cocultures of both cell types, or mast cells incubated with T cell membranes were analyzed by gelatin zymography to detect gelatinase activity. Aliquots (20 μl) of cell supernatants were subjected to electrophoresis under nonreducing conditions in 10% polyacrylamide gels containing 1 mg/ml gelatin type A (Sigma, St. Louis, MO). Gels were washed three times in 2.5% Triton X-100 to renature the gelatinases, then incubated overnight in 50 mM Tris-HCl (pH 7.5) and 5 mM $CaCl_2$. Coomassie blue staining, followed by destaining, allowed visualization of clear zones of lysis against a blue background.

TIMP-1 ELISA

Supernatants of HMC-1 cell cultures were tested for levels of secreted TIMP-1 using a commercial Biotrak ELISA system according to the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

β-Hexosaminidase release

Activity of the secretory granule-associated enzyme β-hexosaminidase was determined by incubating 20-μl aliquots of supernatants and cell lysates for 90 min at 37°C with 50 μl of substrate solution consisting of 1.3 mg/ml *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma) in 0.1 M citrate (pH 4.5). Reactions were stopped by the addition of 150 μl of 0.2 M glycine (pH 10.7). OD was read at 405 nm using an ELISA reader. Results (mean ± SD) were expressed as percentage of total β-hexosaminidase activity present in the cells.

TNF-α bioassay

Released TNF-α was measured in supernatants of HMC-1 cell cultures as previously described (14). Briefly, the supernatants were added to cultures of the TNF-α-sensitive mouse fibrosarcoma cell line L-929. Cell death, caused by TNF-α in the HMC-1 cell supernatants, was quantified by comparison with titration curves of cell death due to the addition of purified TNF-α (PeproTech).

Subcellular fractionation of HMC-1 cells

Fractionation of HMC-1 cells was performed essentially as described elsewhere (27). HMC-1 cells were washed with PBS and resuspended in homogenization buffer comprised of 0.25 M sucrose, 1 mM $MgCl_2$, 800 U/ml DNase I (Sigma), 10 mM HEPES (pH 7.4), 1 mM PMSF, and a protease inhibitor mixture (Complete). Cells were then disrupted by five cycles of freezing and thawing. Unbroken cells and nuclei were removed by sequential filtering through 5- and 2-μm pore size filters (Poretics, Livermore, CA). The final filtrate was centrifuged for 10 min at 500 × *g*, and the supernatant was loaded onto a continuous 0.45–2.0 M sucrose gradient (10 ml) and centrifuged for 18 h at 100,000 × *g*. Thirty fractions were taken from the top of the gradient.

Histamine assay

Histamine content in the gradient fractions was assayed fluorometrically after condensation in alkaline medium with *o*-phthaldialdehyde (28).

SDS-PAGE and immunoblotting

Samples were separated by SDS-PAGE using 10% polyacrylamide gels and transferred to nitrocellulose filters. Blots were blocked for 3 h in TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20) containing 5% skim milk, followed by overnight incubation at 4°C with the indicated primary Abs. Blots were washed three times and incubated for 1 h at room temperature with HRP-conjugated secondary Ab. Immunoreactive bands were visualized using the ECL method according to standard procedures.

Cathepsin D content

Aliquots of the gradient fractions were mixed with 5× Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE and immunoblotting. Blots were processed with anti-cathepsin D Abs. Results were expressed as percentage of total OD as determined by densitometry of the immunoreactive bands using the TINA-PCbas software (Ray test, Isotopenmessgeräte, Germany) (27).

Semiquantitative RT-PCR

HMC-1 cells were incubated for 20 h with cellular membranes isolated from resting or activated Jurkat T cells, then lysed in TRIzol reagent (Life Technologies, Rockville, MD). Total RNA was isolated according to the manufacturer's instructions, and 30 μg was treated with DNase I (Amersham Pharmacia Biotech). RNA samples (5 μg) were reverse transcribed using 20 U avian myeloblastosis virus-reverse transcriptase (Promega, Madison, WI) or 20 U of SuperScript II (Life Technologies) and amplified using specific sets of primers. The linear ranges of PCR amplification were determined for each transcript, and amplification was performed within those linear ranges. PCR samples were subjected to electrophoresis on 1% agarose gels.

The following oligonucleotide primers were synthesized at the Nucleotide Core Facility (Weizmann Institute of Science, Rehovot, Israel): human MMP-9 sense, 5′-GACTCTACACCCGGGACGGCAATGCTG; human MMP-9 antisense, 5′-CGTCCACCGGACTCAAAGGCACAGTAG; GAPDH sense, 5′-CGGAGTCAACGGATTTGGTCGTAT; and GAPDH antisense, 5′-AGCCTTCTCCATGGTTGGTGAAGAC.

The following oligonucleotide primers were synthesized by Life Technologies: human MMP-2 sense, 5′-CTTTTTCAAAGGTGACCGCTAC; human MMP-2 antisense, 5′-TAGAAGTAGGTGTAGGCTGCGTC; human IL-4 sense, 5′-CAGTTCTACAGCCACCATGAGA; human IL-4 antisense, 5′-CATGATCGTCTTTAGCCTTTCC; human IL-6 sense, 5′-CCAGGAGAAGATTCCAAAGATG; human IL-6 antisense, 5′-GGAAGGTTCAGGTTGTTTTCTG; human TNF-α sense, 5′-CTGTACCTCATCTACTCCCAGGTC; human TNF-α antisense, 5′-AGACTCGGCAAAGTCGAGATAGT; human TNF-α sense, 5′-CTGTACCTCATCTACTCCCAGGTC; and human TNF-α antisense, 5′-AGACTCGGCAAAGTCGAGATAGT.

Results

Mast cell-T cell coculture results in mast cell release of MMP-9

Although mast cell exocytosis and release of serine proteases critical in inflammation have been extensively studied, only recently has evidence on the regulation of mast cell MMP synthesis emerged. Soluble mediators, including SCF and TGF-β (20, 21), and phorbol esters (19) have been shown to modulate expression of mast cell MMP-9. However, the role of intercellular communication in modulating such expression, via cell-cell contact, remains unresolved.

We first determined whether mast cell-T cell interactions, which likely occur at sites of inflammation, could potentiate mast cell production of MMP-9. HMC-1 mast cells were incubated overnight with resting or activated peripheral blood-derived human T cells or Jurkat T cells in serum-free media; supernatants were then collected and analyzed for released mediators. Zymographic analysis of supernatants obtained from single cultures of resting or activated peripheral blood T cells or Jurkat T cells showed low basal levels of released MMP-9 (by activated Jurkat T cells) or no release at all (Fig. 1). Moreover, incubation of mast cells with resting T cells had no effect on MMP-9 release. In sharp contrast, coculture with activated peripheral blood-derived human T cells or Jurkat T cells markedly potentiated MMP-9 release by HMC-1

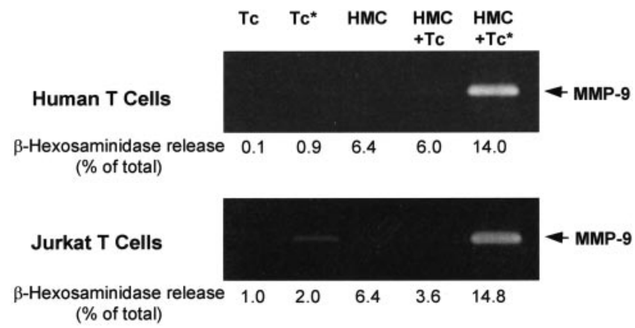

FIGURE 1. MMP-9 and β-hexosaminidase release from mast cells upon interaction with T cells. Resting (Tc) or activated (PMA (50 ng/ml) for 60 min, (Tc*)) peripheral blood-derived human T cells (*upper panel*) or Jurkat T cells (*lower panel*) were either incubated alone or in the presence of equal numbers of HMC-1 mast cells. Supernatants were collected at 20 h of incubation and analyzed for released MMP-9 by zymography and for released β-hexosaminidase by an enzymatic assay as described in *Materials and Methods*. Data are representative of five independent experiments.

cells (Fig. 1). Western blotting using anti-pro/active MMP-9 mAb indicated that the released MMP-9 was exclusively in the active 84-kDa form (data not shown). A 2- to 3-fold increase in HMC-1 cell degranulation, as determined by β-hexosaminidase activity, was found after coincubation with activated, but not resting, both T cell types (Fig. 1). These results suggest that mast cell MMP-9 release is highly regulated by interactions with activated, but not resting, T cells. Since similar results were obtained using either peripheral blood T lymphocytes or Jurkat T cells, subsequent experiments were performed using Jurkat T cells. As has been previously reported by us (14, 15), using a microporous membrane (Transwell; Costar, Cambridge, MA) to separate between the two cell populations, or supernatants from activated T cells did not induce β-hexosaminidase nor MMP-9 release from HMC-1 mast cells (data not shown).

To verify that induction of MMP-9 and β-hexosaminidase release was due to direct cell-cell contact and to enable us to work with a single-cell system (i.e., mast cells), membranes from resting or activated Jurkat T cells were isolated and incubated with HMC-1 cells for 20 h. Membranes obtained from activated, but not resting, Jurkat T cells induced mast cell degranulation, as demonstrated by a pronounced increase (~5-fold) in β-hexosaminidase activity in the supernatants (Fig. 2*A*). Furthermore, MMP-9 release was markedly induced in HMC-1 cells via direct contact with activated, but not resting, T cell membranes (Fig. 2*B*, *upper panel*). This result was also confirmed by Western blotting using anti-MMP-9 mAb (Fig. 2*B*, *lower panel*). Similar results, that is, T cell membrane-induced β-hexosaminidase and MMP-9 release (data not shown), were obtained by using an additional method for isolation of T cell membranes (method 2 in *Materials and Methods*), which has been reported to provide purified plasma membranes with minimal microsomal/lysosomal contamination (26).

Previous studies have demonstrated that MMP-9 production in HMC-1 cells and mast cells from other origins is regulated by induction of MMP-9 gene expression (19–21). Thus, to gain a better understanding of MMP-9 secretion in this system, we examined whether induction of MMP-9 release by coincubation of mast cells with activated T cell membranes results from an up-regulation of MMP-9 gene expression. Although MMP-9 mRNA expression was not observed in resting HMC-1 cells or in HMC-1 cells incubated with resting T cell membranes, incubation with activated T cell membranes resulted in an up-regulated expression of MMP-9 mRNA (Fig. 2*C*). In contrast, MMP-2 was found to be

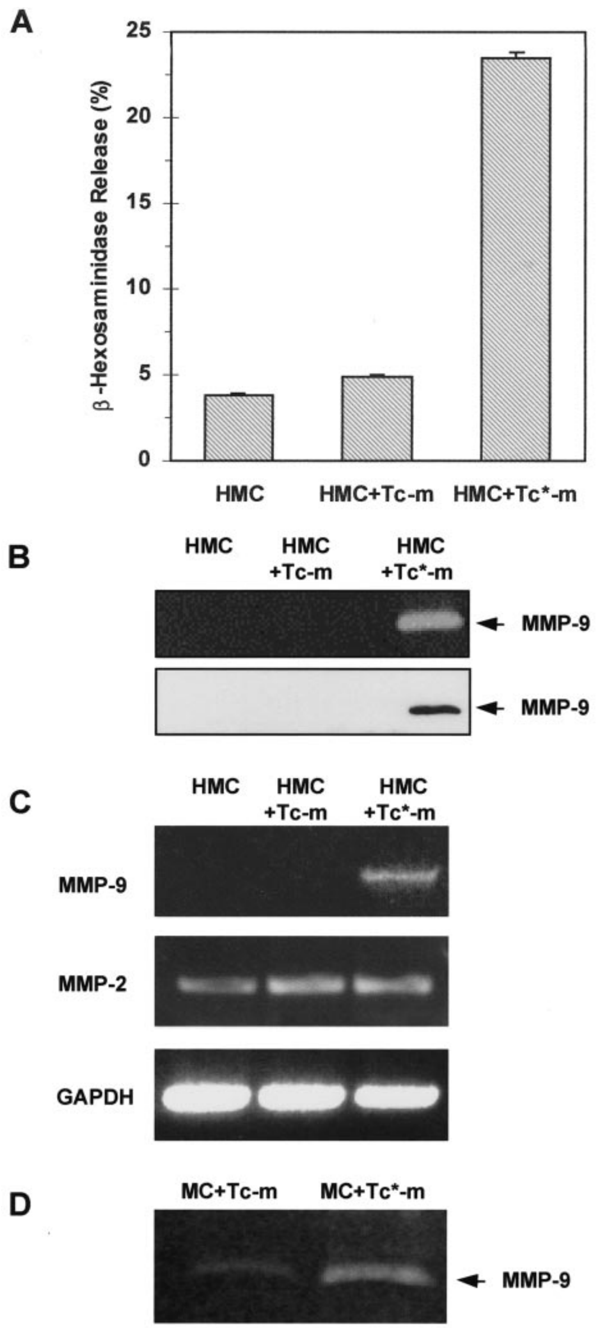

FIGURE 2. Membranes of activated T cells stimulate β-hexosaminidase and MMP-9 release. HMC-1 mast cells were incubated for 20 h with cell membranes isolated from an equal number of resting (Tc-m) or activated (Tc*-m) Jurkat T cells. Supernatants were collected after coincubation for measurement of β-hexosaminidase release (*A*) or of MMP-9 release by gelatin zymography and Western blotting (*B*). *C*, HMC-1 cells were treated as above and total RNA was isolated from HMC-1 cell lysates for analysis of human MMP-9 and MMP-2 expression by RT-PCR. GAPDH served as a control for constitutive gene expression. Amplified products were subjected to electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. *D*, Primary peripheral blood-derived human mast cells were incubated with T cell membranes as above, and the resultant supernatants were subjected to gelatin zymography for analysis of MMP-9 release. Data are representative of three independent experiments.

constitutively expressed in resting and activated HMC-1 cells (Fig. 2*C*).

Experiments were also performed using mature (9- to 10-wk-old) peripheral blood ($CD34^+$)-derived primary human mast cell cultures. Similar to our findings with the HMC-1 cell line, a marked increase in β-hexosaminidase release (>4-fold; data not shown) was observed as well as a pronounced augmentation in MMP-9 release in response to direct contact of these primary mast cell cultures with activated T cell membranes (Fig. 2*D*). These data provide evidence that direct contact between cell surface molecules on mast cells and on activated T cell membranes is sufficient to transduce the stimulatory signal in mast cells necessary for degranulation and MMP-9 release, independent of T cell intracellular function or production of cytokines and other mediators.

Kinetics of MMP-9 and β-hexosaminidase release

Although both β-hexosaminidase and active MMP-9 were secreted from mast cells upon activation with activated T cell membranes, the rate of their release was not defined. To compare the kinetics of β-hexosaminidase and active MMP-9 release, HMC-1 cells were incubated with activated T cell membranes for various periods of time, and supernatants were collected for analysis of these mediators. β-Hexosaminidase release was first noticed after 4 h of incubation with activated T cell membranes and increased over time, sharply peaking at 22 h (Fig. 3). This very slow pattern of secretion kinetics resembled very much that of MMP-9, except for a later onset of the latter (6 h as compared with 4 h; Fig. 3). The later onset of secretion may be explained by the fact that in contrast to MMP-9, β-hexosaminidase is prestored in mast cell granules (29).

Cell-cell contact induces mast cell release of TIMP-1

Although enzymatic activity of MMPs within an inflammatory milieu is partly determined by their levels of expression, their capacity to degrade ECM is also influenced by the expression of TIMPs, the natural inhibitors of MMP activity. TIMP-1, which is a prevalent TIMP found in body fluids, has been shown to be inducible in immune cells by several factors, including cytokines (30) and phorbol esters (31). To our knowledge, no studies to date have described mast cell synthesis of TIMP-1. Thus, we examined

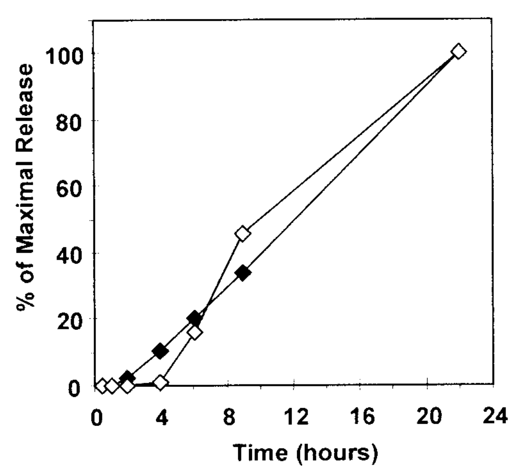

FIGURE 3. Kinetics of MMP-9 and β-hexosaminidase release. HMC-1 mast cells were incubated with activated Jurkat T cell membranes for various time periods and supernatants were collected for measurement of β-hexosaminidase (■) and MMP-9 (□) release. For the purpose of comparison between β-hexosaminidase and MMP-9 release, data are presented as the percentage of maximal release detected during the incubation period by using the specific measurement assay for each mediator. Maximal β-hexosaminidase release (detected at 22 h) was 27.7 ± 1.4%. MMP-9 was quantified by densitometry of the reversed zymogram bands using the TINA-PCbas software. Maximal MMP-9 release (detected at 22 h) was 132,057 arbitrary densitometric units.

whether activation of HMC-1 cells by T cell membranes exerts a stimulatory effect on mast cell production of TIMP-1. Secreted TIMP-1 was measured in supernatants, and the results are presented in Fig. 4. HMC-1 cells alone or HMC-1 cells incubated with resting T cell membranes secreted low levels (<10 ng/ml) of TIMP-1. In contrast, HMC-1 cells incubated with activated T cell membranes demonstrated a significant increase in the TIMP-1 level (120 ng/ml; Fig. 4).

Subcellular distribution of pro- and active MMP-9 in HMC-1 cells

Considering our finding that MMP-9 release, induced by mast cell-T cell interactions, corresponded with β-hexosaminidase release, it was important to determine whether MMP-9 was stored and secreted from the secretory granules of mast cells. MMP-9 gene expression was clearly induced by such interactions (Fig. 2*C*), and several studies have demonstrated induction of MMP-9 mRNA in mast cells from various species (19–21); however, no studies to date have reported whether MMP-9 is transported into, and possibly stored in, the secretory granules.

To examine the cellular localization of the newly synthesized MMP-9, HMC-1 cells were incubated with membranes of activated T cells for 20 h, then subjected to subcellular fractionation using continuous sucrose gradient. Samples of fractions taken from the gradient were subjected to SDS-PAGE and immunoblotting, and analyzed by using anti-human MMP-9 mAb that recognizes both the proenzyme and the active forms of MMP-9. As shown in Fig. 5*A*, the higher molecular mass form of MMP-9, corresponding to the proenzyme of 92 kDa, was predominantly found in fractions 5–10. Densitometric analysis of the Western blot indicated a distinct peak at fraction 8, with ~7-fold more pro-MMP-9 (92 kDa) than active MMP-9 (84 kDa; Fig. 5*B*). In contrast, a second peak consisting only of active MMP-9 (84 kDa), the predominant form seen by gelatin zymography and Western blotting (Figs. 1 and 2), was found in fractions 17–22 (Fig. 5, *A* and *B*). These results suggest that the proenzymes and active enzymes are distributed in distinct subcellular compartments upon induction of expression and protein synthesis. Of note, MMP-9 was absent from sucrose gradient fractions of control HMC-1 cells or cells incubated with nonactivated T cell membranes (data not shown).

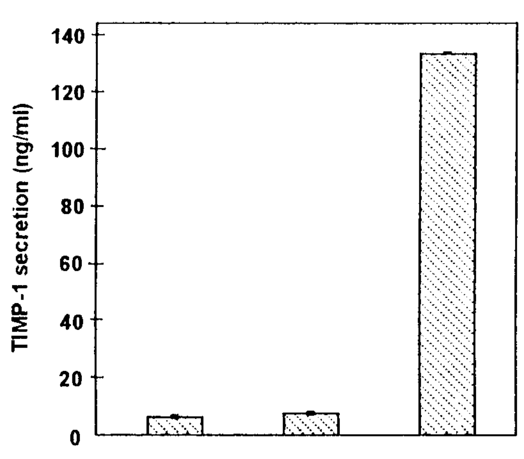

FIGURE 4. TIMP-1 release by mast cells. HMC-1 mast cells (1×10^6/ml) were incubated for 20 h alone or with cell membranes isolated from an equal number of resting (Tc-m) or activated (Tc*-m) Jurkat T cells. Supernatants were collected for analysis of TIMP-1 release using an ELISA kit specific for TIMP-1 detection. Data are mean ± SD of three independent experiments.

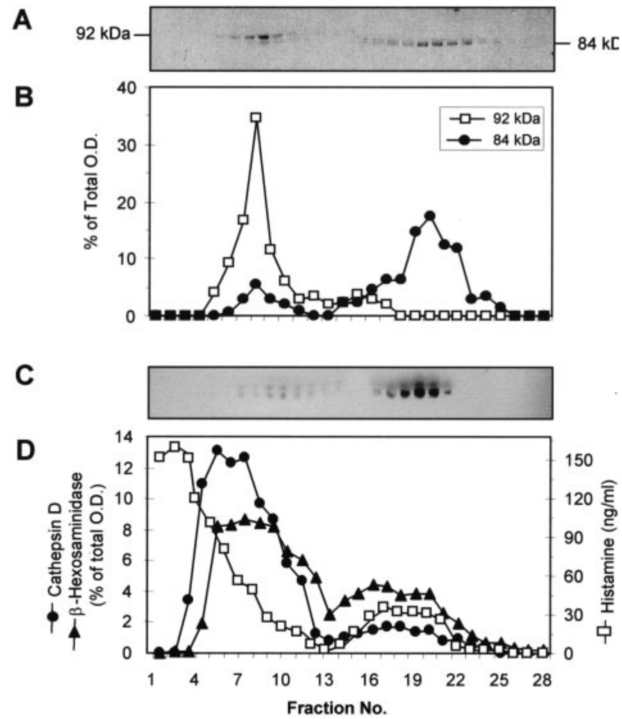

FIGURE 5. Subcellular localization of MMP-9 in HMC-1 cells. Subcellular fractions were isolated from HMC-1 cells incubated with activated T cell membranes by separation of the cell lysates on continuous sucrose gradients. Samples from the fractions were analyzed for MMP-9, β-hexosaminidase, histamine, and cathepsin D. *A,* MMP-9 was visualized by SDS-PAGE and immunoblotting with anti-pro/active human MMP-9 mAb. The 92-kDa inactive and 84-kDa active forms of MMP-9 are indicated. *B,* The intensity of the bands corresponding to pro-MMP-9 (92 kDa, □) and active MMP-9 (84 kDa, ●) was quantified by densitometry and is presented as percentage of total OD units measured. *C,* Tryptase was visualized by SDS-PAGE and immunoblotting with anti-human mast cell tryptase mAb. *D,* Histamine (□), β-hexosaminidase (▲), and cathepsin D (●) were measured as described in *Materials and Methods*.

To identify the putative subcellular fractions containing the inactive and active forms of MMP-9, the presence of histamine, tryptase, β-hexosaminidase, and the lysosomal enzyme cathepsin D in the gradient fractions was determined. A major peak of the mature form of cathepsin D was found in fractions 4–10. Yet a smaller peak (~15% of the total cathepsin D) was found in fractions 15–21 (Fig. 5*D*). β-Hexosaminidase was also distributed between these two peaks, with 60% of the total activity present in fractions 5–12. Histamine, which is a major constituent of mast cell secretory granules, migrated particularly at fractions 15–21, along with the remaining β-hexosaminidase activity (Fig. 5*D*). Histamine was also found at the top of the gradient, but this probably reflected the cytosolic pool and the content of secretory granules that were released during cell disruption. Tryptase, another prototypic granule-associated enzyme, was present in fractions 16–21 (Fig. 5*C*). It has previously been reported that fractions containing very high amounts of cathepsin D and β-hexosaminidase are likely to represent lysosomal/endosomal compartments (fractions 5–10, Fig. 5*C*), whereas fractions containing tryptase and histamine, as well as β-hexosaminidase (fractions 15–21; Fig. 5, *C* and *D*), likely represent the secretory granule compartment of HMC-1 cells (27). Taken together, it appears that the proenzyme of MMP-9 (92 kDa) may be transported to both lysosomes/endosomes and secretory granule compartments. However, only at the secretory granules is the enzyme converted to the 84-kDa active form.

Expression of cytokines induced by cell-cell contact

Since certain cytokines are known modulators of MMP production by leukocytes (31–33), we sought to investigate whether mast cell activation by T cell membranes results in cytokine expression. Therefore, the expression of the inflammatory cytokines IL-4 and IL-6 by mast cells was investigated. IL-4 gene expression was not detected, by RT-PCR, in control cells or cells incubated with resting T cell membranes. However, direct contact with cell membranes from activated T cells induced IL-4 expression (Fig. 6). Low levels of IL-6 mRNA were detected by RT-PCR in control cells or cells incubated with resting T cell membranes. Yet, direct contact with cell membranes from activated T cells markedly increased IL-6 expression (Fig. 6). Thus, MMP-9 gene expression appeared to be concomitantly induced with that of IL-4 and IL-6.

Kinetics of MMP-9 and TNF-α release

We have previously reported that in addition to stimulating mast cell degranulation, activated T lymphocytes also stimulate human mast cells to secrete TNF-α upon heterotypic aggregation (14). Release of TNF-α was likely independent of the mechanism leading to mast cell degranulation, since the phosphatidylinositol 3-kinase inhibitor wortmannin blocked β-hexosaminidase release, but not TNF-α secretion (14). TNF-α is considered a potent stimulator of MMP-9 release in leukocytes (30–32). We therefore studied the kinetics of TNF-α secretion by mast cells, induced by activated T cell membranes, compared with that of MMP-9. As can be seen in Fig. 7, TNF-α release started as early as 4 h following the incubation with activated T cell membranes, reaching a maximum at 6 h. These TNF-α kinetics are similar to those reported for IgE-mediated mast cell activation (34). In contrast, the release of active MMP-9 started only after 6 h of incubation, with a prolonged and steady rise to a peak at 22 h of incubation (Fig. 7). Accordingly, the release of TNF-α clearly precedes MMP-9 release and thus TNF-α may be a key factor in the regulation of MMP-9 expression and release.

Anti-TNF-α mAb inhibits MMP-9 induction and release

To confirm the dependency of MMP-9 induction on cytokine production, HMC-1 cells were cultured with activated T cell membranes for 20 h in the presence or absence of neutralizing mAb against human TNF-α, IL-4, and IL-6. Supernatants were collected for analysis of MMP-9 secretion and β-hexosaminidase release. Abs to TNF-α, IL-4, and IL-6 had no effect on β-hexosaminidase release induced by incubation with T cell membranes (data not shown). On the other hand, anti-TNF-α mAb inhibited both expression and release of MMP-9 induced by activated T cell membranes (Fig. 8, *A* and *C*), whereas anti-IL-4 and anti-IL-6 mAb did not alter levels of released MMP-9 (Fig. 8*B*). MMP-2 was unaffected by the Abs tested (Fig. 8*A*). Interestingly, experiments using HMC-1 cells incubated with increasing doses (1–20 ng/ml) of purified TNF-α indicated that soluble TNF-α alone is not sufficient to induce mast cell release of MMP-9 (data not shown).

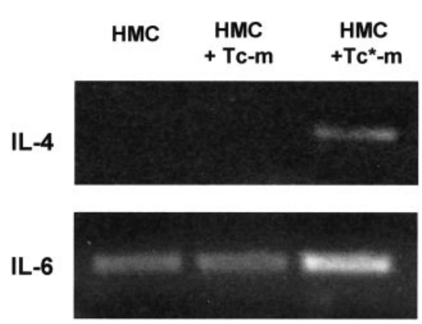

FIGURE 6. RT-PCR analysis of cytokine gene expression. HMC-1 mast cells were incubated with or without membranes isolated from either resting (Tc-m) or activated (Tc*-m) Jurkat T cells. Total RNA was isolated from HMC-1 lysates for analysis of the expression of human IL-4 and IL-6 by RT-PCR. Amplified products were subjected to electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. Gels shown are representative of four experiments.

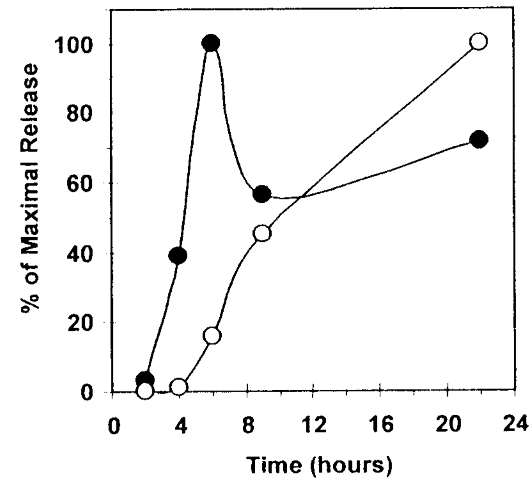

FIGURE 7. Kinetics of TNF-α and MMP-9 release. HMC-1 cells were incubated with activated Jurkat T cell membranes for various time periods, and supernatants were collected for measurements of released TNF−α (●) and MMP-9 (○). Data are presented as the percentage of maximal release, as described in the legend of Fig. 3. Maximal TNF-α release (detected at 6 h) was 213.9 U. Maximal MMP-9 release (detected at 22 h) was 132,057 arbitrary densitometric units.

Thus, heterotypic aggregation of mast cells and T lymphocytes affects multiple-related mast cell activities, including MMP-9 synthesis, degranulation, expression of various cytokines, and specifically TNF-α release. These findings provide further support of a functional mast cell-T cell relationships. Cell-cell transmission of signals is likely leading to important mast cell activities in allergic and T cell-mediated inflammation.

Discussion

The inflammatory milieu may contain several cell types, including monocytes/macrophages, B and T lymphocytes, neutrophils, and mast cells, each with their respective functions in inflammation and in restoring homeostasis. Although they may be specialized in their effector functions, these cells interact with each other and may thereby cause reciprocal modifications of their activities, such as expression of surface receptors, production of inflammatory cytokines, or changes in their activation (14, 35–37). These changes in immune cell activities originate from direct intercellular contact and thus manifest important functions underlying the inflammatory reaction.

Mast cells and T lymphocytes clearly have bidirectional influences on each other, as evidenced during T cell-mediated inflammation (6, 8, 9) and parasitic infections (3, 5). Such influences have primarily been attributed to the biological effects of T cell-derived soluble mediators on mast cell function (38, 39). However, recent investigations of mast cell-T cell interactions have revealed a novel intercellular communication exclusively involving the binding of cell surface molecules. Mast cells have been shown to degranulate and produce TNF-α upon direct contact with activated T cells (14), a process that may be regulated by LFA-1-ICAM-1 interaction (15). Considering that such T cell-dependent mast cell

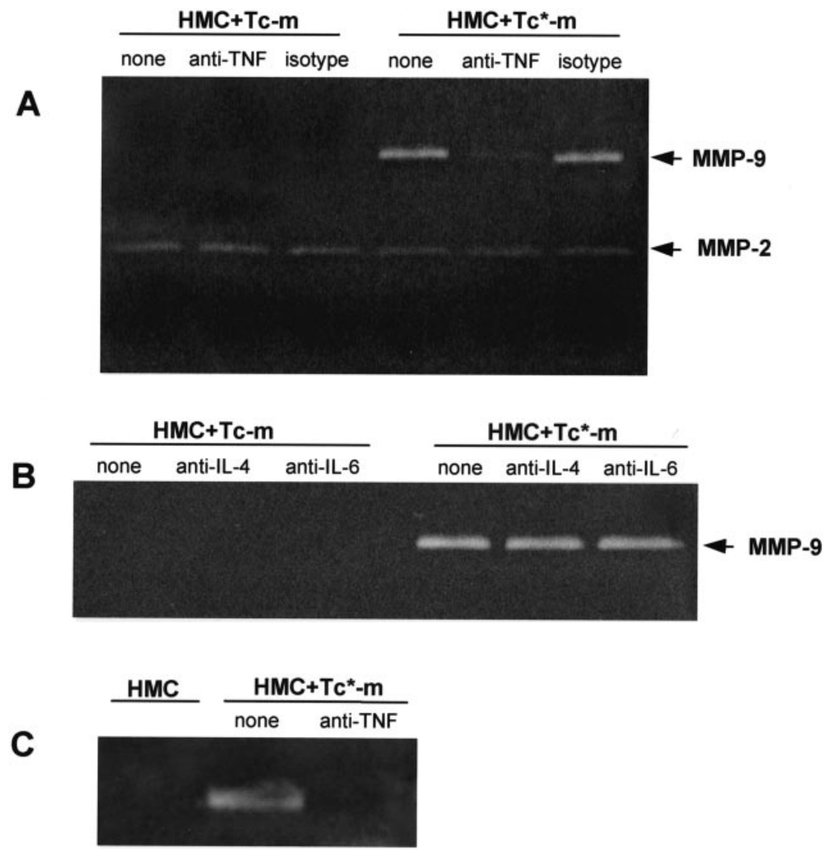

FIGURE 8. Anti-TNF-α mAb inhibits MMP-9 induction. HMC-1 mast cells were incubated with either resting (Tc-m) or activated (Tc*-m) Jurkat T cell membranes in the presence or absence of anti-human TNF-α neutralizing mAb (10 μg/ml, *A*) and control mouse IgG1 isotype Abs (10 μg/ml, *A*) and anti-human IL-4 (1 μg/ml, *B*) and anti-human IL-6 (1 μg/ml, *B*) mAb. Ab concentrations were according to the manufacturer's recommendations for maximal inhibition. Supernatants were collected after 20 h and analyzed by gelatin zymography for MMP-9 release. *C*, Inhibition of MMP-9 expression by anti-human TNF-α-neutralizing mAb as measured by RT-PCR.

activation may also involve other alterations in mast cell functions, we undertook the present study to investigate the role of mast cell-T cell interactions in regulating mast cell production of MMP-9 and TIMP-1, important mediators of ECM degradation. We now show that activated T cells cause a marked increase in the release of MMP-9 from human mast cells (Fig. 1). We further demonstrate that this effect is mediated by a direct contact of activated T cell membranes with mast cells (Fig. 2). This effect was established by using two types of human mast cells: the HMC-1 cell line (Fig. 2*B*) and primary cultures of mast cells isolated from $CD34^+$ peripheral blood cells (Fig. 2*D*).

Several lines of evidence have implicated cell-cell interactions between inflammatory cells as a mode of either bilaterally or directionally regulating MMP expression via specific receptors and counterreceptors. Endothelial cells can directly up-regulate expression of MMP-9 in monocytes (40) and in T cells (22) via ICAM-1-LFA-1 interactions. T cells are capable of directly inducing MMP-9 expression in fibroblasts (41), neutrophils (42), and monocytes (13, 43) through cell-cell contact. In addition to stimulating monocyte production of MMP-9 (43), interaction between CD40 on monocytes and CD40 ligand (gp39) on T cells was shown to stimulate monocyte expression of various cytokines and adhesion molecules (44).

To our knowledge, the results presented herein are the first evidence of MMP-9 regulation in mast cells by direct contact with another leukocyte population. Such contact with T lymphocytes correlated with induction of degranulation (Figs. 1 and 2*A*), release of TNF-α, and expression of the cytokines IL-4 and IL-6 (Fig. 6). Analysis of the kinetics of TNF-α production indicated that this cytokine was released by HMC-1 cells early (40% of maximal release as soon as 4 h of coincubation, reaching a maximum at 6 h) in response to interaction with activated T cell membranes (Fig. 7). In contrast, MMP-9 release required extended periods of incubation (22 h) with T cell membranes before a peak of secretion was attained (Figs. 3 and 7). These very slow kinetics of MMP-9 release appears to be correlated with that of the secretory granule exocytosis, as indicated by secretion of the granule associated enzyme β-hexosaminidase (Fig. 3). It seems therefore plausible that TNF-α may be an early mediator that is produced in response to cell-cell contact, thereby leading to MMP-9 expression. The newly released TNF-α may be specifically involved in the up-regulation of MMP-9 expression, but not in the secretion of β-hexosaminidase. Thus, these findings further support the notion that TNF-α may be required early for an autocrine regulatory pathway of MMP-9 gene expression, but not for the exocytotic degranulation process. TNF-α has been shown to be a potent stimulator of MMP-9 production (31, 32). However, although neutralization of TNF-α during incubation of mast cells with activated T cell membranes blocked MMP-9 production (Fig. 8), exogenous TNF-α was not sufficient to stimulate HMC-1 cell expression of MMP-9 (data not shown). This may be ascribed to a need for a preactivation state of the mast cells, which is achieved by incubation with activated T cells or T cell membranes and which is absent when the cells are incubated with soluble TNF-α alone.

Recently, the regulation of mast cell MMP activation by endogenous proteinases has been investigated. Dog mastocytoma cells were shown to constitutively secrete the zymogen and the active forms of MMP-9 and the active form of MMP-2. Endogenous α-chymase, released upon degranulation, was shown to be responsible for the activation of these MMP enzymes (17). Activation of MMP-9 and MMP-2 has also been attributed to the dog mastocytoma cell MMP-3, which is preactivated with endogenous tryptase or chymase (18). Our data on the subcellular distribution of MMP-9 in HMC-1 cells upon incubation with activated T cell membranes (Fig. 5) suggest that the mechanism of cell-cell stimulation plays a major role in the release of active MMP-9 from

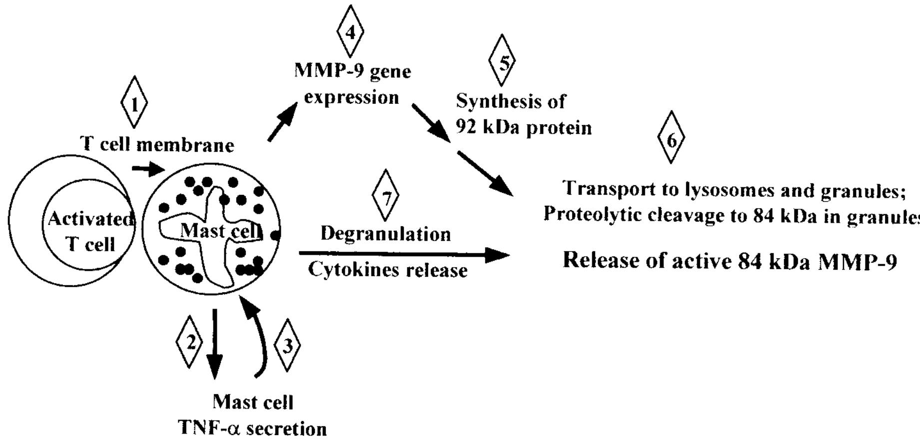

FIGURE 9. Schematic model of mast cell-T cell interactions. Step-by-step model for release of active MMP-9 from mast cells during interactions with T cells, as depicted by step numbers enclosed in diamonds. *1,* Activated T lymphocytes may come in direct cell-cell contact with tissue-resident mast cells during inflammation via as yet unidentified cell surface molecules. *2,* Such contact induces relatively rapid mast cell secretion of TNF-α. *3,* TNF-α released from mast cells may, in turn, regulate gene expression in these cells. *4,* MMP-9 gene expression is up-regulated in response to TNF-α. *5,* The 92-kDa MMP-9 protein is synthesized and transported to lysosomes/endosomes and secretory granules. *6,* The 92-kDa proenzyme is proteolytically cleaved in the secretory granules into the 84-kDa active enzyme. *7,* Heterotypic adhesion between mast cells and activated T cells may also result in the release of granule-associated mediators and various cytokines.

within mast cell granules. Such localization of MMP-9 in the granular fractions of human mast cells, predominantly as an active enzyme (84 kDa), is a novel finding, since previous studies only demonstrated activation of MMP-9 by extracellular α-chymase (17). As illustrated in Fig. 9, it is conceivable from the data presented herein that upon contact with activated T cells, mast cell expression of MMP-9 is up-regulated by either endogenous soluble TNF-α or T cell membrane-bound TNF-α. The newly synthesized zymogen (92 kDa) is then transported into endosomes/lysosomes and into the secretory granules as well. α-Chymase, tryptase, or other proteinases prestored at the secretory granules may convert the zymogen into its active 84-kDa form. This model of intracellular MMP-9 localization/activation is further supported by the observations that 1) direct contact with activated T cell membranes concomitantly stimulates mast cell degranulation in similar time kinetics as MMP-9 release and 2) active MMP-9 cofractionated with histamine and tryptase in the granular fraction of subcellular components. Thus, we propose that mast cells may receive stimulatory signals while interacting with activated T lymphocytes, that may serve to influence local MMP-9 release, as well as other mast cell activities within the inflammatory microenvironment. We currently focus on identifying the specific stimulatory signals expressed by activated T cells that lead to enhancement of MMP synthesis and release.

The exact role of secreted TIMP-1, which is also induced upon mast cell-T cell membrane interactions (Fig. 4), in regulating the activity of MMP-9 is not yet evident. Correlative up-regulation of MMP and TIMP-1 release by cytokines or other stimuli has been described in other leukocytes (30, 31). Considering the robust responses of mast cells to direct contact with activated T cells, it is probable that TIMP-1 is also induced to counterbalance the substantial levels of active MMP-9 released from secretory granules. This form of regulation may aid in limiting and defining the degree of localized MMP-9 degradation of ECM components.

In summary, the present study provides support for the concept of a functional relationship between mast cells and activated T cells involving either soluble mediators secreted from both cell populations or direct cell-cell contact. Herein, we show evidence that direct contact between the two cell types stimulates mast cells to produce and release several granule-associated mediators, cytokines and MMP-9, which possess immunoregulatory and/or immunomodulatory properties. These mast cell activities may support reciprocal activities by other leukocytes, such as cell migration, as well as promote structural and biochemical changes in the ECM microenvironment during T cell-mediated inflammation.

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