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Human Mast Cells Augment Fibroblast Proliferation by Heterotypic Cell-Cell Adhesion and Action of IL-4

Axel Trautmann,1* Georg Krohne,† Eva-B. Bröcker,* and C. Eberhard Klein*

Mast cells have been implicated in the pathogenesis of fibrosis because of their increased number in chronic inflammatory reactions. In a previous study, we had shown that human mast cells readily attach and form heterotypic cell-cell contacts when seeded on top of fibroblast monolayers. Here, we report that human mast cells stimulate fibroblast proliferation after cell-cell contact. Proliferation was measured by 5-bromo-2'-deoxyuridine or [3H]thymidine uptake of subconfluent fibroblast monolayers after attachment of mast cells that had been preincubated with mitomycin C. An 18-h coculture of the human mast cell line HMC-1 doubled proliferation of normal skin fibroblasts. Moreover, normal mast cells prepared from neonatal foreskin doubled fibroblast proliferation.

The stimulatory effect was dependent on heterotypic cell-cell contact since it was not transferred by tissue culture supernatants from mast cells. We hypothesized that mast cell cytokines secreted after heterotypic cell-cell contact stimulate fibroblast proliferation. Several mast cell-derived cytokines were tested for effects on fibroblast proliferation. Only IL-4 was able to double fibroblast proliferation. Additional experiments revealed that: 1) the stimulatory effect of IL-4 as well as of the mast cell coculture could be completely abrogated by preincubation of fibroblasts with an anti-IL-4R mAb blocking ligand binding; 2) mast cell-derived IL-4 acts as a second signal for fibroblasts since it amplifies the action of low doses of obligatory fibroblast growth factor such as fibroblast growth factor or platelet-derived growth factor.

Preparation of human mast cells

Suspensions of human skin mast cells were prepared by enzymatic digestion of human foreskin obtained after circumcision. Skin was immediately placed in HEPES buffer (137 mM NaCl, 5.6 mM D-glucose, 10 mM HEPES, 2.7 mM KCl, 0.4 mM NaH2PO4, 0.5 mM MgCl2, 1.0 mM CaCl2, pH 7.3), stored at 4°C, and processed within 1 h. The skin was cut with scissors; tissue fragments were washed twice in HEPES buffer containing 0.1% BSA (HEPES-BSA). After incubation with collagenase A from Clostridium histolyticum (1.5 mg/ml, Boehringer, Mannheim, Germany) and hyaluronidase from bovine testes (0.75 mg/ml, Boehringer) in HEPES-BSA for 2 h at 37°C in a shaking water bath, the resulting cell suspension was filtered through gauze; undigested tissue was incubated with fresh enzyme for a second period. Dispersed cells were washed twice in HEPES-BSA, recovered by centrifugation, and stored at 4°C in PIPES-A (137 mM NaCl, 5.6 mM D-glucose, 10 mM PIPES, 2.7 mM KCl, 0.1% human serum albumin, pH 7.4) before use.

Proliferation assay

The proliferation of fibroblasts was determined using a 5-bromo-2'-deoxyuridine (BrdU)2 labeling procedure (all reagents from Boehringer). Cells grown in 96-well microtiter plates were incubated with 10 μM BrdU for 18 h. After fixation with 0.5 M ethanol/HCl, cells were incubated with nucleases to partially digest the DNA. Incorporated BrdU was detected using monoclonal anti-BrdU Ab (Fab fragments). The bound conjugates were visualized with the soluble chromogenic substrate ABTS and measured by an ELISA reader. In confirming experiments, the “classical” [3H]thymidine incorporation assay was used.

Fibroblasts, 5 × 105 cells/well, were plated in 96-well flat-bottom plates and maintained (37°C, 5% CO2) for at least 48 to 72 h in culture. Then mast cells, preincubated with mitomycin C (2 h, 15 μg/ml) (Boehringer), were seeded (1000/well) into the plates. The effect of the mast cell coculture with fibroblasts on fibroblast proliferation was assessed in RPMI medium with or without 10% FCS. In some experiments, fibroblasts and mast cells were separated using the Transwell coculture system: 24-well cluster...
plate; 6.5-mm-diameter insert; membrane thickness, 10 μm; pore size, 3.0 μm (Costar, Cambridge, MA).

In a series of experiments, we compared the effect of the mast cell/fibroblast coculture on fibroblast proliferation with that of several relevant cytokines (IL-1β, IL-4, IL-6, IFN-γ, platelet-derived growth factor (PDGF) A/B, basic fibroblast growth factor (bFGF; Boehringer) under the same conditions in the absence of mast cells. Again, fibroblast proliferation was measured after 18 h of incubation.

In inhibition assays, mAbs were added to the fibroblast monolayer 2 h before the mast cells were seeded into the plate: CDw124 (monoclonal mouse IgG1 anti-human IL-4 receptor, 0.1–10 μg/ml, Genzyme, Cambridge, MA); 22221.3 (monoclonal mouse IgG2A anti-human TNF-α receptor, 1–10 μg/ml, R+D Systems, Abingdon, U.K.); A2 (monoclonal mouse IgG1 anti-human β2 integrin, 1–10 μg/ml). In an inhibition assay, a neutralizing anti-IL-4 Ab (anti-human IL-4, polyclonal neutralizing Ab, goat IgG, 0.1–10 μg/ml, R+D Systems) was added to the mast cell-fibroblast coculture together with the mast cells.

**Flow cytometry analysis**

Cell surface expression of the IL-4 receptor on the fibroblasts was analyzed by flow cytometry. Briefly, cells (5 × 10^5) were washed with PBS + 1% BSA, incubated with primary Abs for 30 min, washed again (three times), and subsequently incubated with FITC-labeled F(ab’), fragments of rabbit anti-mouse or rabbit anti-rat Ig (Dako, Glostrup, Denmark) for 30 min. After final washes (three times), resuspended cells were analyzed using a FACScan (Becton Dickinson, Mountain View, CA).

**Mast cell stimulation and release of IL-4**

To analyze under which circumstances HMC-1 mast cells release IL-4, cells were stimulated in 24-well polystyrene plates with PMA (25 ng/ml) or the calcium ionophore A23187 (0.2 μM) (Sigma, St. Louis, MO) for 24 h at 37°C. IL-4 was measured in supernatants as well as in cell lysates after detergent solubilization (0.5% Nonidet P-40) and the addition of proteinase inhibitors (2 mM PMSF, 4 μg/ml aprotonin; all from Sigma) by using a commercial ELISA assay.

**Electron microscopy**

Mast cell/fibroblast cocultures seeded on glass coverslips were fixed with 7% buffered glutaraldehyde (0.1 M phosphate, pH 7.4) at 4°C for 12 to 18 h and were then dehydrated in acetone and critical point dried with CO2. Cocultures grown on plastic tissue culture plates were directly dehydrated in ethanol and incubated for 60 min in hexamethyldisilazane (Sigma) (2 × medium change) and then air dried. Specimens were sputtered with 20 to 30 nm gold. Photographs were taken with a Zeiss scanning electron microscope (DSM 962, Zeiss, Oberkochen, Germany).

**Cell viability**

To exclude toxic effects of the experimental procedures on fibroblasts or mast cells, we monitored their viability by trypan blue exclusion and in the case of fibroblasts by examining their integrity in the monolayers using an inverted phase contrast microscope. At the concentrations and time periods used, none of the treatments changed cell viability or cell shape.

**Statistical analysis**

Data are expressed as mean ± SEM. Statistical analysis was performed by the Student t test. p values of 0.01 or less were considered significant.

**Results**

Previously, we have demonstrated that human mast cells form intimate cell-to-cell contacts with fibroblasts (9). These contacts are not mediated by any of the known integrin receptors expressed on the cells. In the present study, we investigated the effects of mast cells on fibroblast proliferation after allowing them to attach on top of fibroblast monolayers.

**Mast cells enhance fibroblast proliferation**

Microscopic and ultrastructural observation of the human mast cell line HMC-1 normally growing in suspension revealed that the cells, when seeded on top of fibroblasts, readily adhere and spread (9) (Fig. 1). We now investigated how the human mast cell line HMC-1 as well as normal human mast cells isolated from foreskin affect fibroblast proliferation. To exclude that the proliferation rate of the mast cells interferes with the measurements, mast cells were arrested in the cell cycle by preincubation with mitomycin C. Mitomycin C-treated HMC-1 cells as well as mast cells from human foreskin showed no difference in attachment to fibroblast monolayers compared with nontreated cycling mast cells.

Proliferation was quantified in a nonradioactive proliferation assay using the BrdU labeling procedure and the [3H]thymidine uptake assay. We found that the human mast cell line HMC-1 as well as normal human mast cells prepared from neonatal foreskin doubled F135-60-86 fibroblast proliferation (Fig. 2A). Three of five human fibroblast cell lines tested showed increased proliferation after cell-cell contact with mast cells (Table I). When mast cells and responder fibroblasts were cocultured in a Transwell system, not allowing cell-cell contact, the increase of fibroblast proliferation was not observed (Fig. 2B).

Exchange of information between mast cells and fibroblasts may depend on the production of soluble factors that can act on neighboring cells in a paracrine fashion. Various cytokines are known to display a variety of activities to modulate fibroblast functions (11). Therefore, we investigated several cytokines to determine whether they would similarly enhance fibroblast proliferation in our assay system as the mast cell/fibroblast coculture. Among them were PDGF and FGF, known to be potent stimulators of fibroblast proliferation, which were used as positive controls. Interestingly, not only PDGF A/B and bFGF but also IL-4 readily enhanced fibroblast proliferation in a dose-dependent manner (Fig. 3). The increase of fibroblast proliferation by IL-4 was seen only when the medium contained FCS. No increased fibroblast proliferation was seen with IL-6, IL-1β, and IFN-γ (Fig. 3).

Neutralizing anti-human IL-4 receptor Abs inhibit the increase of fibroblast proliferation induced by mast cells

To assess whether the stimulatory effect of the mast cell-fibroblast coculture is mediated by IL-4 secreted by mast cells, fibroblast monolayers were preincubated with saturating amounts of an anti-IL-4 receptor Ab blocking ligand binding. The mAb abrogated the increase of fibroblast proliferation induced by the coculture with HMC-1 or normal mast cells in a dose-dependent manner (Fig. 4). This indicated that IL-4 indeed is the relevant mediator which apparently is secreted into the intercellular space by mast cells after cell-cell contact with fibroblasts. However, the addition of a neutralizing anti-IL-4 Ab had no inhibitory effect on the increase of fibroblast proliferation induced by the mast cell-fibroblast coculture (data not shown).
All five fibroblast cell lines were analyzed for the surface expression of the IL-4 receptor and were found to be positive (range of mean fluorescence intensity, 412 to 583; FACScan software, Becton Dickinson) (Table I). The incubation of F135–60–86 fibroblasts with bFGF (0.1 or 0.25 ng/ml) did not up-regulate IL-4 receptor levels on the cell surface (FACS analysis) after 18 h of incubation (data not shown).

IL-4 is synthesized and can be released by human mast cells

To prove that the mast cell line HMC-1 is capable of synthesizing IL-4, lysates of HMC-1 cells were tested by ELISA. Lysates contained 55.3 ± 6.7 pg IL-4/10^6 cells. IL-4 was released after PMA stimulation but not after treatment with the calcium ionophore A23187 (Fig. 5). IL-4 protein was not detectable in culture supernatants of untreated HMC-1 cells. Furthermore, coculture of mast cells with F135-60-86 fibroblasts did not induce IL-4 release from the mast cells (HMC-1 cells, skin mast cells) into the culture supernatants (data not shown).

IL-4 acts as a second signal requiring the presence of low doses of obligatory fibroblast growth factors

Based on our observation that IL-4 was capable to increase fibroblast proliferation only in the presence of FCS we hypothesized that IL-4 may act as a second signal requiring low doses of primary factors regulating fibroblast proliferation. Experiments in the absence of FCS with supplementation of low doses of obligatory fibroblast growth factors in addition to IL-4 indeed showed that

<table>
<thead>
<tr>
<th>Fibroblast Cell Line</th>
<th>Source</th>
<th>Passage</th>
<th>Increased Proliferation After Cell-Cell Contact</th>
<th>IL-4R on the Cell Surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>F135-60-86</td>
<td>Fetal skin</td>
<td>5–10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FRF</td>
<td>Adult foreskin</td>
<td>6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FGS</td>
<td>Child foreskin</td>
<td>5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FZSN</td>
<td>Child foreskin</td>
<td>3–4</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>FPJ</td>
<td>Adult foreskin</td>
<td>5</td>
<td>−</td>
<td>+</td>
</tr>
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</table>

*Expression of the IL-4 receptor on the cell surface of the fibroblasts as determined by flow cytometry (range of mean fluorescence intensity 412 to 583; FACScan software, Becton Dickinson).
this is the case. The supplementation of low doses of bFGF alone (0.1 or 0.25 ng/ml) which by itself only marginally increased fibroblast proliferation was sufficient to induce the maximally possible proliferative response of the fibroblast cultures in this assay when IL-4 (5 ng/ml) was present (Fig. 6). By supplementation of bFGF alone, maximal fibroblast proliferation required 10 to 50 times higher doses of bFGF (up to 5 ng/ml) than in the presence of IL-4 (Fig. 6). Again, IL-4 alone under serum-free conditions did not increase fibroblast proliferation.

Discussion

Mast cells produce a number of multifunctional cytokines (1, 8). Such molecules are potentially important mediators in mast cell/fibroblast interactions. In this study, we provide direct evidence that mast cells may regulate the proliferation of neighboring fibroblasts in tissues by secretion of IL-4. This signal is directly transmitted by the IL-4 receptor expressed on the fibroblast surface. Moreover, release of IL-4 by mast cells appears to be strictly regulated and is dependent on heterotypic cell-cell contact.

There is evidence that fibroblasts influence mast cell maturation/differentiation (12). In this context, stem cell factor produced by fibroblasts seems of major importance (13). Thus far, very few studies provide direct evidence that mast cells may influence functions of terminally differentiated fibroblasts, although this has often been postulated, particularly in the context of emergence of fibrosis in chronic inflammation (2–4). Our study conducted under defined conditions demonstrates direct action of mast cells on fibroblast proliferation and elucidates the nature of this activity.

Most of the information on regulation of fibroblast activity, especially of the metabolism of structural proteins by cytokines and growth factors, has been obtained from in vitro studies of cultured cells. It is well accepted that mediators, such as TGF-β, PDGF, IFN-γ, IL-1, bFGF, IL-6, epidermal growth factor, and TNF-α are...
involved in physiologic and pathologic processes, relevant for wound healing and/or the development of fibrosis (11). Monroe et al. first demonstrated that IL-4 stimulates fibroblast proliferation (14). Our results demonstrate that release of IL-4 by mast cells induced by heterotypic cell-cell contact between mast cells and fibroblasts may be of prime importance for the regulation of fibroblast proliferation in chronically inflamed tissues containing increased numbers of mast cells.

IL-4 has many effects that promote chronic inflammation, including stimulation of T cell proliferation and TH2 cell development (15), induction of LFA-1 and LFA-3 on B cells (16), activation of macrophages (17), induction of VCAM-1 on endothelial cells (18), and as demonstrated in this article the proliferation of fibroblasts. It is our hypothesis that the release of IL-4 from mast cells provides a relevant stimulus for fibroblast proliferation. Thus, in addition to being the source of histamine and eicosanoids, which are responsible for most of the symptoms of the early phase of an allergic response, mast cells may play an important role in chronic inflammation leading to fibrosis by the production and release of IL-4.

A reciprocal influencing of both cell types may be taken for granted in a coculture of mast cells and fibroblasts, reference having already been made to the evident effects of fibroblasts on the maturing and differentiation of mast cells. It may be considered that fibroblasts activate c-kit+ mast cells via their membrane-bound stem cell factor, consecutively leading to the secretion of IL-4 into the intracellular space (19). However, it could not be shown that neutralizing anti IL-4 Abs added to the medium could disrupt the induction of increased fibroblast proliferation by mast cells after cell-cell contact. Moreover, IL-4 could not be detected in the supernatant after cell-cell contact of both cell types. Nevertheless, IL-4 is expressed at low quantities by the HMC-1 cell line and can be released by phorbol ester treatment. Taking into account that preincubation of fibroblasts with a mAb blocking the ligand-binding site of the human IL-4 receptor resulted in the full obliteration of the increase of fibroblast proliferation, our experiments strongly suggest that mast cell-derived IL-4 secreted into the intracellular space guided by fibroblast-derived activation signals induced by cell-cell contact with mast cells up-regulates fibroblast proliferation. We hypothesize that IL-4 is secreted by mast cells in low amounts only and strictly to cell-cell contacts with fibroblasts. The IL-4 not immediately attached to the receptors of the fibroblasts may be degraded by proteases or attached to other components of the mast cell granules, e.g., heparin, and thus no longer detectable. Our notion that IL-4 is the most important signal secreted by mast cells into the intracellular space is further substantiated by our observation that IL-4 alone in the absence of mast cell coculture may induce the same up-regulation of fibroblast proliferation when cultured in FCS-containing medium. Our experiments cannot exclude, however, that other mast cell-derived mediators, e.g., TGF-β1 and trypsin, also have the capability of up-regulating fibroblast proliferation (20, 21).

Our experiments under serum-free conditions further support the concept that mast cell-derived IL-4 is important for the regulation of fibroblast proliferation (22), since it was capable of inducing maximal fibroblast proliferation in spite of only low, suboptimal concentrations of bFGF, the obligatory FGF. Interestingly, we found that only three of five fibroblast cell lines responded with increased proliferation after cell-cell contact with mast cells. Thus far, there is no good explanation for this observation. Studies of cell surface expression of the IL-4 receptor indicated that all five fibroblast cell lines highly expressed the receptor. We hypothesize that heterogeneity in IL-4-induced signal transduction exists, determining predisposition for fibrotic reactions in chronic inflammation.

Based on this and other reports, it has now become evident that mast cells may influence several fibroblast functions relevant for fibrosis, particularly proliferation as well as the production and deposition of extracellular matrix components (5, 8, 20–22).

References