

Mast Cell-Fibroblast Interactions Induce Matrix Metalloproteinase-9 Release from Fibroblasts: Role for IgE-Mediated Mast Cell Activation¹

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Mast cells adhere to fibroblasts, but the biological effects of adhesion are not well understood. We hypothesized that these adhesive interactions are important for tissue remodeling through the release of matrix metalloproteinases (MMP). Murine bone marrow cultured mast cells (BMCMC) were cocultured with NIH-3T3 fibroblasts or murine lung fibroblasts (CCL-206) and supernatants analyzed for MMP-9 release by gelatin zymography. Coculture of BMCMC for 24 h with NIH-3T3 or CCL-206 fibroblasts increased the release of MMP-9 from fibroblasts by 1.7 ± 0.2 and 2.0 ± 0.7 -fold, respectively. Coculture of BMCMC and fibroblasts in the presence of IgE increased further MMP-9 release, which was released by fibroblasts. MMP-9 release was dependent on TNF released from IgE activated BMCMC and on adhesive interactions between BMCMC and fibroblasts. Increased MMP-9 release was also p44/42-dependent, as was MMP-9 up-regulation during coculture of fibroblasts with resting BMCMC. Finally, IgE injection into the mouse ear increased MMP-9 content of the ear tissue in the absence of Ag, indicating that IgE-mediated remodeling may play a pathogenic role in allergic conditions even in the absence of exposure to allergens. In conclusion, mast cell-fibroblast interactions induce the release of proteases important for tissue remodeling, such as MMP-9. MMP-9 release was further increased in the presence of IgE during coculture, suggesting a role for mast cell-fibroblast interactions in atopic conditions. *The Journal of Immunology*, 2008, 180: 3543–3550.

A part from their extensively studied role in immediate hypersensitivity reactions, mast cells are also involved in other inflammatory conditions including fibrosis. Mast cell numbers increase in scleroderma (1), pulmonary fibrosis (2), and hypertrophic scars (3). Mast cell hyperplasia is also evident in animal models of lung fibrosis (4, 5). Furthermore, allergic asthma, a condition characterized by mast cell activation, shows increased production of collagens and extracellular matrix (ECM)³ proteins as well as subepithelial fibrosis (6). Fibrosis also occurs in the skin of patients with atopic dermatitis (7) and in mastocytosis patients (8).

Histological studies in rats suggest that mast cell degranulation is followed by changes in fibroblast proliferation and in the consistency of connective tissue ground substances (9). Mast cell mediators, such as tryptase (10), heparin (11), TNF, and TGF- β (12) increase fibroblast proliferation and stimulate collagen mRNA synthesis. Mast cells are also able to synthesize ECM components

(13) and may this way affect proliferation, survival, and activation status of fibroblasts. Despite all this information on the effects of soluble mast cell mediators on fibroblast functions, little is known about direct interactions between the two cell types (14).

Our hypothesis is that direct and/or indirect interactions between mast cells and fibroblasts promote tissue inflammation and remodeling. Interactions between these two cell types are known to have proinflammatory effects (15–17), but less is known about mediators involved in tissue remodeling. Matrix metalloproteinases (MMP) are zinc and calcium-dependent proteases that digest most ECM components. MMP are produced by structural cells such as fibroblasts, endothelial and epithelial cells, and by many inflammatory cells including macrophages, eosinophils, neutrophils, and mast cells (reviewed in Ref. 18). They are secreted as latent proenzymes and converted to the active form by proteolytic cleavage of an amino-terminal domain (19). MMP are important in tissue remodeling in the airways through their ability to affect the integrity of the basal lamina and the degree of infiltration by inflammatory cells.

MMP-9 in particular plays an important role in lung inflammation and remodeling through its potential to facilitate tissue infiltration by inflammatory cells (20, 21) and/or activate other proinflammatory mediators (22, 23). MMP-9 is an important mediator of inflammation in a murine model of asthma (24). Asthmatic individuals also show abundant expression of MMP-9 in the airways (25). MMP-9 levels are increased in sputum (26) and bronchoalveolar lavage fluid (27) of asthmatic individuals and increase further in status asthmaticus (28). Furthermore, MMP-9 is also increased in other inflammatory obstructive lung diseases, such as in chronic obstructive pulmonary disease patients during exacerbations (29).

Tissue fibrosis comprises another manifestation of tissue remodeling in chronic asthma and is the cardinal manifestation of remodeling in fibrotic diseases of the lung. MMP-9 is increased in the bronchoalveolar lavage (30) and epithelial lining fluid (31) of

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Received for publication March 29, 2007. Accepted for publication December 17, 2007.

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¹ This work was supported by grants from the Canadian Institutes of Health Research, Alberta Heritage Foundation for Medical Research, and The Lung Association of Alberta and Northwest Territories. H.V. is an Alberta Heritage Foundation for Medical Research Scholar.

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³ Abbreviations used in this paper: ECM, extracellular matrix; MMP, matrix metalloproteinase; FN, fibronectin; HA, hemagglutinin; BMCMC, bone marrow cultured mast cell; TIMP, tissue inhibitor of metalloproteinase.

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patients with idiopathic pulmonary fibrosis. Both alveolar macrophages (31) and fibroblasts (32) from patients with idiopathic pulmonary fibrosis make higher amounts of MMP-9 than those from normal individuals. Finally, lung fibrosis in IL-13 transgenic animals is dependent on the potential of MMP-9 to activate latent TGF β_1 in the lungs of these mice (22, 33).

In this study, we show that mast cell adhesive interactions with fibroblasts lead to MMP-9 release from fibroblasts mediated through c-kit-stem cell factor-dependent adhesion and p44/42 (Erk1/2) activation in fibroblasts. Furthermore, IgE-mediated activation of mast cells, in the absence of Ag, enhances MMP-9 release through both adhesive interactions and the release of TNF from mast cells. Finally, we show that IgE can increase MMP-9 tissue content in the mouse ear *in vivo* in the absence of Ag, using potentially similar mechanisms to the ones we studied *in vitro*.

Materials and Methods

Materials

RPMI 1640, penicillin/streptomycin, HEPES, L-glutamine, nonessential amino acids (BioWhittaker); DMEM (American Type Culture Collection, Manassas, VA); plasma fibronectin (FN), lipofectamine (Invitrogen Life Technologies); murine recombinant stem cell factor and IL-3 (PeproTech); anti-murine c-kit (clone ACK45) and anti-murine TNF mAb, OptEIA Mouse TNF Set (BD Pharmingen); mouse monoclonal anti-tissue inhibitor of metalloproteinases-2 (TIMP-2) Ab clone 3A4 (Medicorp); goat anti-mouse IgG IRDye800 conjugated Ab (Rockland); anti-hemagglutinin (HA) mAb, clone HA-11 (Covance Research Products); rabbit polyclonal anti-p44/42, anti-phospho-p44/42 (Thr202/Tyr204), p44/42 MAP Kinase Assay Kit (Cell Signaling Technology); LI-COR blocking buffer for Western blots (LI-COR); flat-bottom 96-well plates (Linbro, Flow Laboratories); calcein AM (Molecular Probes); piceatannol, U0126, SB203580, wortmannin (Calbiochem); murine IgE anti-DNP (clone SPE-7), human serum albumin-conjugated DNP, Coomassie Brilliant Blue G-250 and gelatin (Sigma-Aldrich); PVDF Immobilon-P transfer membranes (Millipore); Hyperfilm (Amersham Pharmacia Biotech) were purchased as shown. The pcDNAIII vector carrying HA-tagged ERK-2 was a gift from Dr. J. S. Gutkind, (National Institute on Dental Research).

Cell cultures

Bone marrow cultured mast cells (BMCMC) were obtained as described (34) from male BALB/c mice (obtained from Charles River Laboratories) and cultured in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 4 mM L-glutamine, 25 mM HEPES, 100 μ g/ml penicillin/streptomycin, 50 μ M 2-ME, and 10% FBS (cRPMI). The medium was also supplemented with 10 ng/ml murine recombinant stem cell factor and mIL-3 and was changed every 4 days. BMCMC were used after 3–5 wk in culture when >98% of the cells were mast cells as determined by flow cytometry for c-kit expression. BMCMC were also obtained in a similar fashion from MMP-9 knock out mice (FVB.Cg-Mmp9 tm1Tvu/J, obtained from The Jackson Laboratories) and littermate controls (FVB/NJ). University of Alberta Health Sciences Laboratory Animal Ethics Board approved all experiments using mice.

NIH-3T3 fibroblasts and CCL-206 murine lung fibroblasts (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% FBS. Fibroblasts were subcultured using trypsin when they were 85–90% confluent.

Coculture experiments

NIH-3T3 or CCL-206 murine fibroblasts were grown in 12-well plates until confluent. BMCMC (5×10^5 or 10^6) were added in 0.5 ml of DMEM and cells were cocultured for up to 24 h. Supernatants were collected and kept frozen at -70°C until analyzed for MMP-9 or TNF content. For inhibition experiments, both cell types were incubated for 30 min with the indicated concentrations of inhibitors or Abs before initiating coculture.

Gelatin zymography

SDS-PAGE gelatin zymography was performed using 7% polyacrylamide gels containing 0.2% gelatin (35). Supernatants from BMCMC and/or fibroblasts cultured alone or together were assayed. Following electrophoresis at 4°C , gels were washed in 2% Triton X-100 at room temperature and then incubated in 50 mM Tris-HCl buffer (pH 7.6) supplemented with 0.15 M NaCl, 5 mM CaCl_2 , and 0.05% NaN_3 at 37°C for 24 h. Following

incubation, the gels were stained for 1 h with 0.05% Coomassie Brilliant Blue G-250 and then destained overnight in 20% isopropanol/10% acetic acid.

The gels were photographed using Alpha imager 2200 (Alpha Innotech), and then the pictures inverted to be analyzed by densitometry. These inverted pictures are shown in the figures in this paper.

Western blot analysis

Supernatants from BMCMC, fibroblasts, or cocultures were separated on a 10% SDS gels and then transferred to PVDF Immobilon-P membranes. Western blot was performed with a mouse monoclonal anti-TIMP-2 Ab (2 μ g/ml) in PBS mixed 1/1 with LI-COR blocking buffer. The membranes were subsequently incubated with goat-anti-mouse IgG IRDye800-conjugated secondary Ab (1/10,000 dilution) for 1 h at room temperature. Proteins were visualized using the Odyssey Infrared Imaging System (LI-COR) and analyzed using Odyssey Software Release v1.1 (LI-COR).

Densitometry

Optical densities of zymograms and Western blot films were measured using the Sigma Gel Analysis program (Jandel Scientific, version 1.0.5.0, 1995). Band intensities of zymograms were expressed as fold of coculture or otherwise activated cells over the band intensity of fibroblasts cultured alone under the same conditions (average $\pm$ SEM). Only samples run in the same gel were compared.

Cytokine measurement

TNF was measured in BMCMC or coculture supernatants by ELISA using Ab-matching pairs from BD Pharmingen, as per the manufacturer's instructions.

Adhesion assay

A fluorescent adhesion assay was used as described (36). To study BMCMC adhesion to FN, flat-bottom 96-well plates were coated with plasma FN (20 μ g/ml). To study BMCMC adhesion to fibroblasts, NIH-3T3 cells were grown in the same 96-well plates until confluent and then used for adhesion assays.

Calcein-loaded BMCMC were added in each well alone (for spontaneous adhesion) or together with activating agents for 1 h. The nonadherent cells and medium were then aspirated and the wells washed three times. Fluorescence was measured using a Millipore Cytofluor 2350 plate reader before washing the plates (total fluorescence) and following the washing procedure.

The % adhesion was calculated using the following formula:

$$\% \text{ cell adhesion} = (\text{fluorescence in adherent cells} / \text{total fluorescence}) \times 100$$

All assays were performed in duplicate. One value, the mean of the two measurements, was calculated for each condition in each independent experiment. The results in the text are given as the mean $\pm$ SEM of *n* independent experiments.

p44/42 *in vitro* kinase assay

To study the activation of signal transduction pathways, fibroblasts were transfected with pcDNAIII vectors carrying HA-tagged ERK-2 (37). Transfection was conducted in 6-well plates using 10- μ l lipofectamine and 4 μ g of DNA per transfection, which were determined to be the optimal conditions. Cells were incubated in complete medium for 24 h after transfection and then used in activation experiments.

Transfected NIH-3T3 cells were cocultured with BMCMC for the indicated times. HA-tagged ERK-2 was immunoprecipitated with anti-HA mAb and an *in vitro* kinase assay, using recombinant transcription factor Elk-1 as substrate for active ERK-2, was performed with an assay kit as per the manufacturer's instructions. Then, proteins were separated on 10% SDS gels and transferred to a nitrocellulose membrane. Western blot was performed with an anti-phospho-Elk-1 rabbit polyclonal Ab (1/1000 dilution) in tris-buffered saline with 0.05% Tween 20 and 5% BSA. The membranes were subsequently incubated with goat-anti-rabbit IgG HRP-conjugated secondary Ab (1/5,000 dilution) for 1 h at room temperature. Proteins were visualized by ECL on Hyperfilm.

In vivo IgE administration in the ear

Murine IgE anti-DNP (25 μ g) or normal saline was injected into the ear pinna in 25 μ l of PBS. Mice were sacrificed 24 h later and the ears were removed. Ear tissue was homogenized following freezing in liquid nitrogen

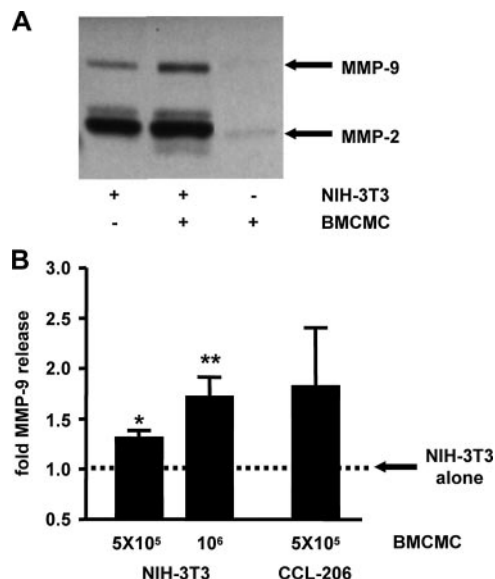


FIGURE 1. MMP-9 up-regulation following mast cell fibroblast coculture. BMCMC (5×10^5 or 10^6 cells) were cocultured with murine fibroblasts (NIH-3T3 or CCL-206 lung fibroblasts) for 24 h. Supernatants were analyzed for MMP-9 release by gelatin zymography. **A**, Representative gelatin zymography for MMP-9 and MMP-2 release from NIH-3T3 and BMCMC cultured alone or together for 24 h. **B**, MMP-9 release following coculture of NIH-3T3 or CCL-206 fibroblasts with 5×10^5 or 10^6 BMCMC for 24 h ($n = 11$ and 12 respectively). Release is shown as fold increase over the amount of MMP-9 released by NIH-3T3 cultured alone under the same conditions (*, $p = 0.001$ and **, $p = 0.0085$, respectively as compared with NIH-3T3 cells cultured alone).

and total protein was measured in the homogenate with a Bradford assay. Then, $25 \mu\text{g}$ of protein were separated on a 7% polyacrylamide zymography gel, and zymography performed as described before.

Statistical analysis

Statistical differences between treatment groups were determined using one-way ANOVA. Paired Student *t* test was used to analyze the results for statistical significance when only two conditions were compared. A value of $p < 0.05$ was considered statistically significant.

Results

Coculture of mast cells and fibroblast up-regulates MMP-9 release

Resting NIH-3T3 fibroblasts released both MMP-9 and MMP-2 in culture, while BMCMC under the same conditions did not release detectable levels of MMP-9 and only very low levels of MMP-2 (Fig. 1A). Coculture of NIH-3T3 fibroblasts with BMCMC increased the amount of MMP-9 released. In all cases, the bands corresponded to proMMPs. In some experiments we could also detect activated forms of MMPs following mast cell fibroblast cocultures, but this was not a consistent finding.

To quantitate this increase, we compared MMP-9 release during coculture to the release of fibroblasts alone, because BMCMC alone did not show any significant MMP-9 release. Coculture of NIH-3T3 with 5×10^5 or 10^6 BMCMC increased MMP-9 release by 1.31 ± 0.07 -fold ($p = 0.001$, $n = 11$) and 1.72 ± 0.19 -fold ($p = 0.0085$, $n = 12$) respectively (Fig. 1B). We also used a murine lung fibroblast cell line, CCL-206. Coculture of BMCMC with CCL-206 fibroblasts showed a trend for increased MMP-9 release (2.03 ± 0.66 -fold increase, $n = 5$, $p > 0.05$). Coculture of mast cells with NIH-3T3 or CCL-206 fibroblasts had no effect on MMP-2 release (Fig. 1A, quantitation not shown). Survival of BMCMC in coculture experiments was comparable to that of

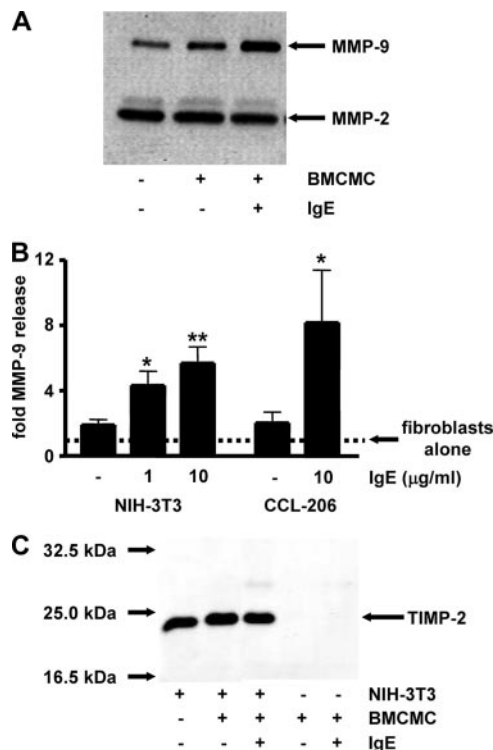


FIGURE 2. MMP-9 release up-regulation following fibroblast coculture with IgE-activated BMCMC. **A**, Representative gelatin zymography for MMP-9 and MMP-2 release from NIH-3T3 alone and NIH-3T3 cocultured with resting or IgE-activated BMCMC (10^6) for 24 h. **B**, MMP-9-fold induction following BMCMC coculture with NIH-3T3 or CCL-206 fibroblasts in the presence of various concentrations of IgE for 24 h (*, $p < 0.01$ and **, $p < 0.001$). **C**, Western blot analysis of TIMP-2 release from NIH-3T3 fibroblasts and BMCMC cultured alone or together in the presence or absence of IgE (a representative blot is shown).

BMCMC in the presence of IL-3 and SCF (flow cytometric analysis using Annexin V showed $79.5 \pm 6.4\%$ vs $79.9 \pm 2.8\%$ live cells respectively, $n = 6$).

IgE-mediated mast cell activation further increases MMP-9 release following coculture with fibroblasts

Recent studies have shown that IgE is sufficient to induce mast cell activation without the need for FcεRI cross linking with specific Ag (38, 39). Activation of BMCMC with IgE anti-DNP (clone SPE-7, $10 \mu\text{g/ml}$) further increased coculture induced MMP-9 release (Fig. 2A). When BMCMC were cultured alone they did not release detectable levels of MMP-9 following activation with IgE or IgE and Ag (data not shown). IgE ($10 \mu\text{g/ml}$) induced a 5.67 ± 1.02 -fold increase in MMP-9 release compared with MMP-9 release from NIH-3T3 alone ($p < 0.001$, $n = 8$) while IgE at $1 \mu\text{g/ml}$ a 4.32 ± 0.88 -fold increase ($p < 0.01$, $n = 7$) (Fig. 2B). Although there is a trend for higher effect of $10 \mu\text{g/ml}$ compared with $1 \mu\text{g/ml}$, this difference was not statistically significant. We also examined whether IgE activation of BMCMC up-regulated MMP-9 release in cocultures with lung fibroblasts. When we activated BMCMC with IgE ($10 \mu\text{g/ml}$), MMP-9 release increased by 8.56 ± 3.6 -fold compared with the release from CCL-206 fibroblasts alone ($n = 5$, $p < 0.01$) (Fig. 2B).

In contrast to its effect on MMP-9 release, coculture of NIH-3T3 fibroblasts with BMCMC had no effect on TIMP-2 release (Fig. 2C), whether we cocultured them in the presence or absence of IgE, indicating that the increased release of MMP-9 is not followed by increased release of MMP inhibitors. Mast cells when cultured

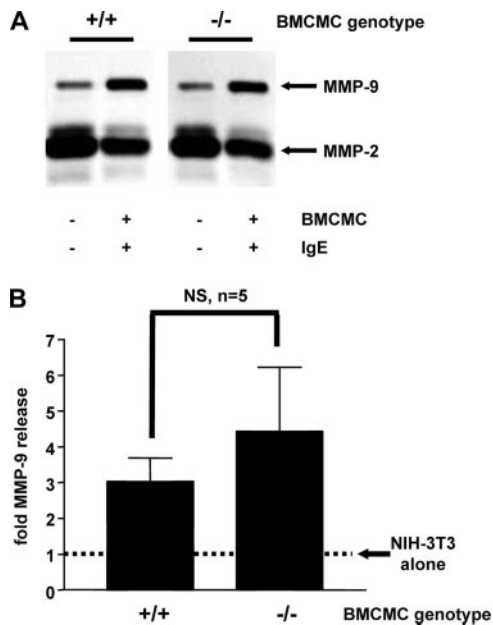


FIGURE 3. MMP-9 release up-regulation following fibroblast coculture with IgE-activated BMCMC from MMP-9 knock out and control mice. *A*, Representative gelatin zymography for MMP-9 and MMP-2 release from NIH-3T3 cocultured with IgE-activated ($1 \mu\text{g/ml}$) BMCMC (10^6) from MMP-9 knock out (-/-) and littermate control (+/+) mice for 24 h. *B*, MMP-9-fold induction following coculture of NIH-3T3 with BMCMC from MMP-9 knock out (-/-) or littermate control (+/+) mice in the presence of IgE ($1 \mu\text{g/ml}$) for 24 h ($n = 5$) compared with fibroblasts alone.

alone, in the presence or absence of IgE, did not release detectable amounts of TIMP-2 under similar conditions (Fig. 2C).

The results so far do not indicate which cell type released MMP-9 following coculture. Although BMCMC under the conditions used in these experiments do not release detectable levels of MMP-9 either resting (Fig. 1A) or after IgE-mediated activation (data not shown) there is a possibility they may release MMP-9 following coculture with fibroblasts. To exclude this possibility we cultured BMCMC from MMP-9 knock out mice or from their littermate controls with NIH-3T3 fibroblasts and compared the effect of IgE-mediated BMCMC activation in the MMP-9 release following coculture (Fig. 3A). Coculture of NIH-3T3 fibroblast with BMCMC from MMP-9 knock out mice in the presence of IgE ($1 \mu\text{g/ml}$) induced the release of MMP-9 (4.43 ± 4.0 -fold over NIH-3T3 fibroblasts alone) as did coculture of fibroblasts with BMCMC from +/+ mice, (3.03 ± 1.48 -fold increase compared with fibroblasts alone) (Fig. 3B). However, there was no difference in the amount of MMP-9 released whether we used MMP-9 knock out or +/+ derived BMCMC (Fig. 3B). These results would indicate that the MMP-9 released following coculture comes from fibroblasts and not mast cells, because the effect is unchanged in case where mast cells are unable to make MMP-9.

TNF partially mediates the up-regulation of MMP-9 release following coculture of fibroblasts with IgE activated mast cells

IgE-mediated BMCMC activation for 24 h induced the release of TNF in a dose dependent fashion in the absence of Ag crosslinking (Fig. 4A), as has been shown before (40). Under the same conditions there was no β -hexosaminidase release indicating that monomeric IgE did not cause degranulation (data not shown). The amount of TNF released was the same whether BMCMC were cultured in suspension, adherent to fibronectin, or adherent to NIH-3T3 cells (data not shown). Therefore, we studied whether TNF

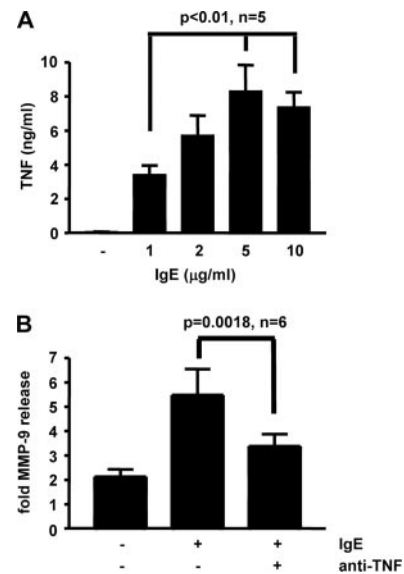


FIGURE 4. Role of TNF in MMP-9 up-regulation. *A*, Dose response of IgE-induced TNF release from BMCMC following 24 h incubation. *B*, Effect of anti-TNF Ab on MMP-9 release from NIH-3T3 fibroblasts following coculture with IgE-activated BMCMC ($n = 6$).

mediated the effects of IgE on MMP-9 up-regulation following BMCMC coculture with fibroblasts.

Anti-TNF Ab ($10 \mu\text{g/ml}$) completely inhibited the effect of recombinant TNF (up to 100 ng/ml) on MMP-9 release from NIH-3T3 fibroblasts (data not shown). The same anti-TNF Ab ($10 \mu\text{g/ml}$) partially inhibited the effects of IgE-activated BMCMC on the release of MMP-9 following coculture with fibroblasts (Fig. 4B). Anti-TNF Ab decreased MMP-9 release from 5.48 ± 1.07 to 3.38 ± 0.5 -fold ($n = 6, p = 0.0018$). Isotype control Abs had no effect on MMP-9 release. Therefore, the effect of IgE-activated BMCMC on MMP-9 release following coculture with fibroblasts is partially mediated through TNF release from mast cells, because it is not completely inhibited by anti-TNF Abs.

Role of adhesion on MMP-9 up-regulation

Mast cells have been shown to adhere to fibroblasts at least partially through c-kit-SCF interactions (41). BMCMC adhered to NIH-3T3 fibroblasts spontaneously. In particular, $20.4 \pm 1.3\%$ of BMCMC adhered to fibroblasts following 1 h coculture ($n = 4, p < 0.05$). BMCMC activation with IgE had no effect on the adhesion of BMCMC to fibroblasts (data not shown). However, similar with what has been shown before (40), activation of BMCMC with IgE induced a dose dependent mast cell adhesion to fibronectin (data not shown). Therefore, we investigated whether adhesive interactions between BMCMC and fibroblasts are required for MMP-9 up-regulation during coculture.

Anti-c-kit Abs ($10 \mu\text{g/ml}$) decreased MMP-9 release following coculture of IgE-activated BMCMC and NIH-3T3 fibroblasts from 5.51 ± 1.31 to 3.43 ± 0.66 -fold ($n = 5, p = 0.0317$) (Fig. 5). This Ab inhibited mast cell adhesion to fibroblasts by $>70\%$ ($n = 3$), but did not affect mast cell survival during the 24 h of coculture (data not shown). Isotype control Abs had no effect on MMP-9 release. Furthermore, the anti-c-kit Ab had no effect on the release of TNF following IgE-mediated mast cell activation ($1824 \pm 846 \text{ pg/ml}$ without anti c-kit and $1471.2 \pm 884.2 \text{ pg/ml}$ in the presence of anti c-kit, $n = 5, p > 0.05$). These results indicate that TNF is not the only mechanism of MMP-9 up-regulation. Other stimuli induced by adhesion, whether soluble mediators or direct adhesion-induced cell activation, may also be involved.

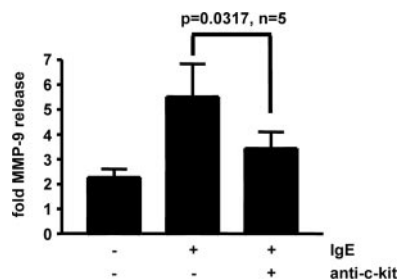


FIGURE 5. Role of adhesion in MMP-9 up-regulation. Effect of anti-c-kit Ab on MMP-9 release following BMCMC coculture with NIH-3T3 fibroblasts ($n = 5$).

Signaling mechanism of MMP-9 up-regulation during coculture experiments

We next studied the role of various signaling molecules, known to be involved in FcεRI signaling, in coculture-induced MMP-9 up-regulation. Only the p44/42 inhibitor U0126 (12.5 μM), but not the Syk inhibitor piceatannol (20 μM), PI3-kinase inhibitor wortmannin (5×10^{-8} M) or the p38 inhibitor SB203580 (600 nM), inhibited MMP-9 release following coculture of fibroblasts with IgE activated BMCMC ($n = 5$) (Fig. 6A). The p44/42 inhibitor U0126 also inhibited TNF production by IgE activated BMCMC. The Syk inhibitor piceatannol had only a small effect, while wortmannin and SB203580 had no effect on TNF release (Fig. 6B).

We also studied the signal transduction pathways leading to up-regulation of MMP-9 release following coculture of fibroblasts with resting BMCMC. Baseline MMP-9 release from fibroblasts was not affected by p44/p42 inhibitors. However, the up-regulation seen after coculture with BMCMC was abolished following preincubation with the p44/42 inhibitor U0126 ($n = 4$, $p < 0.05$) (Fig. 7A). Piceatannol, wortmannin, and SB203580 had no effect on either baseline MMP-9 release from fibroblasts or coculture induced MMP-9 release (data not shown).

Because p44/42 inhibitors down-regulated the effect of BMCMC-fibroblast coculture on MMP-9 release, we studied whether the coculture induced p44/42 activation. To be able to differentiate between p44/42 activation in fibroblasts and

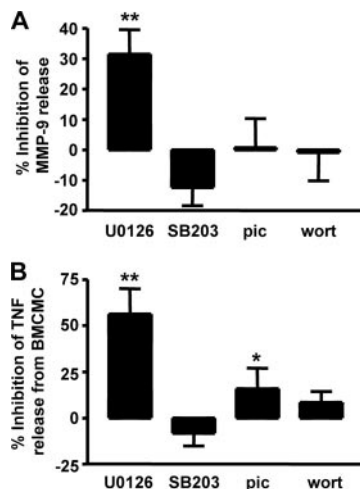


FIGURE 6. Signaling pathways of MMP-9 up-regulation. Effect of inhibitors of p44/42 MAPK (U0126, 12.5 μM), p38 MAPK (SB203580, 600 nM), Syk kinase (piceatannol, 20 μM), and PI3K (wortmannin, 5×10^{-8} M) on MMP-9 release (A) or TNF release (B) following coculture of IgE-activated BMCMC with NIH-3T3 fibroblasts (*, $p < 0.05$ and **, $p < 0.01$).

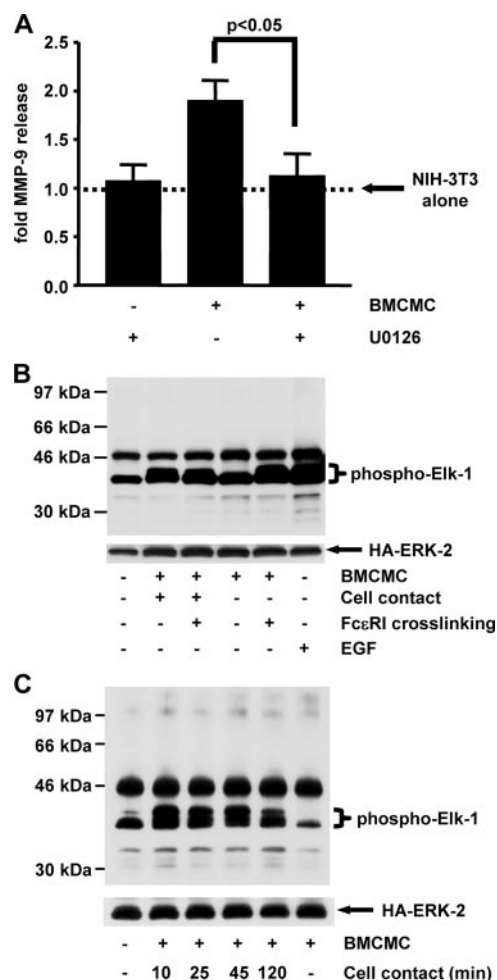


FIGURE 7. ERK-2 activation following mast cell-fibroblast adhesion. A, Effect of preincubation with U0126 (12.5 μM) on MMP-9 release following coculture of NIH-3T3 cells with 10^6 BMCMC for 24 h ($n = 4$). B, In vitro kinase assay of ERK-2 activity in NIH-3T3 fibroblasts alone (lane 1) or following adhesion of BMCMC (lanes 2 and 3) or separated by a filter (lanes 4 and 5) with (lanes 3 and 5) or without (lanes 2 and 4) FcεRI crosslinking. Lane 6 shows ERK-2 activity following fibroblast activation with Epidermal Growth Factor as positive control. Lower panel shows western blot with anti-HA Ab as control for equal loading. C, Time course of ERK-2 activation following mast cell adhesion to NIH-3T3 fibroblasts. Lane 1 shows fibroblasts alone, lanes 2–5 shows different time points after adhesion between mast cells and fibroblasts and lane 6 shows the two cell types separated by a filter. Lower panel shows Western blot with anti-HA Ab as control for equal loading.

BMCMC, we transfected the fibroblasts with HA-tagged ERK-2 and then immunoprecipitated the tagged ERK-2 with an anti-HA mAb. Coculture of NIH-3T3 cells with BMCMC induced ERK-2 activation in the fibroblasts (Fig. 7B). This activation was cell-to-cell contact dependent because when a semipermeable membrane separated the two cell types to inhibit cell-to-cell contact, there was no fibroblast ERK-2 activation (Fig. 7B). ERK-2 could also be activated following FcεRI-mediated BMCMC activation without cell contact (Fig. 7B), presumably through soluble mediators induced by FcεRI activation. ERK-2 activation following adhesion was rapid and prolonged, reached maximum at 10 min, and lasted for over 120 min (Fig. 7C). These data indicate that BMCMC adhesion to fibroblasts induces p44/42 activation and this activation is at least in part responsible for coculture-mediated MMP-9 up-regulation.

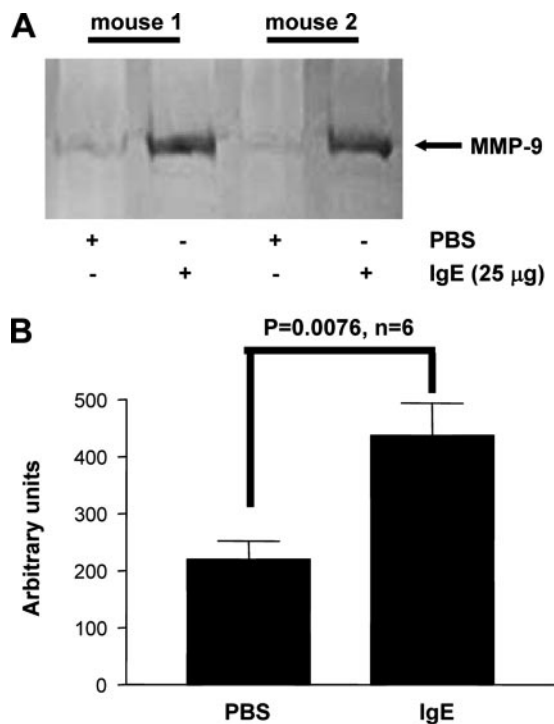


FIGURE 8. MMP-9 content of ears injected with IgE or PBS. **A**, Representative gelatin zymography for MMP-9 content of ears injected with IgE or PBS. **B**, MMP-9 content in ears injected with IgE or PBS ($n = 6$, $p < 0.0076$).

In vivo effects of monomeric IgE without allergen

To study whether IgE could up-regulate MMP-9 release in vivo in the absence of allergen, we administered monomeric IgE (25 μ g) into the ear pinna of naive mice. The same volume of saline was administered to the other ear as control. The ears were removed

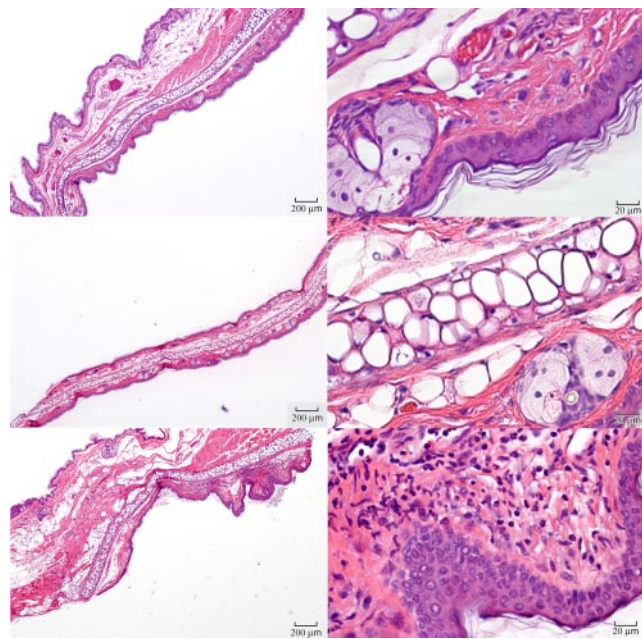


FIGURE 9. Representative sections from IgE- and saline-injected ears. Representative photographs from saline injected ears (top panels), IgE injected ears without inflammatory changes (middle panels), and IgE-injected ears with mild inflammation (lower panels) at $\times 92$ (left panels) and $\times 920$ (right panels) magnification are shown.

24 h later, homogenized, and analyzed by zymography for the presence of MMP-9. Administration of IgE without Ag increased the MMP-9 content of the ear by 2-fold compared with the ears receiving PBS (Fig. 8). PBS did not change the ear MMP-9 content compared with noninjected ears (data not shown). We also performed histological analysis of IgE and saline injected ears from three mice. Out of the three ears injected with IgE, two appeared completely normal histologically and were indistinguishable from the ears injected with saline (Fig. 9, middle panels compared with top panels). These IgE-injected ears had no evidence of infiltrating inflammatory cells and the tissue showed no evidence of edema. However, one ear injected with IgE showed mild infiltration with inflammatory cells (Fig. 9, lower panels).

Discussion

We have shown that coculture of mast cells and fibroblasts leads to MMP-9 release that is dependent on p44/42 activation in fibroblasts following c-kit-mediated mast cell-fibroblast adhesive interactions. Using BMCMC from MMP-9 knock out mice, we showed that the MMP-9 released during coculture comes primarily from fibroblasts and not mast cells. Furthermore, coculture-induced MMP-9 release was enhanced when mast cells were activated by a highly cytokinergic IgE clone in the absence of Ag. This increase was partially mediated by TNF release from IgE-activated mast cells. Adhesive interactions between mast cells and fibroblasts still played a role in MMP-9 release, independently from TNF release from mast cells.

MMP-9 plays an important role in the pathophysiology of inflammatory and fibrotic diseases of the lung. However, the physiological stimuli that induce MMP-9 production during inflammatory conditions and the role of particular cells in MMP-9 production in the lung are not fully understood. Mast cells, and in particular tryptase and TNF released from mast cells, have been implicated in the up-regulation of MMP in human aortic endarterectomy specimens (42) and in endometrial stroma cells (43), but there is no available information about the direct role of mast cells in MMP-9 release in lung or airway inflammatory conditions. Our results indicate that direct adhesive interactions between mast cells and fibroblasts, as well as mast cell mediators such as TNF, may be important for release of MMP-9 in airway inflammatory conditions. The balance between proteases and anti-proteases in the lung also plays an important role in inflammatory diseases, including asthma (44). In our case, mast cell activation induced the release of MMP-9 from fibroblasts without significant effect on TIMP-2. Therefore, the increased production of MMP-9 is not counteracted by increased production of its inhibitors and may lead to increased inflammation and/or remodeling.

Lung fibroblasts are known to express MMP-9 (32, 45) and MMP-9 synthesis and release is up-regulated by TNF (45). Our results indicate that MMP-9 is released from fibroblasts during coculture with mast cells. Of note, under the conditions used in our experiments mast cells alone did not release detectable levels of MMP-9. Previous reports have shown MMP-9 production by BMCMC (46, 47). We believe that we were unable to detect MMP-9 release from mast cells cultured alone or in the presence of IgE, because our culture conditions included SCF, which down-regulates the release of MMP-9 from mast cells (46). Furthermore, in that report, BMCMC were also cultured in much higher cell concentrations for up to 3 days before MMP-9 was detectable in the supernatant. However, it is still possible that under the coculture conditions we used, soluble mediators or direct cell-to-cell contact induced the release of small amounts of MMP-9 from mast cells, although the bulk of MMP-9 came from fibroblasts.

Our results also indicate that mast cell adhesive interactions with fibroblasts activate signal transduction pathways in fibroblasts without the need for other activation signals. It would be interesting to evaluate whether mast cell-dependent activation of fibroblasts has synergistic effects with other fibroblast-specific activation stimuli. In that case, mast cell adhesive interactions with fibroblasts may represent a priming event for fibroblasts that come in close contact with mast cells, especially in cases of mast cell hyperplasia.

Recently, IgE was shown to induce mast cell survival, proliferation, and cytokine production (38, 39) without Ag-induced Fc ϵ RI cross-linking, but through low-level spontaneous receptor dimerization. Our experiments further indicate that IgE-induced mast cell activation in association with cell-to-cell interactions with other cells, may be sufficient to induce the release of factors involved in tissue remodeling, such as MMP-9, even in the absence of specific Ag. This suggests that the atopic state, characterized by high IgE levels, may be sufficient to induce changes leading to airway remodeling and inflammation before the development of overt allergic inflammation and the development of manifestations of asthma or other allergic diseases. IgE binding to mast cells may lead to increased local MMP-9 concentration in the absence of specific allergen. The released MMP-9 would then increase the local concentration of activated inflammatory mediators and the ability of inflammatory cells to be recruited into these tissues. Consequently, the release of other proinflammatory mediators following exposure to specific allergen will find the tissues primed for induction of the characteristic inflammatory changes seen in asthma or other inflammatory conditions. This observation would also imply that anti-IgE effectiveness in allergic inflammation might also be, at least partially, attributed to the down-regulation of remodeling irrespective of Ag presence in the airways or other tissues of allergic individuals.

The physiological relevance of this observation is also underscored by the *in vivo* results presented in this study. Administration of IgE into the mouse ear increased the MMP-9 content of the tissue, indicating the initiation of inflammatory and remodeling pathways even though the animal did not come in contact with any Ag. There is very little known regarding Ag-independent *in vivo* effects of IgE. IgE promotes skin sensitization to haptens independently of Ag specificity (48), an effect that is mast cell dependent, although does not require mast cell degranulation. It is likely that mechanisms similar with the one presented here, release of MMP-9 or other proinflammatory molecules through Fc ϵ RI occupancy, may mediate these Ag-independent *in vivo* IgE effects.

IgE alone, in the absence of Ag-mediated Fc ϵ RI cross-linking, stimulates MAPK p44/42 and p38 activation in mast cells (38). It also induces IL-6 release, which is mediated by p44/42, p38, and PI3K activation (38). IgE alone also mediates mast cell adhesion to fibronectin that is dependent on PI3K but not p44/42 or p38 activation (40). Finally, Fc ϵ RI activation is linked to Syk kinase activation (49). In our experiments p44/42, but not p38, PI3K or Syk, was involved in MMP-9 release in cocultures of fibroblasts with IgE-activated mast cells. Inhibitors of p44/42 also down-regulated the release of TNF from IgE-activated BMCMC, indicating that TNF down-regulation may be the reason for decreased MMP-9 release. However, the same p44/42 inhibitor abolished the increased MMP-9 release seen following coculture of fibroblasts with resting mast cells, indicating that p44/42 also plays a direct role in MMP-9 up-regulation after coculture.

In conclusion, mast cells induce MMP-9 release from fibroblasts both directly through cell to cell contact and indirectly through the release of soluble mediators and this release is increased in cases of high occupancy of the Fc ϵ RI on mast cells. These interactions

may play an important role in initiating tissue remodeling in a variety of conditions with mast cell hyperplasia and/or increased levels of circulating IgE.

Acknowledgments

We thank Dr. Lakshmi Puttagunta for help with histological analysis and Dr. Nancy Arizmendi for help with the mast cell survival studies. We thank Drs. Dean Befus, Redwan Moqbel, Paige Lacy, and Lisa Cameron for useful discussions and critical reading of the manuscript. We thank Dr. Dean D. Metcalfe for discussions and encouragement during the initial phases of this project.

Disclosures

The authors have no financial conflict of interest.

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