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Promotion of mouse fibroblast proliferation by IgE-dependent activation of mouse mast cells: Role for mast cell tumor necrosis factor- α and transforming growth factor- β 1

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Abstract

TOP

Pathologic fibroblast proliferation or tissue fibrosis develops in certain chronic allergic diseases and in a wide array of other inflammatory disorders in which mast cell activation is also a prominent feature. In this study we investigated a number of potential mechanisms by which IgE-dependent

activation of mouse mast cells might influence the proliferation of mouse fibroblasts in vitro. We found that supernatants from in vitro-derived mast cells that had been activated by IgE and specific antigen (but not those from quiescent mast cells) promoted the proliferation of mouse embryonic skin or 3T3 fibroblasts, and we showed that this effect was detectable in the absence of fetal calf serum. We analyzed the kinetics with which the fibroblast-proliferative activity was secreted from bone marrow–derived cultured mast cells and found that it was released both rapidly (i.e., in 30 minutes or less) and for a more prolonged period (i.e., for more than 2 hours) after IgE-dependent mast cell activation. We then measured the levels at which the mast cells produce a number of cytokines that are known to affect fibroblasts (IL-1, IL-6, transforming growth factor- β 1 [TGF- β 1], and tumor necrosis factor- α [TNF- α]) and assessed their relative effects, as recombinant cytokines, on fibroblast proliferation. Our mast cells secreted high levels of TGF- β 1 and TNF- α , intermediate amounts of IL-6, and low levels of IL-1. We titrated the fibroproliferative effects of each of these cytokines and determined that at a dose of 50 pg/ml their rank order of activity was TGF- β 1 > TNF- α > IL-1 > IL-6, with all but IL-6 having significant effects. The ability of supernatants from activated bone marrow–derived cultured mast cells to promote fibroblast proliferation was partially diminished by absorption with neutralizing antibodies against either TNF- α or TGF- β 1, and absorption of the supernatants with a combination of antibodies against TNF- α and TGF- β 1 reduced their ability to induce fibroblast proliferation by approximately 50% ($p \leq 0.001$, $n = 5$). These findings show that IgE-dependent activation of mouse mast cells can result in the release of mediators that promote fibroblast proliferation in the absence of any other cell type and suggest that mast cell–derived TNF- α and TGF- β 1 contribute substantially to this effect. They also suggest that these cytokines exert their effects through synergistic interactions with other mast cell mediators. (J Allergy Clin Immunol 1997;99:113-23.)

BMC MC	Bone marrow–derived cultured mast cells
DMEM	Dulbecco's minimum essential medium
DNP₃₀₋₄₀HSA	DNP-substituted HSA
FcϵRI	High affinity surface receptor for IgE
FCS	Fetal calf serum
2-Me	2-Mercaptoethanol
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide
NRS	Normal rabbit sera
SCF	Stem cell factor
TGF	Transforming growth factor
TNF	Tumor necrosis factor

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Key Words: [Mast cell](#), [fibroblast](#), [fibrosis](#), [TNF- \$\alpha\$](#) , [TGF- \$\beta\$ 1](#), [cytokines](#), [proliferation](#), [inflammation](#)

Tissue fibrosis and fibroblast proliferation are components of the pathology of many chronic inflammatory diseases that are not thought to involve IgE antibodies,^{1 2} as well as of certain allergic disorders such as allergic asthma and atopic dermatitis.^{3 4} Many of these diseases also exhibit morphologic evidence of tissue mast cell degranulation.^{1 2} Indeed, the frequent association of mast cell activation with fibroblast proliferation and tissue fibrosis has suggested that mast cells may contribute directly to these processes.^{1 2}

Studies in a number of experimental systems have established that mast cell degranulation or mast cell mediators can induce fibroblast proliferation—these range from experiments with rat mesentery spreads challenged with mast cell activators in the presence of high levels of fetal calf serum (FCS)⁵ to studies with cloned fibroblasts challenged with purified mast cell mediators.^{6 9} However, such experiments typically are performed in the presence of FCS, which itself may be mitogenic for fibroblasts. Moreover, some effects of mast cells on fibroblasts may require physical contact between the cells. For example, the coculture of growth factor–dependent mouse mast cells with fibroblasts in medium containing IL-3 and FCS for 2 to 6 weeks resulted in augmented fibroblast growth, in the absence of overt mast cell activation, by a mechanism that required contact between the mast cells and the fibroblasts.¹⁰

A number of mast cell secretion products including heparin,⁶ tryptase,⁷ and in some cases, IL-4,^{8 9} can induce proliferation when added to cultures of fibroblasts. Moreover, mast cells can also secrete a wide array of other cytokines, including IL-1, IL-6,¹¹ transforming growth factor- β 1 (TGF- β 1),^{12 14} and tumor necrosis factor- α (TNF- α),^{15 17} that have potential to affect fibroblast proliferation.¹⁸ However, the relative amounts of each of these cytokines released by mast cells after activation via the high affinity surface receptors for IgE (Fc ϵ RI) and their relative contributions to the fibroproliferative activities of mast cells have not been explored.

In the studies reported here we examined first whether mediators secreted by activated mouse mast cells can themselves induce fibroblast proliferation (i.e., in the absence of co-mitogenic serum components). We then evaluated the levels at which a panel of cytokines (IL-1, IL-6, TGF- β 1, and TNF- α) are secreted by mast cells, as well as the relative impact of these cytokines on fibroblast proliferation. Finally, on the basis of the relative levels at which mast cells produce each of these cytokines and their relative impact on primary cultures of mouse embryonic fibroblasts, we assessed the relative contributions of mast cell–derived TNF- α and TGF- β 1 to bone marrow–derived cultured mast cell (BMCMC)–dependent fibroblast proliferation.

Methods

TOP

Mice

BALB/c mice were purchased from the Animal Resources Centre of the University of

Saskatchewan. All animal treatments were performed while the mice were lightly anesthetized with methoxyfluorane, according to the "Guidelines For the Care and Use of Experimental Animals" established by the Canadian Council on Animal Care. The mice were euthanized with methoxyfluorane.

Cytokines and antisera

We purchased recombinant mouse IL-1, IL-6, and TNF- α from Genzyme (Cambridge, Mass.), and purified human TGF- β 1 and affinity-purified chicken anti-porcine TGF- β 1 IgG antibodies from R&D Systems (Minneapolis, Minn.). Normal rabbit sera (NRS) and rabbit anti-TNF- α antisera were prepared as reported in detail previously.¹⁶ The IgG was purified from these sera by affinity chromatography on Avid AL (BioProbe International, Inc., Tustin, Calif.) columns according to the manufacturer's recommendations, and the protein concentrations of the eluted IgG were determined by using a Coomassie Blue assay (Bio-Rad Laboratories, Mississauga, Ontario). The purified anti-TNF IgG was titrated against recombinant mouse TNF- α and was determined to have a neutralizing capacity of approximately 5×10^5 neutralizing units/ml.

Cells

The derivation and/or maintenance of the growth factor-independent mouse mast cell line Cl.MC/C57.1, growth factor-dependent mouse BMCMC, and L929 cells have been reported.^{16 17} BALB/c 3T3 fibroblasts, generously provided by J. Gilchrist (VIDO, Saskatoon, Canada), were originally obtained from the American Type Culture Collection (ATCC, Rockville, Md.) and were maintained according to the recommendations of that organization.

Mouse embryonic skin fibroblasts were generated from 13-day BALB/c mouse embryos, as reported previously.¹⁴ The adherent outgrowth cells from the minced skin were cultured in Dulbecco's minimum essential medium (DMEM) (Gibco, Grand Island, N.Y.) supplemented with 10% FCS (Gibco), 2.5% essential amino acids mixture (100 $\times$ stock; Gibco), 10 mmol/L L-glutamine, 5×10^{-5} mol/L 2-mercaptoethanol (2-Me; Sigma Chemical Co., St. Louis, Mo.), and 1% antibiotic/antimycotic mixture (100 U/ml penicillin G sulfate, 100 μ g/ml streptomycin sulfate, 250 ng/ml amphotericin B [final concentration]; Gibco). The cells were passaged with use of trypsin/versene, maintained in a subconfluent state, and expanded for 10 passages before being cryopreserved in liquid nitrogen. After thawing, the cells were maintained in RPMI 1640 (Gibco) containing 10% FCS, 10 mmol/L L-glutamine, 5×10^{-5} mol/L 2-Me, and 1% antibiotic/antimycotic (complete RPMI-10% FCS) and then, just before each experiment, they were transferred to similar RPMI 1640 medium, except that it was supplemented with 1% FCS (unless otherwise stated, complete RPMI-1% FCS). All experiments reported here were performed with embryonic skin fibroblasts at passages 12 to 20.

The embryonic skin fibroblasts display a doubling time (in 10% FCS) of approximately 36 hours and exhibit typical fibroblast structure. When cultured in medium containing 2.5% or more FCS, the cells contained high levels of messenger RNA for type α -1 (I) collagen (as assessed by in situ hybridization or northern blotting) and secreted high levels of collagen (not shown). By contrast, in medium containing FCS at concentrations of 1% or less, they contained very low levels of type α -1 (I) collagen messenger RNA and secreted only background levels of collagen (data not shown).

Although numerous batches of FCS (from three different sources) were tested for their ability to support fibroblast proliferation, only one batch was used for the experiments reported here.

Activation of cells

Mast cells

Mast cells were activated via the FcεRI as reported.^{16 17} Briefly, the cells were sensitized with a monoclonal IgE anti-DNP antibody (H1-DNP-ε-26; generously provided by Drs. Fu-Tong Liu and D. Katz¹⁹) for 30 minutes and then washed, resuspended in DMEM supplemented with 1% FCS (unless otherwise noted), and either stimulated with antigen (IgE/Ag), namely, DNP-substituted HSA (DNP₃₀₋₄₀HSA; 50 ng/ml; Sigma) or left untreated (IgE/-). At varying times thereafter, the cell supernatants were harvested from the cultures. In some experiments, the cells were placed back in culture each time the culture supernatant fluids were collected, so that we obtained serial samples containing the mediators secreted between 0 and 0.5 hour, 0.5 and 1 hour, 1 and 2 hours, and 2 and 4 hours after cellular activation. All supernatants were either used fresh or aliquotted and stored at -80° C before assay. Maintenance of the mast cells in DMEM with or without 1% FCS during the cellular activation period had no adverse effects on the viability or secretory activity of the cells, as determined by monitoring the release of serotonin and TNF-α of the cells in response to FcεRI signaling (not shown).

Fibroblasts

Subconfluent monolayers of embryonic skin or 3T3 fibroblasts were trypsinized, replated into 96-well tissue culture plates (Corning Inc., Corning, N.Y.) and then held for 24 hours in complete RPMI-10% FCS. The cells were then washed, the medium was replaced with complete RPMI-1% FCS (unless otherwise stated), and various activators were added to the cells. For assay of the effects of mast cell secretion products on embryonic skin fibroblasts or 3T3 cell proliferation, supernatants from IgE-sensitized mast cells that had been stimulated with antigen or supernatants from time-matched unstimulated mast cells were added in quadruplicate to the fibroblasts. Fibroblast proliferation was measured 48 hours later by using an MTT dye uptake assay, essentially as noted,¹⁶ with the exception that we used a 590 nm wavelength filter to measure the optical density of the solubilized dye. In brief, the dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/ml; Sigma), in 20 μl of DMEM, was added to the cultures, and 45 minutes later the cell-associated dye was solubilized in acidified isopropanol and quantified with an ELISA plate reader. The percent enhancement of cellular proliferation (y) for each sample was calculated by using the equation

$$y = \left(\frac{\text{OD}_{590} \text{ stimulated cells} - \text{OD}_{590} \text{ unstimulated cells}}{\text{OD}_{590} \text{ unstimulated cells}} \right) \times 100$$

In a series of preliminary dose-response experiments, we titrated the effects of the mast cell supernatants (at concentrations ranging from 0.1% to 100% of the final culture concentration) against those of recombinant murine IL-1, IL-6, and TNF-α and purified human TGF β1; for each cytokine, the concentrations utilized ranged between 0.01 and 20 ng/ml. We determined that (1) the fibroblast responses increased in a linear fashion with increasing levels of activated mast cell supernatants, with maximal responses occurring at supernatant concentrations of 50% or higher (vol/vol); (2) the fibroblast proliferative responses to each of the tested cytokines were dose-

dependent, with maximal responses to TNF- α and TGF- β 1 being observed at 20 and 10 ng/ml, respectively, and at concentrations of 1 ng/ml or lower, only IL-1, TGF- β , and TNF- α retained significant activity; and (3) there was a linear correlation between the fibroblast cell numbers and MTT dye uptake under the conditions used in these experiments ($r^2 = 0.98$). In the experiments reported here we added the mast cell supernatants to the fibroblast cultures at a 50% final concentration.

Inhibition of mast cell-dependent fibroblast proliferation with anti-cytokine antibodies

Supernatants from 2-hour antigen-activated or time-matched unstimulated BMCMC were preincubated for 3 hours on ice with selected anti-cytokine IgG antibodies (7.5 μ l of a 1 mg/ml IgG solution/50 μ l mast cell supernatant), including anti-TGF- β 1 IgG, anti-TNF- α IgG, or NRS IgG. After the 3-hour preincubation, the supernatant-antibody mixtures were added to subconfluent monolayers of embryonic skin fibroblasts, and the levels of fibroblast proliferation were determined 48 hours later, as noted above.

Cytokine assays

IL-1 bioassay

For the IL-1 bioassay we used the LM-1 subclone of the ATCC cell line D10.G4.1 (a kind gift from the Veterinary Infectious Diseases Organization, Saskatoon, Canada), which is sensitive to IL-1 in the absence of concanavalin A. These cells were maintained in Click's medium (Gibco) supplemented with 10% FCS, L-glutamine, antibiotics/antimycotics, 10% concanavalin-stimulated mouse splenocyte-conditioned medium,¹⁶ and 5×10^{-5} mol/L 2-Me. For the assay, the LM-1 cells were not fed fresh medium (i.e., they were IL-1-starved) for 4 to 7 days before use and were washed and dispensed into 96-well plates at 4×10^5 cells/ml in Click's medium but without concanavalin A-stimulated splenocyte-conditioned medium. Test samples (usually 1 to 10 μ l/well) or control agents (e.g., recombinant mouse IL-1 at 2.5 to 5000 pg/ml) were added to the wells, and the plates were incubated at 37° C for 3 to 4 days before cell proliferation was determined by the MTT uptake method, as noted earlier.¹⁶ The LM-1 cell proliferation responses could be blocked entirely with anti-IL-1 antibodies.

IL-6 bioassay

IL-6 activity was detected by using an IL-6-driven cell proliferation assay, employing the IL-6-dependent 7TD1 cell line (ATCC). In brief, the 7TD1 cells were maintained in RPMI 1640 supplemented with 80 pg/ml recombinant human IL-6, 10% FCS, 5×10^{-5} mol/L 2-Me, and 1% antibiotics/antimycotics, at a cell concentration of approximately 10^5 cells/ml. For the assay, log growth-phase 7TD1 cells were plated into 96-well plates at 2.5×10^4 cells/ml of IL-6-deficient medium, and the samples or recombinant IL-6 standards were added to the wells. After 3 to 4 days the cell proliferation in each well was determined by MTT dye uptake as described earlier. The 7TD1 cells are sensitive to IL-6 levels as low as 1 pg/ml, and their proliferation in the assay could be completely blocked with anti-IL-6 antibodies.

TNF- α bioassay

TNF- α was detected by using an MTT cytotoxicity assay with L929 cell targets, as reported previously.^{16 17} The specificity of the assay for TNF- α was confirmed by use of TNF- α antisera, and internal standards of recombinant TNF- α were used in each assay.^{16 17}

TGF- β 1 assays

We used both bioassays and immunoassays for the detection of TGF- β 1. For the biologic detection of TGF- β 1 activity, we used the Mv1Lu cell growth inhibition assay.²⁰ Briefly, monolayers of Mv1Lu cells were trypsinized, washed, and resuspended in DMEM–10% FCS and plated in 96-well plates at 1×10^4 cells/well.⁴ The following day the medium was replaced with DMEM–1% FCS and serial dilutions of transiently acidified mast cell supernatants or purified human TGF- β 1 standards. After 48 hours of culture the cell densities in the cultures were then determined by MTT dye uptake, as indicated above. The TGF- β 1 was activated by addition of 1.5 μ l of 6N HCl per 200 μ l of supernatant and incubation for 5 minutes at 22° C; the pH was then neutralized by the addition of 30 μ l of a solution of 0.5 mol/L HEPES/0.5 mol/L NaOH.²¹

Because the results of Mv1Lu bioassays can be quite dependent on the precise methodology employed and can vary substantially from one laboratory to another, we confirmed the results of our bioassay with a commercial TGF- β 1 ELISA (Quantikine TGF- β 1, R&D Systems, Inc.), used according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed with the software program Statview (version 4.1; Abacus Concepts, Berkeley, Calif.). Two group comparisons were made by Student's *t* test (two-tailed). More extensive group data were subjected to analysis of variance testing to determine the overall impact of sample treatments within an experiment, with additional post hoc testing by using the Fisher Protected Least Significant Difference (PLSD) test to determine the statistical significance of individual sample treatments on the parameters in question. The analysis of variance results are reported as significant only if both the analysis of variance and the Fisher PLSD tests yielded a probability (*p*) value of 0.05 or lower. Correlation analyses were also performed with the Statview package.

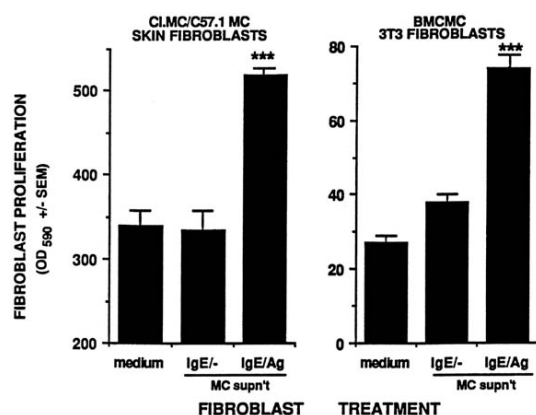
Results

TOP

Fc ϵ RI- and mast cell–dependent fibroblast proliferation occurs in the absence of fetal calf serum

Supernatants from mast cells that had been activated for 2 hours via the Fc ϵ RI in medium containing no FCS and that were also tested with fibroblasts in medium containing no FCS, significantly enhanced fibroblast proliferation (Fig. 1).

Fig. 1. Mast cell– and FcεRI-dependent fibroblast proliferation occurs in the absence of FCS. Cl.MC/C57.1 cells (*left panel*) or BMCMC (*right panel*) were sensitized with IgE anti-DNP antibody and then either incubated for 2 hours with DNP₃₀₋₄₀HSA (IgE/Ag) or left unstimulated (IgE/-). Supernatants from these cultures or culture medium alone (medium) were then added to subconfluent monolayers of embryonic skin fibroblasts (for Cl.MC/C57.1 cells) or 3T3 fibroblasts (for BMCMC), and 48 hours later, the fibroblast proliferation was measured by using an MTT assay. The results are expressed as the optical density (at a wavelength of 590 nm) of the solubilized formazan dye in the fibroblasts. Supernatants from the unstimulated mast cells had no significant effect on fibroblast growth ($p > 0.05$ vs medium alone; $n = 4$), whereas with both the Cl.MC/C57.1 cells/embryonic skin fibroblast and the BMCMC/3T3 fibroblast combinations, the supernatants from the activated mast cells induced highly significant levels of fibroblast proliferation. $***, p \leq 0.001$ for activity of supernatants from activated mast cells versus activity of medium alone or versus activity of supernatants from unstimulated mast cells. $n = 4$.



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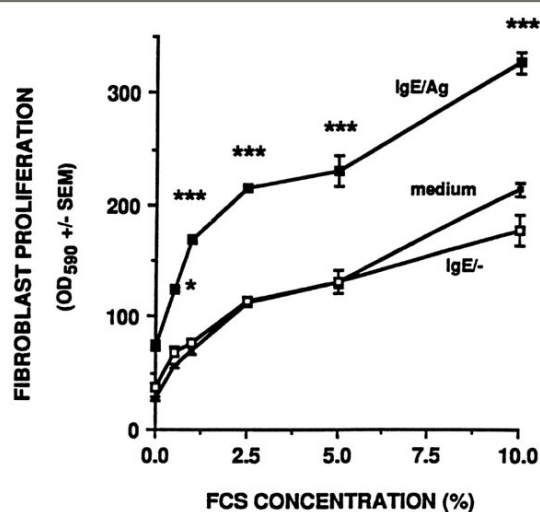
Specifically, supernatants from activated Cl.MC/C57.1 cells augmented embryonic skin fibroblast proliferative responses by $50\% \pm 3\%$ relative to culture medium alone and by $52\% \pm 3\%$ relative to time-matched supernatants from unstimulated Cl.MC/C57.1 cells. In addition, activated BMCMC released mediators that augmented 3T3 fibroblast proliferation by $164\% \pm 15\%$ relative to medium alone and by $95\% \pm 11\%$ relative to time-matched supernatants from unstimulated BMCMC (in each case, $p \leq 0.001$ vs values for medium alone or for supernatants from unstimulated mast cells; $n = 4$). Thus supernatants from IgE- and antigen-activated, but not quiescent, mast cells were strongly mitogenic for the fibroblasts, and this proliferative response did not require co-mitogenic

signals from factors present in FCS.

FCS potentiates mast cell–dependent fibroblast proliferation and is mitogenic for mouse fibroblasts

At final concentrations of 1% or less, FCS had no statistically significant effect on the growth of BALB/c 3T3 fibroblasts (Fig. 2) or embryonic skin fibroblasts (not shown), but at concentrations greater than 1%, the FCS by itself was mitogenic for the fibroblasts in a dose-dependent manner ($F_{[5, 18]} = 262.5$; $p \leq 0.001$).

Fig. 2. Effect of FCS on the in vitro proliferation of normal mouse skin fibroblasts and on mast cell–augmented fibroblast proliferation. Medium alone (medium) or supernatants from unstimulated (IgE/-) or activated (IgE/Ag) BMCMC were added to 3T3 fibroblasts, and the levels of cellular proliferation were determined 48 hours later, as in Fig. 1 ■. The FCS by itself was highly mitogenic for the fibroblasts, and there were no statistically significant differences between the fibroblast responses with medium alone or with supernatants from unstimulated mast cells ($p > 0.05$). However, at all concentrations of FCS, the supernatants from the activated mast cells induced significantly greater fibroblast proliferation. * and ***, $p \leq 0.05$ and $p \leq 0.001$, respectively, for activity of supernatants from activated mast cells versus activity of medium alone.



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Furthermore, supernatants from activated (IgE/Ag), but not from quiescent (IgE/-), BMCMC strongly potentiated the mitogenic effects of FCS for 3T3 fibroblasts (Fig. 2 ■). The relative effect of the activated mast cell supernatants on the fibroblasts was greatest with 1% FCS, at which

concentration the fibroblast growth increased by $141\% \pm 4\%$ or $119\% \pm 4\%$ compared with values for fibroblasts exposed to medium alone or to supernatants from unstimulated mast cells, respectively. However, at all concentrations of FCS, the fibroblast-proliferative responses to activated mast cell supernatants were significantly augmented relative to those observed with either medium alone or with unstimulated mast cell supernatants (Fig. 2 ■).

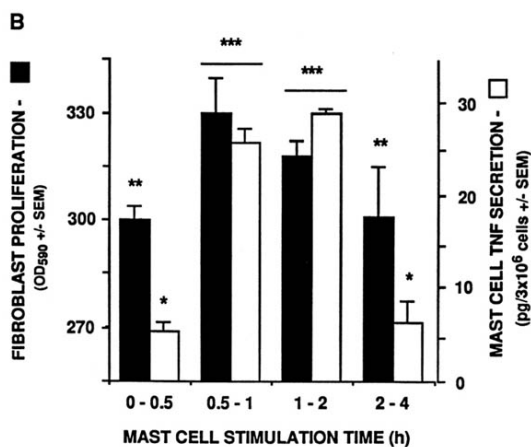
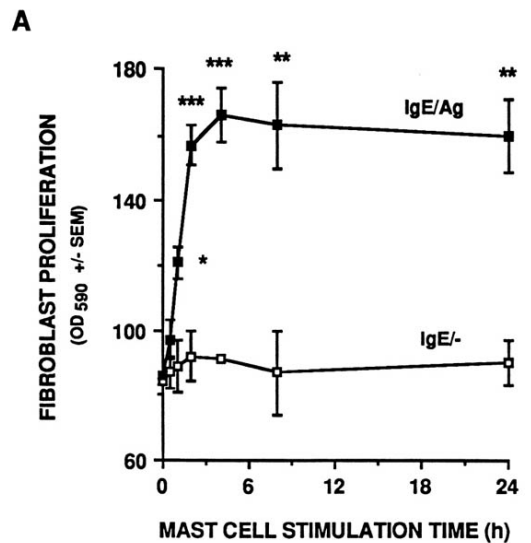
Mast cells secrete products that augment fibroblast proliferation both immediately and for a prolonged period after cellular activation

After IgE-dependent mast cell activation, the secretion of different mast cell–derived mediators can exhibit distinct kinetics.^{22 23} The secretion of certain of the preformed, granule-associated mediators (e.g., histamine, serotonin) is completed very rapidly (in less than 10 minutes) on anaphylactic degranulation of the cells, and the arachidonic acid metabolites (e.g., prostaglandin D₂ and leukotriene C₄) are synthesized de novo over a longer time frame, whereas some of the granule-associated mediators, such as heparin, may undergo solubilization very slowly.²² Mast cell–associated cytokines (e.g., TNF- α ¹⁷) can be secreted either rapidly (within approximately 30 minutes) from a preformed pool or over a more prolonged period (approximately 2 hours) from a de novo–synthesized pool.^{23 24} Thus the time frame over which biologic activities are detectable in mast cell supernatants can provide clues concerning the nature of the mediators involved.

When we examined the production of fibroblast proliferative activities by stimulated mast cells, we found that relatively low levels were released immediately but that the secretion levels increased substantially thereafter (Fig. 3, A).

Fig. 3. A, Time course of release of fibroblast proliferation factor(s) from mast cells that had been stimulated via the Fc ϵ RI (IgE/Ag) or from time-matched, unstimulated mast cells that had been sensitized with IgE but not challenged with antigen (IgE/-). BMCMC were treated, and their supernatants were tested on 3T3 fibroblasts as in Fig. 1 ■, except that all culture media contained 1% FCS. The results are expressed as in Fig. 1. B ■, Comparison of the secretion of TNF- α and fibroproliferative activities of BMCMC stimulated via the Fc ϵ RI. Supernatants of IgE and Ag-activated BMCMC were harvested at the indicated time intervals after cellular activation by antigen and were then assayed for fibroproliferative activity (*black bars*) as in Fig. 1 ■ and for TNF- α content (*white bars*) by MTT bioassay. Fibroblast proliferation data are reported as above, whereas the TNF- α results are presented as pg TNF per 3×10^6 BMCMC. The secretion kinetics for the two activities are not strongly correlated ($r^2 = 0.354$, $n = 4$). *, **, and ***, $p \leq 0.05$, $p \leq 0.01$, and $p \leq$

0.001, respectively, for activity of supernatants from activated mast cells versus activity of supernatants from unstimulated BMCMC (not shown). $n = 4$.



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Specifically, within 30 minutes of activation via the FcεRI, BMCMC released sufficient activity to augment fibroblast growth $15\% \pm 9\%$ over that observed with supernatants from unstimulated mast cells, but by 2 hours after activation, the supernatants of the activated BMCMC augmented fibroblast proliferation by $87\% \pm 7\%$ relative to values for unstimulated mast cell supernatants. Time-matched supernatants from unstimulated BMCMC had no significant effect on fibroblast proliferation (Fig. 3, A■). When we examined the release of fibroblast-proliferative activities by CI.MC/C57.1 cells, the results were virtually identical to those obtained with BMCMC (data not shown).

Because the kinetics of TNF-α release from mast cells¹⁷ and the potential for TNF-α to affect fibroblast proliferation¹⁸ are well documented, we next compared the release, from activated BMCMC, of both TNF-α and the fibroblast growth-enhancing activity (Fig. 3, B■). BMCMC were again activated via the FcεRI, and samples of the mediators released between 0 and 0.5 hour, 0.5 and 1 hour, 1 and 2 hours, and 2 and 4 hours after stimulation were collected and analyzed. TNF-α secretion followed the pattern we reported previously for mast cells,¹⁷ with relatively low levels of

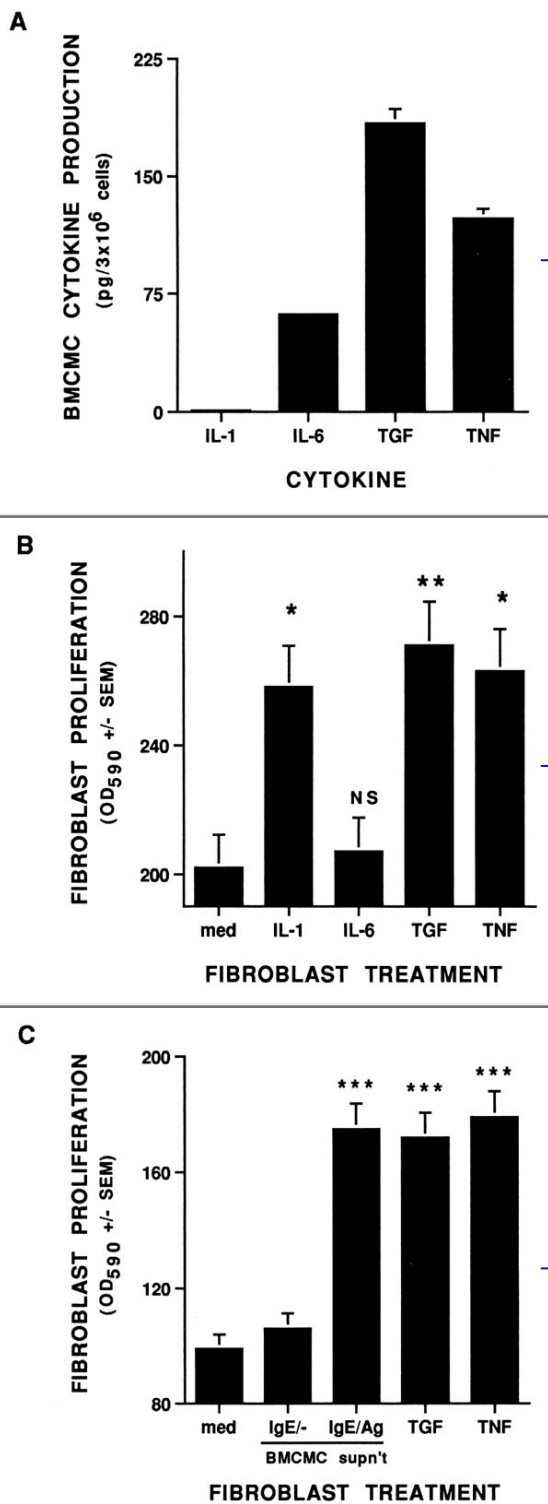
cytokine released initially (between 0 and 0.5 hour) and increasing amounts of TNF- α found in the supernatants between 0.5 and 1 hour and between 1 and 2 hours; TNF- α secretion declined somewhat during the 2- to 4-hour period after antigen challenge (Fig. 3, **B**[■]). The BMCMC cumulatively secreted approximately 60 pg TNF- α per 3×10^6 cells within the first 2 hours after activation. Assessment of parallel time-frame supernatants collected from BMCMC not challenged with antigen confirmed that the repeated medium changes did not affect activation of these cells; their mean TNF release across all time frames was 1.27 ± 1.16 pg per 3×10^6 cells. By contrast, fibroblast-proliferative activity continued to be released in substantial amounts in each of the four periods tested, although that released in the first period (from 0 to 0.5 hour after antigen stimulation) was lower than that observed in the subsequent three intervals (Fig. 3, **B**[■]). Correlational analysis indicated that there were differences in the production kinetics of TNF- α and the fibroblast-proliferative activities ($r^2 = 0.354$; $n = 4$). As might be expected, these results strongly suggested that the fibroblast-proliferative activity in these activated mast cell supernatants did not reflect their TNF- α content solely.

IL-3-dependent bone marrow-derived mouse mast cells secrete TGF- β 1 and TNF- α as their primary fibroblast-reactive cytokines

We have previously described the capacity of mast cells to secrete a wide array of cytokines,²³⁻²⁵ and many of these, including IL-1, IL-6, TGF- β 1, and TNF- α , reportedly have the capacity to activate or otherwise interact with fibroblasts.^{7,9,18} Therefore we examined the capacity of our mast cells to secrete each of these cytokines (Fig. 4, **A**).

Fig. 4. A, Mast cells secrete multiple potentially fibroblast-reactive cytokines after activation via the Fc ϵ RI. BMCMC were activated via the Fc ϵ RI as in Fig. 1[■], and the cell supernatants collected at 4 hours after challenge were analyzed for IL-1, IL-6, TGF- β , and TNF- α . **B**, IL-1, IL-6, TGF- β , and TNF- α exert differential effects on mouse embryonic skin fibroblast proliferation. Each cytokine was added to subconfluent cultures of mouse embryonic skin fibroblasts at 50 pg/ml, and the fibroblast-proliferative responses were measured as in Fig. 1[■]. **C**, Comparison of the fibroblast proliferation induced by supernatants from unstimulated (IgE/-) and activated (IgE/Ag) BMCMC with that induced by optimal doses of TNF- α and TGF- β . Supernatants from BMCMC treated as in Fig. 1[■] (at 50% final concentration), TNF- α (10 ng/ml), or TGF- β (20 ng/ml) were added to embryonic skin fibroblasts, and their relative effects on fibroblast proliferation were monitored as above. Supernatants from unstimulated BMCMC had no significant effect on fibroblast proliferation ($p > 0.05$ vs values for

medium [RPMI-1% FCS] alone). *, **, and *** represent $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively, versus values for medium alone or values for supernatants from unstimulated BMCMC. $n = 4$. The results are expressed as in Fig. 1■.



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Over a 4-hour period after stimulation via the FcεRI, BMCMC released only negligible amounts of IL-1 (less than 3 pg per 3 × 10⁶ cells) but substantial amounts of IL-6 (57.1 pg per 3 × 10⁶ cells), TGF-

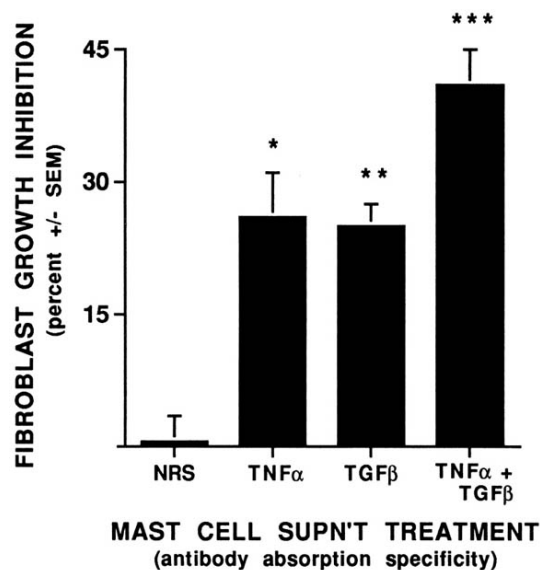
β 1 (184 pg per 3×10^6 cells), and TNF- α (128 pg per 3×10^6 cells) (Fig. 4, A ■). Similarly Cl.MC/C57.1 mast cells challenged in this fashion released 4.4 pg per 3×10^6 cells of IL-1, 59 pg per 3×10^6 cells of IL-6, 165 pg per 3×10^6 cells of TGF- β 1, and 210 pg per 3×10^6 cells of TNF- α over 4 hours. The differences observed in TNF- α production by these two populations of mast cells reconfirm our earlier observations about their relative abilities to produce this cytokine.¹⁷

We also assessed the relative impact of each of these cytokines on the proliferation of embryonic skin fibroblasts, by performing a series of dose-response experiments with recombinant or purified cytokines. When used at the limiting dose of 50 pg/ml, these cytokines differentially affected fibroblast proliferation (Fig. 4, B ■). Their rank order of activity in this assay was TGF- β 1 > TNF- α > IL-1; at doses of 1 ng/ml or lower, IL-6 did not induce statistically significant levels of cellular proliferation. Because neither BMCMC nor Cl.MC/C57.1 cells produced substantial amounts of IL-1 and because the fibroblasts were not significantly reactive to IL-6 at the levels found in the mast cell supernatants, our data suggested that, among the cytokines tested, TGF- β 1 and TNF- α could be two mast cell mediators of some importance for mast cell-dependent fibroblast-proliferative responses. Indeed, when we compared the fibroblast-proliferative responses induced by supernatants of activated BMCMC (at a final concentration of 50%) with those induced by optimal levels (10 to 20 ng/ml) of TNF- α or TGF- β 1 (Fig. 4, C ■), we found that there were no significant differences between these stimuli ($F_{[3,12]} = 0.23$, $p = 0.88$). It is noteworthy that the levels of TGF- β 1 and TNF- α in the activated BMCMC supernatants (approximately 128 to 184 pg/ml; Fig. 4, A ■) were approximately two orders of magnitude less than the concentration of either cytokine that was required to induce fibroblast responses equivalent to those mediated by the BMCMC supernatants (10 to 20 ng/ml; Fig. 4, C ■). These results suggested that the putative effects on fibroblast proliferation of mast cell TGF- β 1 and TNF- α would probably arise through synergistic interactions of these two cytokines with each other and/or other mast cell mediators.

Absorption of activated BMCMC secretion products with anti-TGF- β 1 or anti-TNF- α antibodies significantly reduces their ability to induce fibroblast proliferation

To determine the extent to which BMCMC TNF- α and TGF- β 1 actually contribute to mast cell-dependent fibroblast proliferation, we examined the effects of neutralizing anti-TNF- α and anti-TGF- β 1 antibodies on the mast cell-driven fibroblast responses. Supernatants from unstimulated or activated BMCMC were preincubated with purified anti-TNF- α or anti-TGF- β 1 IgG or with equivalent levels of NRS IgG and were then tested for their remaining abilities to stimulate fibroblast proliferation (Fig. 5).

Fig. 5. Inhibition of mast cell-mediated fibroblast proliferation by anti-TNF- α and anti-TGF- β 1 IgG antibodies. Supernatants from activated BMCMC were incubated with neutralizing doses of purified rabbit IgG antibodies specific for TNF- α , TGF- β , or a combination of anti-TNF- α and anti-TGF- β (TNF- α + TGF- β) IgGs, or with equivalent amounts of purified normal rabbit serum IgG (NRS). The supernatant-antibody mixtures were added to subconfluent monolayers of fibroblasts, and the cellular proliferation over 48 hours was assessed as above. The percent inhibition of proliferation associated with the various antibodies was compared with that observed with NRS. *, **, and *** represent $p < 0.02$, $p < 0.01$, or $p < 0.001$, respectively. The results are presented as the percent inhibition of fibroblast proliferation as judged from parallel untreated supernatants from activated BMCMC.



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These levels of IgG were sufficient to inhibit the TNF- α and TGF- β 1 bioactivities of the BMCMC supernatants by $98\% \pm 3\%$ and $97\% \pm 0.1\%$, respectively. As reported previously,^{16 17} the NRS IgG was without detectable effect in the TNF- α assay and had no discernible effect in the TGF- β 1 bioassay (data not shown). The treatment with control NRS IgG also had no significant effect on the ability of activated mast cell supernatants to stimulate embryonic fibroblast proliferation, whereas the anti-TNF- α and anti-TGF- β 1 treatments both reduced the fibroblast responses significantly ($p < 0.01$ for both anti-TNF- α and anti-TGF- β 1 vs NRS IgG; $n = 5$). The effect of combining the anti-TNF- α and anti-TGF- β 1 treatments was greater than that of either antiserum alone, but even this combination did not result in greater than approximately 50% inhibition of fibroblast proliferation

(Fig. 5■). These results demonstrate that mast cell–derived TNF- α and TGF- β 1 each contribute significantly to the mast cell–dependent fibroblast proliferation observed in our experiments, probably through synergistic or additive interactions with other mast cell mediators.

Discussion

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We found that mouse mast cells activated by IgE-dependent mechanisms, but not unstimulated mast cells, release soluble products that can promote fibroblast proliferation in vitro and that this effect can be observed in the complete absence of FCS. Moreover, our mast cell cytokine secretion analyses combined with our assessment of the differential effects of these cytokines on fibroblast proliferation suggested that mast cell TGF- β 1 and TNF- α could be important to mast cell–dependent fibroblast proliferation, and our antibody blocking studies confirmed that this was the case. In addition, our experiments also suggest that mast cell IL-1 and IL-6 probably did not contribute greatly to this effect.

These results extend our understanding of how mast cell activation might promote fibroblast proliferation. For example, previous studies that indicated that mast cell activation could be followed by fibroblast proliferation employed agents, such as compound 48/80, that do not activate mast cells as selectively as do IgE and specific antigen or were performed in media containing high levels (i.e., up to 20%) of FCS.⁵ In such situations, it may be difficult to determine whether products of mast cells directly stimulate proliferation of fibroblasts or are mitogenic because they act synergistically with or modify the activity of potentially mitogenic constituents of the calf serum. Furthermore, during allergic or inflammatory responses in vivo in which both mast cell activation and fibroblast proliferation are observed, it might be argued that the fibroblast responses are attributable to factors in the augmented vascular ultrafiltrates associated with the accompanying tissue edema or to mediators secreted by other cells involved in the responses. However, mouse mast cells have been identified as a source of TNF- α ,^{15 16} and TGF- β 1,^{13 14} and we and others have proposed that the production of these and other cytokines by mast cells may represent one potential link between mast cell activation and fibroblast proliferation.^{1 14 23} Indeed, the data reported here represent the best evidence to date indicating that cytokines produced by activated mast cells can actually promote fibroblast proliferation.

Mast cells exhibit a complex bidirectional relationship with fibroblasts, and some of these interactions require cell-cell contact, whereas others do not. Fibroblasts represent a source of both membrane-associated and soluble forms of the c-kit ligand, stem cell factor, and either form of stem cell factor can promote mast cell development in vitro or in vivo.²⁶ However, several lines of evidence suggest that the membrane-associated form of stem cell factor may be a more important mast cell development factor in vivo than is the soluble form of the growth factor.²⁶ In the case of mast cell–dependent regulation of fibroblast proliferation, the 3T3 cell proliferation that was observed when the cells were cocultured with BMCMC in IL-3 and FCS required prolonged contact between the mast cells and the fibroblasts.¹⁰ By contrast, the fibroblast proliferation that we observed as a result of products released by mast cells activated through IgE-dependent mechanisms was detectable rapidly. Although mast cells may significantly influence fibroblast proliferation under some circumstances by either contact-dependent or contact-independent mechanisms, morphologic observations indicate that most tissue mast cells are not in direct contact

with fibroblasts in situ but are separated from them and other potential cellular targets by components of the extracellular matrix.

Alterations in fibroblast numbers that are associated with mast cell activation in vivo clearly may reflect direct or indirect effects of mast cell activation. This study has confirmed that mast cells can affect fibroblast proliferative responses without the participation of either other cell types or serum components, and thus, in this sense, the mast cell can have direct effects on fibroblast responses. However, we have previously shown that the infiltration of leukocytes into IgE-dependent cutaneous reactions in the mouse is entirely mast cell–dependent and is mediated in large part by TNF- α .²⁷ We and others have suggested that cytokines and other products of the recruited leukocytes would contribute to the progression of these mast cell–induced responses.^{1 23 25} For example, eosinophils, which can be prominent in the leukocyte infiltrates that develop after mast cell activation,²⁸ represent potential sources of additional TNF- α ²⁹ or TGF- β 1.^{30 31} Thus from this perspective, during responses involving the recruitment of additional sources of fibroblast-activating mediators, the mast cell would also exert indirect effects on the fibroblast. The array of mediators secreted by mast cells could also stimulate fibroblast proliferation through the ability of these mediators to induce the secondary production of autocrine growth factors (e.g., platelet-derived growth factor) by the fibroblasts or through mediators that overtly induce fibroblast cell cycling. For example, either IL-1 or TGF- β 1, both of which have been identified as mast cell products,^{11 14} can induce fibroblasts to produce platelet-derived growth factor,¹⁸ whereas other mast cell products such as TNF- α and IL-4^{23 25} could well exhibit direct effects on the fibroblasts. Overall, the effect of mast cells on fibroblast proliferative responses is probably a complex one that involves both direct and indirect effects.

Although we have demonstrated here that mast cell TNF- α and TGF- β 1 do significantly affect fibroblast proliferation, even a mixture of antibodies against these two cytokines inhibited the fibroblast proliferation induced by supernatants of activated mast cells by approximately 50% only (Fig. 5■). This finding indicates that these cytokines almost certainly do not account for all of the ability of activated mast cell supernatants to augment fibroblast proliferation in vitro. Furthermore, the observation that the amounts of TNF- α and TGF- β 1 in the activated mast cell supernatants that induced optimal fibroblast responses were significantly (by several orders of magnitude) lower than the levels of recombinant or purified cytokine required for equivalent responses suggests that mast cell TNF- α and TGF- β 1 might interact synergistically with other mast cell mediators that also express fibroblast mitogenic activities (e.g., tryptase, IL-4^{7 9 18}). Thus it will be of some interest to determine which other products of mast cell or fibroblast origin contribute to the mast cell–dependent fibroblast proliferation observed in this system.

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