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Human Intestinal Fibroblasts Prevent Apoptosis in Human Intestinal Mast Cells by a Mechanism Independent of Stem Cell Factor, IL-3, IL-4, and Nerve Growth Factor¹,²

Gernot Sellge,* Axel Lorentz,* Thomas Gebhardt,* Francesca Levi-Schaffer,§ Hueseyin Bektas,† Michael P. Manns,* Detlef Schuppan,§ and Stephan C. Bischoff³*

In rodents, fibroblasts (FBs) mediate stem cell factor (SCF)-dependent growth of mast cells (MCs). In humans, SCF is mandatory for MC differentiation and survival. Other factors such as IL-3, IL-4, and nerve growth factor (NGF) act in synergism with SCF, thus enhancing proliferation and/or preventing apoptosis in MCs. In this study, we studied in vitro interactions between human MCs and human FBs, both isolated from the intestine and purified to homogeneity. In coculture with FBs, MCs survived for up to 3 wk, whereas purified MCs cultured alone died within a few days. TNF-α and IL-1β, which both did not affect MC survival directly, enhanced FB-dependent MC growth. We provide evidence that FB-derived MC growth factors are soluble, heat-sensitive molecules which down-regulate MC apoptosis without enhancing MC proliferation. However, only low amounts of SCF were measured in FB-conditioned medium (<0.2 ng/ml). Moreover, blocking of SCF/c-kit interaction by anti-SCF or anti-c-kit Abs and neutralization of IL-3, IL-4, and NGF did not affect MC survival in the coculture system. In conclusion, our data indicate that human FBs promote survival of human MCs by mechanisms independent of SCF, IL-3, IL-4, and NGF. Such interactions between MCs and FBs may explain why MCs accumulate at sites of inflammatory bowel disease and intestinal fibrosis. The Journal of Immunology, 2004, 172: 260–267.

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In the current study, we analyzed the functional interactions between human intestinal MCs and human intestinal FBs using a coculture system. In contrast to previous reports using other cell sources, we could demonstrate, for the first time, that human intestinal FBs prevent MC apoptosis by a novel yet unraveled mechanism which seems to be independent of the known human MC growth factors SCF, IL-3, IL-4, and nerve growth factor (NGF).

**Materials and Methods**

**Isolation, purification, and culture of human intestinal MCs**

MCs were isolated from surgical tissue specimens (macroscopically normal tissue) using a four-step enzymatic dispersion method as described previously (19–23). After overnight culture in culture medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 25 mM HEPEs, 2 mM glutamine, 100 μg/ml streptomycin, 100 μg/ml gentamicin, 100 U/ml penicillin, and 0.5 μg/ml amphotericin; all from Life Technologies, Karlsruhe, Germany), MCs were enriched from nonadherent cells by positive selection of c-kit-expressing cells using magnetic cell separation (MACS system; Miltenyi Biotec, Bergisch-Gladbach, Germany), and the anti-c-kit mAb YBS.5B (5 ng/ml; BD PharMingen, Hamburg, Germany) as described (19–23). The fraction containing the c-kit-positive cells (MC purity 50–90%) was cultured (1–2 × 10^5 MC/ml) in the presence of recombiant SCF (rSCF; 50 ng/ml; Amgen, Thousand Oaks, CA). After 2–4 wk, MC purity increased to 98–100%. In some experiments, MCs were not enriched before culture. In such cell preparations MC purity increased from 1 to 5% at start to 70 to 99% after 2–4 wk. Only cell preparations with a purity above 97% were used for additional experiments. Weekly, half of the culture medium was exchanged and rSCF were supplemented again.

**Isolation, purification, and culture of human intestinal FBs**

Human intestinal FBs were obtained from the adherent cell fraction isolated from surgical specimens (see above). Cells were maintained in culture medium for 1–2 wk until they formed a subconfluent layer. For subculturing, cells were detached by trypsin/EDTA treatment for 5–20 min (0.05%/0.02%; Biochrom, Berlin, Germany). Then, the cells were seeded into fresh plates at a density of 1 × 10^4 cells/ml. Such passages were repeated two to four times. The culture medium was changed every 3–4 days. To assess FB purity, cells were analyzed for their expression of vimentin, cytokeratin, and anti-smooth muscle actin by immunocytochemistry. Vimentin was expressed in all cells confirming their mesenchymal origin (24). The epithelial cell marker cytokeratin was generally not expressed, with the exception of two of seven analyzed FB cultures displaying 4 and 9.5% positive cells, respectively. In three of seven FB preparations, we found 19–42% anti-smooth muscle actin positive-cells indicating the presence of myofibroblasts in these cultures. FB preparations were negative for CD31 and von Willebrand factor as assessed by flow cytometry and thus were not contaminated by endothelial cells (Ref. 23 and data not shown).

**MC/FB coculture assay**

The principal experimental plan of all experiments is summarized in Fig. 1. For MC/FB coculture or MC culture in the presence of FB-conditioned medium or FB sonicates we used precultured 97–100% pure MCs washed two times to remove rSCF. For coculture, 1 × 10^5 MC were seeded onto confluent FB monolayers in 24-well plates (Nalge Nunc International, Roskilde, Denmark) with or without separation of the two cell types using Transwell membranes (0.2-μm pore size; Nalge Nunc International). Half of the culture medium was changed every 4 days. For cell counting and preparation of cytospin smears, cells were harvested after trypsin/EDTA treatment. For MC mediator release assay and FACS analysis, cells were treated with EDTA (2.5 mM) to detach MCs, and then resuspended using a cell scraper to avoid digestion of surface receptors by trypsin.

For further evaluation of MC/FB interaction, MCs were cultured in the presence of FB-conditioned medium (FB supernatants) and FB sonicates. FB supernatants were harvested from confluent FB monolayers cultured for 24 h in 3 ml of culture medium using 25-cm² culture flasks (Nalge Nunc International) with or without supplementation of IL-1β (10 ng/ml) or TNF-α (10 ng/ml). To produce FB sonicates, FB monolayers were harvested using EDTA and a cell scraper and were lysed after washing by resuspension of 4–8 × 10^7 FBs in 130 μl of H_2O followed by one freezing-thawing cycle and ultrasound treatment for 5 min. Complete lysis of the FBs was confirmed by trypan blue staining and subsequent culture of the cell lysates. FB-conditioned medium and FB sonicates were stored at −80°C. Precultured MCs were then cultured in 96-well culture plates (2–4 × 10^4 MC per well) in the presence or absence of 150 μl of FB-conditioned medium or 7 μl of FB lysates, each obtained from 2 to 4 × 10^7 FBs (1:1 MC/FB ratio), adjusted to a total volume of 200 μl with culture medium. For some experiments, MCs were cultured in the presence of heat-treated FB supernatants (95°C, 45 min) or subfractionated FB supernatants and FB lysates. For subfractionation, 200 μl of FB supernatants and 9.3 μl of FB lysates adjusted to a total volume of 200 μl with culture medium were passed through a 10/50/100-kDa molecular mass filter (Microcon 10/30/50/100; Amicon, Bedford, MA) by centrifugation (14,000 × g, 15 min). The eluate was collected and the filtrate was washed once with 506 μl of PBS (14,000 × g, 15 min). Then, the filtrate was collected and adjusted to 200 μl with culture medium. MCs were cultured in the presence of 150 μl of eluate or filtrate, respectively, and 50 μl of additional culture medium.

Cell numbers and viability were determined using trypan blue staining (Sigma-Aldrich, Taufkirchen, Germany). MC and FB purity was assessed by May-Grumwald/Giemsa staining (Merck, Darmstadt, Germany) and immunocytochemistry for tryptase, a marker for MCs. Recovery rates were expressed as a percent of cell numbers at start of the culture. For blocking experiments, we used the competitive human IL-4 antagonist RY at 100 nM (19), or neutralizing Abs directed against human c-kit (1D6, rat mAb, SR1, Ref. 22), SCF (25 μg/ml, AB255-NA), IL-3 (20 μg/ml, MAB203), IL-3R (20 μg/ml, MAB301), and NGF (20 μg/ml, MAB256; all from R&D Systems, Abingdon, U.K.).

The content of soluble SCF (sSCF) in FB supernatants and FB sonicates was measured by ELISA (Quantikine human SCF ELISA, detection limit <7 pg/ml; R&D Systems).

**Mediator release assay**

MC histamine release upon FcεRI cross-linking was investigated as recently described (19–21). Briefly, MCs were stimulated using the mAb 22E7 (100 ng/ml, 60 min, 37°C; Hoffmann-La Roche, Nutley, NJ) directed against the high-affinity FcεRI α-chain. Histamine release into supernatants was measured by commercial RIA (Coulter-Immunotech, Krefeld, Germany) and expressed as specific histamine release according to the following formula: (histamine release upon FcεRI cross-linking − spontaneous histamine released)/total cellular histamine content determined by MC lysis.

**RNA isolation and RT-PCR**

RNA preparation, reverse transcription, and PCR were performed as recently described (20). Specific sense and antisense primers for the cDNAs of IL-3 (5′-CCTTGAGACAAGCTGGTGTT-3′; 5′-ATTCATTCCAGTACCGTCC-3′), IL-4 (5′-GGACACAATGTCAGCTTCA-3′; 5′-CCAAGCTACTCCTGGTGTTCC-3′), and GAPDH (5′-ACCAGTCATCGCCAATCAC-3′; 5′-TCACACACCTTGGTTGCAGTA-3′) were used.

**Immunocytochemistry**

Cytospin smears were prepared using 5–20 × 10^4 cells and were fixed in aceton (10 min, 4°C). Immunocytochemistry was performed using Abs against human tryptase (mAb, 230 ng/ml; Chemikon, Temecula, CA), chymase (mAb, 500 ng/ml; Chemikon), vimentin (clone LN-6, 1/200; Sigma-Aldrich), cytokeratin (clone 1A4, 1/200; Sigma-Aldrich), anti-smooth actin (clone KS-1A3, 1/100; Sigma-Aldrich), the human nuclear cell prolifera tion-associated Ag Ki-67 (mAb MIB-1, dilution 1/1; Dianova, Hamburg, Germany), and appropriate isotype control Abs (mouse IgG1; mouse
IgG2b, and mouse IgM; Southern Biotechnology Associates, Birmingham, AL) as primary Abs (overnight incubation at 4°C) and the streptavidin-biotin detection system (Histostain-Plus kit; Zymed Laboratories, San Francisco, CA) as described (19, 20, 22).

**Scanning electron microscopy**

FBs were cultured on glass cover slips for 4 days to allow subconfluency of the cells. Then, MCs were seeded onto FBs and cocultured for 4 h. The adherent MCs were washed twice with PBS buffer (37°C), fixed with Karnovsky’s fixative (2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4) for 2 h, and rinsed overnight in 0.1 M cacodylate buffer. The cells were dehydrated in graded series of ethanol concentrations (30, 50, 70, and 90%, two times for 5 min each, and 100% four times for 5 min) and dried at room temperature. Cells were coated with gold-paladium before analysis using a scanning electron microscope to detect the surface structure (model S05; Phillips, Eindhoven, The Netherlands).

**Flow cytometry**

FBs were detached by EDTA treatment (2 mM, 20 min, 37°C) and were harvested using a cell scraper. FACS staining was performed as described recently (22). FBs were labeled with primary Abs directed against mSCF (Ref. 25, clone 4B10), CD31 (clone HC1/6), von Willebrand factor, or CD68 (clone clone H9/108, Novocastra Laboratories, Newcastle-upon-Tyne, U.K.) as primary Abs (overnight incubation at 4°C), washed with PBS, and incubated with FITC- or PE-conjugated goat anti-mouse IgG1 and IgG2b and biotinylated secondary Abs (FITC-conjugated goat anti-mouse IgG1 and IgG2b, and biotinylated secondary Abs for MHC class II). The secondary Abs were detected using FITC- or PE-conjugated streptavidin (Zymed Laboratories, San Francisco, CA) as described (19, 20, 22).

**Adhesion assay**

Adhesion assays were performed as described previously (22, 23). Briefly, precultured MCs were harvested, washed with PBS, and 5 × 10^3 MCs in 200 μl of culture medium were seeded over a FB monolayer cultured in 96-well plates (Nalge Nunc International). After 15–360 min, nonadherent cells were removed by washing the plates twice with PBS. Adherent cells were fixed by adding 150 μl of H₂O₂ and one subsequent freeze-thaw cycle. Adhesion rates were determined according to the following formula: histamine in the lysates of the adherent fraction/histamine in total cell lysates = adhesion rate (%).

**Detection of apoptosis and proliferation**

Apoptosis of MCs were determined by annexin V staining analyzed by flow cytometry and the Apo-ONE Homogeneous Caspase 3/7 Assay (Promega, Madison, WI). Cell proliferation of MCs and FBs was analyzed by Ki-67 staining on cytosin smears.

**Statistics**

All data in text and figures are expressed as mean ± SEM if not indicated otherwise. Significance of differences was assessed by using the Wilcoxon test. A value of p < 0.05 was considered to be statistically significant.

**Results**

**Human intestinal MCs survive in the presence of human intestinal FBs**

Confirming recent observations, we found that MCs cultured in standard medium without cytokine supplementation died within a few days (19, 21, 22). If MCs were cocultured on a FB monolayer about half of the MCs survived if cultured for 8–12 days (Fig. 2A). In some experiments, MCs could be cocultured with FBs for up to 3 wk (data not shown). Interestingly, the number of MCs that survived was similar in experiments allowing a direct contact between MCs and FBs and those in which MCs were separated from FBs by a Transwell insert (50 ± 17% vs 40 ± 11%, p > 0.05). For comparison, we also determined MC recovery in MC monolayers supplemented with human rSCF at suboptimal (5 ng/ml) and optimal (50 ng/ml) concentrations (21). Recovery rates after 8–12 days were 71 ± 15% and 128 ± 37%, respectively. MC survival was confirmed by measurement of intracellular histamine after cell lysis. The histamine content in MCs cocultured with FBs compared with MCs cultured in the presence of rSCF was similar after 3–4 days but was diminished after 8–12 days. The histamine content of MCs cultured in medium alone decreased already after 3–4 days (Fig. 2B). Functional experiments revealed that MCs cocultured with FBs for up to 12 days maintained the capacity to release histamine upon FceRI cross-linking, whereas almost no histamine release could be obtained from MCs cultured for 3–4 days in the absence of growth factors or FBs. These results confirm the pronounced effects of FBs regulating both MC survival and maintenance of MC histamine release upon stimulation in vitro (Fig. 2C).

MCs adhere to FBs in a time-dependent fashion, reaching a maximum after a coculture time of ~2 h (Figs. 2D and 3, A and D). After 8–12 days of coculture, <50% of the MCs were attached to FBs (Fig. 3C). The data suggest that a direct contact between MCs and FBs is not required for MC survival, and thus these experiments confirm the results obtained from the Transwell experiments.

Phase contrast microscopy studies showed that the FB monolayer loses confluency during direct coculture with MCs (Fig. 3, A and B), but not if FBs and MCs were separated by Transwell inserts (not shown). However, FB viability and numbers determined by cell counting (trypan blue staining) and cell differentiation on cytosin smears were slightly but not significantly decreased after 8–12 days of direct MC/FB coculture (73 ± 12% of control, n = 7, p > 0.05). However, FB numbers remained unchanged if MCs and FBs had no direct contact (111 ± 12% of control, n = 7). Interestingly, we found an increased FB proliferation rate determined by Ki-67 staining if FBs were directly cocultured with MCs (FB alone: 1.3 ± 1.2% Ki-67^+; FB/MC coculture (direct): 8.4 ± 6.0% Ki-67^+; FB/MC coculture (Transwell): 2.9 ± 1.9% Ki-67^+). This might be explained by the loss of confluency and, thus loss of
contact inhibition. Proliferation of FBs further indicates that the cells are viable and functional intact. Most of the FBs were anti-smooth muscle actin negative (see Materials and Methods) and the expression rate was not affected by the coculture with MCs (not shown), largely excluding the possibility that FBs become more contractile in the presence of MCs.

Effect of FBs on MC phenotype

Human MCs have been classified into two subtypes according to their expression of proteases. The tryptase-expressing MCs (MC<sub>T</sub>) are found predominantly in the mucosa and the MCs expressing tryptase and chymase (MC<sub>T-C</sub>) are frequently found in connective tissues including intestinal submucosa (1). We examined protease expression by immunocytochemistry. We found that the percentage of MC<sub>T-C</sub> and the intensity of chymase staining (Fig. 3, E and F) was increased if MCs were cocultured with FBs allowing direct contact (28 ± 18% MC<sub>T-C</sub>) or separated by Transwell inserts (24 ± 20% MC<sub>T-C</sub>) for 12 days compared with MCs cultured in the presence of rSCF (13 ± 8% MC<sub>T-C</sub>, all n = 5, mean ± SEM). However, the differences were not statistically significant most likely due to the small numbers of experiments and the high variability of chymase expression in different MC preparations (p > 0.05).

IL-1β and TNF-α enhance FB-dependent growth of MCs

Assuming that FBs may induce MC hyperplasia in chronic inflammatory bowel disease (3), we questioned whether known FB activators such as the proinflammatory cytokines IL-1β and TNF-α (26) may enhance MC survival in a FB-dependent manner. As shown in Fig. 4, MC/FB cocultures in the presence of either one of the two cytokines significantly increased MC survival compared with MC/FB coculture without addition of cytokines.

We showed that a direct cell–cell contact between MCs and FBs is not a prerequisite for MC survival, suggesting (a) soluble factor(s) being involved. Therefore, we tested whether FB-conditioned medium (supernatants) and FB sonicates also promote MC survival. Indeed, both FB supernatants and sonicates caused MC survival, albeit with less efficacy compared with cocultures with vital FBs. FB supernatants or sonicates derived from FBs stimulated with IL-1β or TNF-α for 24 h were more effective in enhancing survival of MCs than supernatants or sonicates from unstimulated FBs (Fig. 4) confirming the data obtained from direct coculture experiments. None of the two cytokines affected MC survival directly, neither alone, nor in the presence of different concentrations of rSCF (0.1–50 ng/ml), nor in the presence of FB supernatants or sonicates derived from unstimulated FBs (n = 3, data not shown).

**FIGURE 3.** Coculture of human intestinal MCs with human FBs. A, Scanning electron microscopy of MCs (arrows) adhering to FBs (arrow heads) after 4 h of coculture. B, Phase contrast microscopy of a monolayer of human intestinal FBs displaying an adherent spindle-shaped cytoplasm. C, Phase contrast microscopy of a MC/FB coculture after 11 days of coculture. Approximately 50% of MCs (open arrows) were attached to FBs (arrow heads) which lost confluency upon coculture with MCs, but by far not all MCs (closed arrows) formed a direct cell-cell contact with FBs. D, Light microscopy of a MC/FB coculture after 30 min of coculture stained with May-Grünwald/Giemsa. Note the pseudopodia formed by MCs in coculture with FBs (arrows). E–G, Immunocytochemistry of MC subtypes using anti-chymase mAb (chymase<sup>+ = MC<sub>T-C</sub></sup>, chymase<sup>− = MC<sub>T</sub></sup>). MCs were precultured in the presence of rSCF (50 ng/ml) for 14 days and then (E) cultured in the presence of 50 ng/ml rSCF, (F) cocultured with FBs allowing direct contact, or (G) cocultured with FBs separated by Transwell inserts.

**FIGURE 4.** IL-1β and TNF-α enhance human intestinal FB-dependent growth of human intestinal MCs. MCs were cocultured for 6 days with FBs (n = 6) with or without IL-1β (10 ng/ml) or TNF-α (10 ng/ml) or cultured for 6 days in the presence of FB supernatants (n = 15) or FB sonicates (n = 10) obtained from confluent FB monolayers that were unchallenged (medium alone) or challenged with IL-1β (10 ng/ml) or TNF-α (10 ng/ml) for 24 h (*, p < 0.05; ***, p < 0.01; ****, p < 0.001 compared with control). Data are shown as means ± SEM.
Characterization of FB-derived MC growth factors

SCF is a unique growth factor for human MCs, because several studies suggest that SCF is absolutely required for MC differentiation and survival (2, 19). In this study, we were interested in whether SCF and possibly other molecules are involved in mediating FB-dependent MC survival. FACS analysis showed that mSCF is produced by 40–70% of human intestinal FBs in vitro (Fig. 5A). The expression of mSCF was not up-regulated upon challenge with IL-1β and TNF-α for 24 h (data not shown). We found only low levels of sSCF produced by intestinal FBs. The amount of sSCF measured in FB supernatants was slightly increased after stimulation with IL-1β or TNF-α but always remained below 200 pg/ml. Moreover, MCs added to the FB cultures did not affect the production of sSCF by FBs (Table I). It is noteworthy that human rSCF at concentrations up to 500 pg/ml had no effect on MC survival. To obtain the MC survival rates similar to that induced by FBs or FB supernatants, rSCF was required at concentrations being 10–100 times higher than those measured in the supernatants (Fig. 5B).

To investigate whether SCF, either sSCF or mSCF, is involved at all in FB-dependent MC survival in our system, we neutralizing Abs directed against c-kit, the SCF receptor, and SCF. We found that both Abs could neutralize the biological activity of 5 ng/ml rSCF to 100% and of 50 ng/ml rSCF to 100% (anti-c-kit) and ~65% (anti-SCF), respectively. Surprisingly, addition of the anti-human c-kit mAb and anti-human SCF mAb to MC/FB cocultures or MCs cultured in the presence of FB supernatants and sonicates did not abrogate survival of MCs after 6 (Table II) or 11 days of culture (data not shown).

We reported previously that IL-3 and IL-4 enhance rSCF-dependent growth of human intestinal MCs (19, 21). Therefore, we tested whether blocking of these cytokines might reduce or even abrogate the effects of FBs on MC survival. However, the competitive IL-4-antagonist neutralizing Abs directed against IL-3 and IL-4 receptor, respectively, failed to reduce MC survival induced by FB supernatants (Table III). At the same time, these antagonists neutralized the effect of 2 ng/ml IL-4 to 100%, 1 ng/ml IL-3 to ~85%, and 10 ng/ml IL-3 to ~60%, respectively, in MCs cultured in the presence of rSCF and recombinant IL-4 or IL-3 (Table III). Confirming these data, we could not detect IL-3 and IL-4 mRNA expression in intestinal FBs (data not shown). Finally, we found that FB supernatants act synergistically with suboptimal concentrations of rSCF (0.5–5 ng/ml) but, in contrast to IL-3 and IL-4 (19, 21), they did not enhance MC survival in the presence of optimal (50 ng/ml) rSCF concentrations (not shown). NGF known to be produced by fibroblast (27) has been shown to prevent apoptosis in human cord blood-derived mast cells in a SCF-dependent manner (28). However, in our experiments a neutralizing anti-NGF mAb did not alter MC survival in the presence of FB supernatants (Table III). Taken together, these data strongly suggest that human intestinal FBs mediate MC survival by mechanisms independent of SCF, IL-3, IL-4, and NGF.

To further characterize the FB-derived MC growth factor(s), we fractionated FB supernatants and FB lysates according to their molecular size using a molecular mass filter. No bioactivity was found in the eluate of a 10-kDa molecular mass cut-off filter, whereas most of the activity was found in the eluate of a 100-kDa filter (Fig. 6A). Approximately 5 and 15% of the bioactivity was found in the eluate fraction of the 30- and 50-kDa filter, respectively (not shown). Heat treatment of FB supernatants derived from unstimulated FBs or TNF-α-stimulated FBs, as well as rSCF, abrogated the effects on MC survival. It is noteworthy that MC survival was not totally abolished if MCs were cultured in heat-treated FB supernatants (Table III). Taken together, these data strongly suggest that human intestinal FBs mediate MC survival by mechanisms independent of SCF, IL-3, IL-4, and NGF.

FBs reduce MC apoptosis rather than enhance MC proliferation

FACS analysis, using annexin V staining, and measurement of the caspase 3/7 activity revealed that MCs cocultured with vital FBs (data not shown) as well as MCs cultured in the presence of FB-conditioned medium and FB lysates exhibited decreased apoptosis

Table I. SCF concentration in FB supernatants and sonicates

<table>
<thead>
<tr>
<th></th>
<th>SCF (pg/ml)</th>
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<tbody>
<tr>
<td>Supernatant (control)a</td>
<td>44 ± 13</td>
</tr>
<tr>
<td>Supernatant (+IL-1β)b</td>
<td>69 ± 27</td>
</tr>
<tr>
<td>Supernatant (+TNF-α)a</td>
<td>60 ± 22</td>
</tr>
<tr>
<td>Sonicate (control)a</td>
<td>&lt;7</td>
</tr>
<tr>
<td>Sonicate (+IL-1β)b</td>
<td>&lt;7</td>
</tr>
<tr>
<td>Sonicate (+TNF-α)a</td>
<td>&lt;7</td>
</tr>
<tr>
<td>Coculture (control)b</td>
<td>63 ± 23</td>
</tr>
<tr>
<td>Coculture (contact)b</td>
<td>56 ± 38</td>
</tr>
<tr>
<td>Coculture (Transwell)b</td>
<td>67 ± 26</td>
</tr>
</tbody>
</table>

a Supernatants and sonicates were obtained from confluent FB monolayers cultured with or without supplementation of IL-1β (10 ng/ml) or TNF-α (10 ng/ml) for 24 h (means ± SEM, n = 4).

b Supernatants from FB cultured for 8–10 days in medium (control), and in coculture with MCs allowing direct contact or separated by Transwell inserts (means ± SEM, n = 4). Experimental conditions as described in Fig. 2.

Table II. Effect of blocking c-kit or SCF on MC survival

<table>
<thead>
<tr>
<th>MC Recovery (%)</th>
<th>Control</th>
<th>mAb c-kit</th>
<th>mAb SCF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.2</td>
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<tr>
<td>rSCF (5 ng/ml)</td>
<td>40.5 ± 10.5</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
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<tr>
<td>rSCF (50 ng/ml)</td>
<td>82.5 ± 17.2</td>
<td>0.5 ± 0.2</td>
<td>27.9 ± 3.1</td>
</tr>
<tr>
<td>Coculture (control)</td>
<td>28.6 ± 11.8</td>
<td>25.4 ± 9.1</td>
<td>26.2 ± 10.4</td>
</tr>
<tr>
<td>Coculture (+IL-1β)</td>
<td>51.8 ± 21.9</td>
<td>54.4 ± 20.2</td>
<td>n.t.</td>
</tr>
<tr>
<td>Coculture (+TNF-α)</td>
<td>57.1 ± 19.5</td>
<td>43.5 ± 14.8</td>
<td>n.t.</td>
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<tr>
<td>Supernatant (control)</td>
<td>31.7 ± 3.1</td>
<td>30.3 ± 3.4</td>
<td>27.2 ± 1.7</td>
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<tr>
<td>Supernatant (+IL-1β)</td>
<td>49.6 ± 10.8</td>
<td>52.3 ± 9.8</td>
<td>50.4 ± 9.6</td>
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<tr>
<td>Supernatant (+TNF-α)</td>
<td>57.7 ± 7.1</td>
<td>62.3 ± 7.6</td>
<td>59.7 ± 7.9</td>
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<tr>
<td>Sonicate (control)</td>
<td>7.0 ± 3.6</td>
<td>11.7 ± 4.5</td>
<td>n.t.</td>
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<tr>
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<td>31.7 ± 5.2</td>
<td>42.9 ± 8.3</td>
<td>n.t.</td>
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<tr>
<td>Sonicate (+TNF-α)</td>
<td>28.7 ± 5.3</td>
<td>40.1 ± 3.9</td>
<td>n.t.</td>
</tr>
</tbody>
</table>

a Effect of blocking mAbs directed against c-kit and SCF on MC survival in coculture with FBs (n = 6) or in the presence of FB supernatants (n = 4), FB sonicates (n = 3), or rSCF (n = 3). Appropriate isotype control Abs did not affect MC survival (data not shown). Experiments were performed as described in Fig. 4. MC recovery was assessed after 6 days of culture. Means ± SEM are shown.

b Not tested.
Table III.  Effect of blocking of IL-3, IL-4, or NGF on MC survival

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>mAb IL-3/IL-3R</th>
<th>RY (IL-4 inhibitor)</th>
<th>mAb NGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>rSCF (2 ng/ml)</td>
<td>18.5 ± 2.7</td>
<td>17.1 ± 5.6</td>
<td>17.5 ± 3.5</td>
<td>n.t.</td>
</tr>
<tr>
<td>+IL-3 (1 ng/ml)</td>
<td>38.1 ± 2.4</td>
<td>21.7 ± 3.0</td>
<td>n.t.^c</td>
<td>n.t.</td>
</tr>
<tr>
<td>+IL-3 (10 ng/ml)</td>
<td>46.2 ± 4.4</td>
<td>30.2 ± 3.4</td>
<td>n.t.^c</td>
<td>n.t.</td>
</tr>
<tr>
<td>+IL-4 (2 ng/ml)</td>
<td>66.2 ± 7.1</td>
<td>n.t.^c</td>
<td>16.0 ± 3.9</td>
<td>n.t.</td>
</tr>
<tr>
<td>Supernatant (control)</td>
<td>18.4 ± 4.0</td>
<td>20.8 ± 5.9</td>
<td>21.2 ± 7.8</td>
<td>17.0 ± 6.7</td>
</tr>
<tr>
<td>Supernatant (+IL-1-β)</td>
<td>66.5 ± 9.2</td>
<td>62.0 ± 10.1</td>
<td>56.4 ± 5.7</td>
<td>56.0 ± 5.7</td>
</tr>
<tr>
<td>Supernatant (+TNF-α)</td>
<td>61.5 ± 9.4</td>
<td>65.2 ± 2.0</td>
<td>65.5 ± 13.0</td>
<td>52.8 ± 0.8</td>
</tr>
</tbody>
</table>

^a Effect of blocking mAbs directed against IL-3/IL-3R and NGF and the IL-4 inhibitor RY on MC survival in the presence of FB supernatants (n = 3), rSCF ± IL-3 (n = 2), or rSCF ± IL-4 (n = 2). Appropriate isotype control Abs did not affect MC survival (data not shown). MC recovery was assessed after 6 days of culture. Means ± SEM are shown.

^b Anti-NGF mAb did not influence rSCF-dependent growth of MC, which was tested in separate experiments (n = 2).

^c Not tested.

rates compared with MCs cultured without growth factors (Fig. 7). Consistent with the data shown in Fig. 4, we found a more pronounced down-regulation of apoptosis in MCs cultured in the presence of FB supernatants and lysates obtained from IL-1β- and TNF-α-stimulated FBs compared with supernatants and lysates obtained from unstimulated FBs.

For the analysis of proliferation, we performed immunocytochemistry, targeting the Ki-67 Ag, an established marker of cell proliferation. We did not find any expression of Ki-67 in MCs cocultured with FBs for 6 days in the absence or presence of IL-1β or TNF-α, or cultured for 6 days in the presence of FB supernatants, or sonicates derived from unstimulated or stimulated (±IL-1-β or TNF-α) FBs (data not shown, n = 3). Confirming recent results, Ki-67 expression was enhanced dose-dependently by rSCF (19, 21). The percentage of Ki-67-positive MCs after 6 days of culture in the presence of 2, 5, and 50 ng/ml rSCF was 0%, 1.2 ± 1.8%, and 9.6 ± 5.2%, respectively (n = 3, mean ± SEM, data not shown).

**Discussion**

Our data show that human intestinal MCs survive in vitro up to 3 wk in coculture with human intestinal FBs, whereas MC monocolonies die within a few days. Compared with other human MC survival factors such as rSCF or EC-derived factors we described earlier, the FB-derived signals were less potent in promoting MC survival (22). We found that FBs, in contrast to ECs and rSCF, failed to induce MC proliferation. However, our data clearly demonstrate that FBs decrease MC apoptosis. This may explain why MC recovery declined with time in the presence of FBs compared with continuous rSCF supplementation at optimal concentrations or coculture with ECs, both of which induce significant MC proliferation. However, we cannot exclude that FB-derived factors at higher concentrations that might occur in vivo are able to induce MC proliferation, too. In accordance with this hypothesis, we found that rSCF at low concentrations (0.5–2 ng/ml) down-regulated MC apoptosis but did not induce MC proliferation.

We have shown recently that ECs support MC growth almost exclusively by membrane-bound factors. In contrast, FBs cause MC survival by releasing soluble growth factors, because Transwell experiments in which MCs were separated from FBs yielded similar results with regard to MC recovery and FceRI-dependent histamine release than experiments allowing a direct contact between MCs and FBs. Furthermore, FB supernatants also promote histamine release than experiments allowing a direct contact between MCs and FBs. Furthermore, FB supernatants also promote
MC growth, whereas FB sonicates had only little effect on MC survival.

Coculture experiments showed that murine 3T3 FBs promote MC development from both human and murine hemopoietic progenitors (1, 12, 13, 16). The 3T3 FB-dependent effects have been attributed to murine SCF. Indeed, murine and human SCF are known to be produced in their soluble or transmembrane form by FBs, ECs, and other stromal cells. SCF is a unique factor for MC development, migration, and survival, because this cytokine induces chemotaxis, adhesion, and proliferation, while suppressing apoptosis in murine and human MCs (1, 2, 16). Previously, the effects of FBs on human lung and foreskin MCs have been studied. Thus, human lung MCs maintained in culture in the presence of murine 3T3 FBs survive for up to 8 days (14). MCs maintained their viability, ultrastructural morphology, and functional activity. The investigators suggested that FB-derived SCF might mediate these effects. However, this hypothesis was not directly proven in these studies, because the effects of SCF-deficient 3T3 FBs or neutralizing Abs against SCF or c-kit were not studied.

We found that human intestinal FBs produce both sSCF and mSCF. Surprisingly, the measured amounts of sSCF were 10–100 times lower than those required for maintaining MC survival in vitro (21). Most interestingly, neutralizing Abs against c-kit and SCF did not change MC survival in our experiments independent of whether MCs were cultured in the presence of FBs, FB supernatants, or FB sonicates. Both the low amount of sSCF found to be produced by intestinal FBs and the failure to block FB-dependent MC survival by the neutralizing Abs against c-kit and SCF support the concept that MC survival was mediated by SCF-independent factors. This finding is intriguing because it has been suggested that SCF is mandatory for the growth of human immature and mature MCs. In previous studies, we and others have identified other growth factors for human MCs such as IL-3, IL-4, or NGF which act in synergism with SCF (19, 21, 28). However, it is rather unlikely that these factors are involved in FB-dependent MC survival because neutralization of either IL-3, IL-4, or NGF did not modulate FB-dependent MC survival. Although we cannot rule out any involvement of SCF in FB-dependent MC survival in vivo, the role of SCF for MC/FB interactions could have been overestimated in the past. In contrast, it seems unlikely that MC development can occur independent of SCF, if one considers that mice and rats deficient of c-kit or SCF almost lack MCs (29). In addition, it is important to note that human FB lines, in contrast to murine 3T3 FBs, do not promote human MC development from progenitor cells suggesting that these cells lines, like intestinal FBs, produce no or only small amounts of SCF (11). However, MC regulation may be different in humans and rodents, and it may be dependent on the body compartment from which the cells are derived.

Human MCs developed in vitro from progenitor cells in the presence of 3T3 FB-conditioned medium or rSCF seem to be less mature according to ultrastructural criteria and exert lower chymase expression than MCs developed in direct coculture with 3T3 FBs (12, 13, 16). This might be the result of membrane-associated factors expressed in FBs. We found an increased intensity of chymase immunostaining in MCs of rSCF and a slight, albeit not significant, up-regulation of the percentage of MCs after coculture with FBs compared with intestinal MCs cultured in the presence of rSCF. The interpretation of this finding is complicated by the fact that MCs used in our studies have been precultured in the presence of rSCF for 2–4 wk.

MC accumulation typically occurs at sites of fibrosis where FBs are supposed to be activated by inflammatory cytokines and other fibrogenic factors. To address the question of whether activated FBs promote MC survival more efficiently, we stimulated FBs with the proinflammatory cytokines IL-1β and TNF-α known to act as FB agonists (26). Indeed, we found that MC survival in coculture with FBs was clearly enhanced in the presence of IL-1β or TNF-α. Because neither IL-1β nor TNF-α affected MC survival directly, it is likely that both cytokines induce or enhance the production of MC survival factor(s) in FBs. These results are in accordance with recent studies showing that IL-1α, platelet-derived growth factor and members of the IL-6 cytokine family (IL-6, IL-11, oncostatin M, and LIF) enhance the growth of mouse bone marrow-derived MCs by an 3T3 FB-dependent mechanism (30–33). In our experiments, IL-1β or TNF-α slightly enhanced the production of sSCF but not that of mSCF in FBs. Similar findings were reported for human synovial FBs that have been stimulated by TNF-α for enhanced SCF production and chemotaxis of the human MC line HMC-1 (34). However, the amounts of sSCF we found in the supernatants of stimulated FBs were still significantly lower than those required to obtain comparable MC survival rates with rSCF. Moreover, blocking of c-kit, SCF, IL-3, IL-4, and NGF did not change MC recovery under these culture conditions. Such findings further suggest that apart from these mediators other MC growth factors are produced by FBs.

According to our preliminary biochemical characterization of the anticipated new FB-derived MC growth factor, the biological activity is mediated by a heat-sensitive soluble molecule with a molecular size between 10 and 100 kDa, characteristics being typical for many cytokines and cell growth factors. We found that MC growth factor(s) in FB supernatants and FB lysates could be passed through a 100-kDa molecular mass filter but not through a 10-kDa filter. This suggests that MC growth factors in FB supernatants and even in FB lysates are soluble molecules and are not associated with membrane debris or vesicles. Little bioactivity was found in the filtrate of 100-kDa filters and in the eluate of 30- and 50-kDa filters, respectively. However, it is not possible to accurately estimate the molecular size of the FB-derived MC growth factor(s) by this method, because membrane materials can nonspecifically bind proteins to varying degrees, which is related to the individual protein character. The fact that MC survival was decreased but not totally abolished in the presence of heat-treated supernatants obtained from IL-1β-stimulated FBs suggests that IL-1β stimulate FBs for the production of a heat stable MC growth factor which is not expressed in unstimulated or TNF-α-stimulated FBs. Clearly, further studies requiring high amounts of supernatants from human intestinal FBs are needed to characterize the factor(s) in more detail.

The findings that human FBs, particularly when activated by proinflammatory cytokines such as TNF-α or IL-1β, promote MC survival by an SCF-independent mechanism and hence MC accumulation at sites of tissue fibrosis may have significant clinical implications. Fibrosis still represents an unsolved clinical challenge. It occurs in the course of many different chronic diseases of inflammatory origin such as chronic asthma, chronic hepatitis, and inflammatory bowel disease, namely Crohn’s disease (35). The latter is characterized by a transmural inflammation associated with an enhanced production of proinflammatory cytokines such as TNF-α and IL-1β (36). Interestingly, a recent study could demonstrate that MCs accumulate in fibrotic lesions of Crohn’s disease (3). The basic mechanisms underlying fibrosis and MC hyperplasia in the course of Crohn’s disease have not been elucidated so far. Our data suggest that MC hyperplasia in fibrotic lesions is mediated, at least in part, by SCF-independent factors released by FBs.
These findings suggest that MC/FB interaction might be of importance for the development of chronic inflammation and intestinal fibrosis.

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References