

Influence of IgE-mediated activation of cultured human mast cells on proliferation and type I collagen production by human dermal fibroblasts

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Background: Mast cells have been suggested to be involved in fibrotic conditions, but it still remains unknown whether IgE-mediated activation of human mast cells promotes fibrogenesis by human fibroblasts.

Objective: The purpose of this study was to determine whether IgE-mediated activation of cultured human mast cells can promote fibrogenesis by cultured human dermal fibroblasts.

Methods: Mast cells derived from human umbilical cord blood cells were incubated with IgE and then activated by anti-IgE, and histamine release was measured. IgE-sensitized mast cells were cocultured with fibroblasts from normal dermis and activated with anti-IgE to induce histamine release, after which proliferation and type I collagen synthesis by the fibroblasts were determined.

Results: Coculture of subconfluent human dermal fibroblasts with IgE-sensitized mast cells did not affect fibroblast proliferation. However, fibroblast proliferation was increased by activated mast cells, and a significant increase was observed in the presence of 10^5 or 3×10^5 mast cells/mL. The promotion of fibroblast proliferation by mast cells (3×10^5 /mL) was partly inhibited by ketotifen at a concentration that significantly reduced histamine release from mast cells. On the other hand, IgE-mediated activation of mast cells did not increase type I collagen production by confluent human dermal fibroblasts.

Conclusion: IgE-mediated activation of cultured human mast cells could increase the proliferation of human dermal fibroblasts, but did not promote type I collagen production by the fibroblasts under the conditions tested. (J Allergy Clin Immunol 2000;106:S72-7.)

Key words: Cultured human mast cells, histamine, ketotifen, human dermal fibroblasts, proliferation, collagen

Mast cells are considered to be central to IgE-mediated allergic reactions, including both the immediate and late-phase responses. Recent studies have suggested that mast cells may also take part in other biologic reactions

Abbreviations used

DMEM: Dulbecco's modified Eagle's medium
HMC-1: Human mast cell 1

that are not essentially dependent on IgE. For example, mast cells frequently accumulate at sites of fibrosis, such as the involved skin of scleroderma patients¹ and fibrotic lung tissue.² Tissue fibrosis and fibroblast proliferation are found in chronic inflammatory diseases and certain allergic disorders, such as allergic asthma,³ which suggests a possible contribution of mast cells to the pathogenesis of various fibrotic conditions.

The 2 major hallmarks in fibrosis are enhanced collagen synthesis by fibroblasts and increased fibroblast proliferation. A number of experimental studies have suggested that mast cell degranulation may induce fibroblast proliferation, but such experiments have usually been performed with rodent mast cells cultured in media containing a high level of FCS,⁴⁻⁶ which may itself be mitogenic for fibroblasts. However, Gordon and Galli⁷ demonstrated that IgE-stimulated mast cell degranulation causes a rapid increase of collagen synthesis by fibroblasts in mast cell-deficient and mast cell-reconstituted mice. Recently, Gruber et al⁸ reported that human mast cells may activate fibroblasts, but they used a cultured human mast cell line, human mast cell-1 (HMC-1),⁹ which does not express the high-affinity surface receptor for IgE. Thus whether IgE-mediated activation of human mast cells actually promotes fibrogenesis by normal human fibroblasts still remains unknown.

Investigation of mast cell function has mainly been conducted with rodent mast cells because such cells are relatively easy to obtain. The problem is that mast cells are heterogeneous^{10,11} and may show functional differences between species. Human mast cells have been isolated from several organ sources for some experiments, but complicated processes were required to obtain these cells, and changes of membrane receptor expression may have occurred during the purification process. On the other hand, recent advances have allowed us to obtain cultured human mast cells from umbilical cord blood cells.¹²⁻¹⁴ The advantages of this method are that a relatively large number of cells can be obtained and that potential changes during the purification process can be

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avoided. Therefore such cells may provide a useful model of human mast cells *in vitro*. In the present study, IgE-sensitized cultured human mast cells were activated by anti-IgE, and we examined whether coculture of these cells with human dermal fibroblasts in the absence of mitogenic serum components could influence fibroblast proliferation and production of type I collagen. Our results indicated that IgE-mediated activation of cultured human mast cells could enhance fibroblast proliferation but not type I collagen production.

METHODS

This study was approved by the Review Board of Gunma University School of Medicine.

Human dermal fibroblast culture

After informed consent was given, normal skin tissue was obtained from surgical specimens resected from the forearm. The skin was cut into small pieces, and the adherent outgrowth cells from the minced skin pieces were cultured in Dulbecco's modified Eagle's medium (DMEM; Nihon Seiyaku, Tokyo, Japan) containing 10% FCS (Cytosystems, Castle Hill, Australia). Incubation was performed at 37°C in a 5% carbon dioxide–humidified incubator, and the culture medium was changed every 3 days. The cells were passaged with trypsin, maintained in a subconfluent state, and expanded for 3 passages before use in the experiments.

Human mast cell culture

Mast cells were obtained from human umbilical cord blood cells by the method described elsewhere.^{13,14} Briefly, fresh heparin-treated umbilical cord blood (approximately 20 mL) was layered over Ficoll-paque (Pharmacia & Upjohn, Uppsala, Sweden) and centrifuged at 400*g* for 20 minutes. The mononuclear cell layer was collected and incubated at 1×10^6 cells/mL in Media I (DMEM containing 10/ μ g/mL insulin, 10 μ g/mL transferrin, 5×10^{-5} mol/L 2-mercaptoethanol, 25 mmol/L Hepes, and 2.6 ng/mL NaSeO₃; IBL Men-eki Seibutsu Kenkyujo, Gunma, Japan) supplemented with 5% FCS, 50 ng/mL human recombinant IL-6 (IL-6), and 80 ng/mL human recombinant stem cell factor (provided by Kirin Brewery Co Ltd, Tokyo, Japan) at 37°C in a 5% carbon dioxide–humidified incubator. The cells were passaged every week and maintained in the same medium. Cell counting was performed by toluidine blue staining. The cells were then fixed with Carnoy's fluid for 15 minutes at room temperature and stained for mast cell tryptase and chymase with monoclonal murine antitryptase or antichymase antibodies, as reported by Irani et al.¹⁵ The purity of the mast cells reached 90% after 10 weeks of culture,¹³ and cells more than 100 days old were used in the present study. Virtually 100% of the cells used were trypan blue–negative and toluidine blue–positive. When the cells were immunocytochemically stained for tryptase, almost 100% were positive. Among the tryptase-positive cells, $30.6\% \pm 9.8\%$ (mean $\pm$ SEM; $n = 6$ experiments) were positive for chymase, and there was some variation in the intensity of the chymase staining.

Activation of human mast cells

Cultured human mast cells were incubated with 1 μ g/mL human myeloma IgE (Chemicon, Temecula, Calif.) for 1 hour at 37°C, washed twice with a cold buffer,¹³ and then resuspended in DMEM without FCS before the experiments described later. The cells were either stimulated with anti-IgE or left untreated. Maintenance of activated mast cells in DMEM without FCS had no adverse effects on their viability or secretion of histamine.

Assay of histamine release from human mast cells

Immunologic histamine release was assessed in duplicate tubes containing 3×10^3 to 10^4 mast cells. IgE-sensitized human mast cells were resuspended in DMEM without FCS and were preincubated for 5 minutes at 37°C, after which the cells were incubated with anti-human IgE (Cappel, Durham, NC) for 24 hours at 37°C in a 5% carbon dioxide–humidified incubator. Culture was terminated by the addition of ice-cold buffer to the cell suspension and centrifugation at 200*g* for 10 minutes at 4°C. Perchloric acid was added to the supernatant at a final concentration of 1%, and the histamine level in the supernatant was measured by high-performance liquid chromatography.¹³ Spontaneous release was evaluated in duplicate tubes without anti-IgE. Total histamine was measured in parallel duplicate tubes after lysing the cells with 2 freeze-thaw cycles and removal of protein by centrifugation. To investigate the effect of ketotifen on histamine release, ketotifen was added to the incubation mixture at 1 minute before the addition of anti-IgE. At the concentrations used in the present study, ketotifen did not affect spontaneous histamine release from cultured human mast cells. The results were analyzed by a data integrator (Hitachi D-2500; Hitachi, Tokyo, Japan), and net histamine release by anti-IgE was expressed as a percentage of total histamine after correction for spontaneous release.

Assay of fibroblast proliferation

Subconfluent monolayers of fibroblasts were trypsinized, seeded into 96-well tissue culture plates at a density of 2.5×10^4 cells/mL in DMEM containing 10% heat-inactivated FCS and cultured overnight at 37°C in a 5% carbon dioxide–humidified incubator to allow adherence. The medium was then replaced with FCS-free DMEM. After 24 hours of serum deprivation, IgE-sensitized mast cells suspended in FCS-free DMEM at 10^2 to 3×10^5 cells/mL were seeded onto the fibroblasts. To assess the effect of mast cell activation on fibroblast proliferation, anti-human IgE was added to quadruplicate cultures. As a control, FCS-free DMEM used for the dilution of anti-IgE was added. After coculture for 24 hours at 37°C in the incubator, the medium was aspirated, and nonadherent cells were removed by washing the plates twice with PBS. In some experiments, fibroblasts were incubated for 24 hours at 37°C in FCS-free DMEM alone or in medium containing histamine (Sigma, St Louis, Mo). To investigate the effect of ketotifen on fibroblast proliferation, ketotifen was added to the cultures 5 minutes before anti-IgE or histamine.

The proliferative response of fibroblasts was measured with [³H]thymidine incorporation. In brief, fibroblasts were incubated with [³H]thymidine (specific activity: 230 Bq/mmol; American Radiolabeled Chemicals, St Louis, Mo) at a final concentration of 10 mCi/mL. After 6 hours of incubation at 37°C, the cells were washed 3 times with cold PBS and 4 times with cold 0.1 N perchloric acid. After the addition of 0.2 mL of 0.1 NaOH/0.1% sodium dodecyl sulfate per well, the radioactivity was measured with a liquid scintillation counter (Beckman LS7000; Beckman Instruments, Fullerton, Calif) after cells were dissolved in 4 mL of Aquasol-2 (New England Nuclear, Boston, Mass). In each experiment, the counts per minute were averaged for 3 determinations.

Assay of type I collagen synthesis

Fibroblasts were seeded into 96-well tissue culture plates at a density of 2.5×10^4 cells/mL in DMEM containing 10% heat-inactivated FCS and were cultured at 37°C in a 5% carbon dioxide–humidified incubator until confluence was reached. The medium was then replaced with FCS-free DMEM. After 24 hours of serum deprivation, IgE-sensitized mast cells in FCS-free DMEM

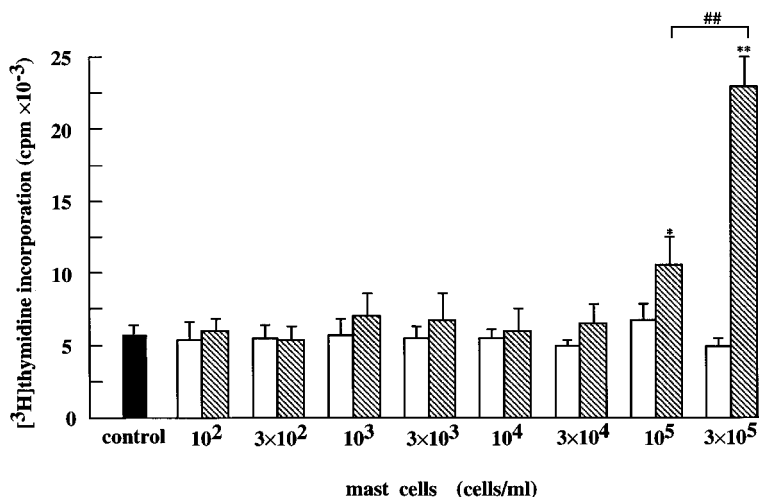


FIG 1. Proliferative response of human fibroblasts cocultured with human mast cells. Mast cells derived from umbilical cord blood cells were sensitized with IgE and seeded onto subconfluent monolayers of fibroblasts from normal dermis. The proliferative response was assessed with [³H]thymidine incorporation, which was measured for fibroblasts incubated with medium alone (*control*) and incubated with IgE-sensitized mast cells in the absence (*open bars*) or presence (*hatched bars*) of anti-IgE. Values shown are the mean $\pm$ SEM of 5 separate experiments. * $P < .05$; ** $P < .01$ versus the untreated control; ## $P < .01$ for the difference between the indicated groups.

(10^2 to 3×10^5 cells/mL) were seeded onto the fibroblasts. To assess the effect of mast cell activation on collagen production, anti-human IgE was added to quadruplicate cultures. As a control, FCS-free DMEM, which was used for the dilution of anti-IgE, was added. After coculture for 24 hours at 37°C, the procollagen type I C-peptide concentration in the culture supernatant was measured as a marker of the activity of type I collagen synthesis.^{16,17} The concentration of this peptide was measured by ELISA with the use of a procollagen type I C-peptide kit (Takara Shuzo, Kyoto, Japan).¹⁸

Statistical analysis

Results are expressed as the mean $\pm$ SEM. Statistical analysis of the effects of mast cells on fibroblasts was performed with Statview software (version 4.0; Abacus Concepts, Berkeley, Calif). Group data were subjected to analysis of variance to determine the overall impact of sample treatments within experiments, with additional post hoc testing with Fisher's protected least significant difference test to determine the statistical significance of individual treatments on the parameters in question. The results of analysis of variance were only reported as significant if both analysis of variance and Fisher's protected least significant difference test yielded a probability value of .05 or less. The effect of ketotifen on [³H]thymidine incorporation by fibroblasts in the presence of histamine and the significance of its effect on histamine release were determined by analysis of variance followed by Dunnett's multiple comparison test; a probability value of .05 or less was considered significant.

RESULTS

Effect of IgE-mediated activation of mast cells on fibroblast proliferation

Preliminary studies showed that histamine release from IgE-sensitized cultured human mast cells was dependent on the concentration of anti-IgE, as reported previously,⁷ and considerable histamine release from the cells was observed after stimulation by an optimal anti-IgE concentration of 10 μ g/mL ($31.5 \pm 8.8\%$, mean $\pm$

SEM; $n = 5$ experiments). Therefore anti-IgE at a concentration of 10 μ g/mL was used in the following experiments. When fibroblast proliferation was assayed with or without mast cells, the peak of [³H]thymidine incorporation was at 24 to 48 hours, so fibroblast proliferation in cultures or cocultures was measured 24 hours after the addition of anti-IgE. At a concentration of 10 μ g/mL, anti-IgE did not influence the proliferation of fibroblasts cultured in the absence of sensitized mast cells.

First, the effect of unactivated IgE-sensitized mast cells (ie, without exposure to anti-IgE) on fibroblast proliferation was assessed. The addition of various numbers of mast cells (10^2 to 3×10^5 cells/mL) to proliferating fibroblasts did not affect their growth, because [³H]thymidine incorporation did not differ between cocultures and control cultures (Fig 1).

Next, the effect of IgE-mediated activation of mast cells on fibroblast proliferation was examined. When IgE-sensitized mast cells (10^5 or 3×10^5 cells/mL) were activated by 10 μ g/mL anti-IgE, they significantly enhanced [³H]thymidine incorporation into fibroblasts compared with control cultures ($P < .05$ at 10^5 cells/mL and $P < .01$ at 3×10^5 cells/mL; Fig 1). The extent of [³H]thymidine incorporation in the presence of 3×10^5 mast cells/mL was significantly higher than in the presence of 10^5 mast cells/mL ($P < .01$).

Subsequently, the effect of ketotifen was examined on histamine release from IgE-sensitized cultured human mast cells stimulated by anti-IgE. Histamine release in the presence of ketotifen was determined as a percentage of control release. Incubation of mast cells with ketotifen caused dose-dependent inhibition of histamine release, which decreased to $90.5 \pm 3.1\%$, $86.4 \pm 6.9\%$, and $54.6 \pm 13.6\%$ at concentrations of 10^{-6} , 10^{-5} , and 10^{-4} mol/L, respectively. At a concentration of 10^{-4} mol/L,

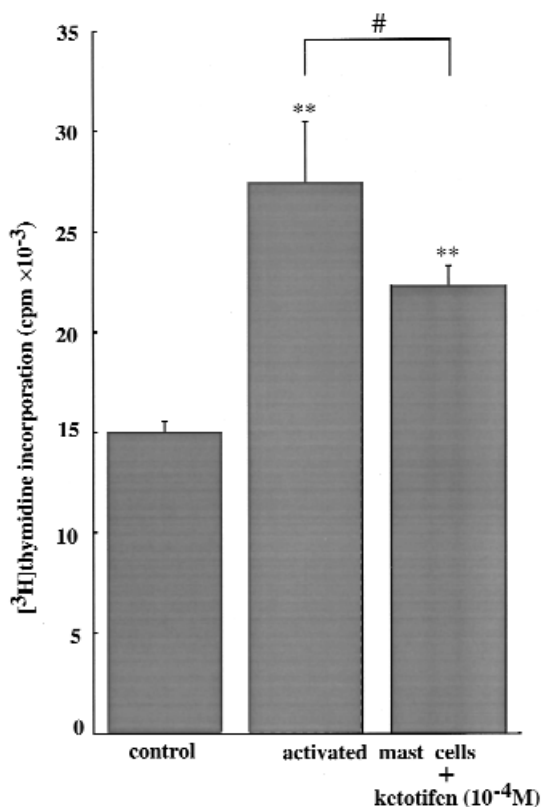


FIG 2. Effect of ketotifen on the proliferative response of human dermal fibroblasts cocultured with human mast cells stimulated by anti-IgE. Incorporation of [³H]thymidine was assessed for fibroblasts incubated with medium alone (*control*) and incubated with anti-IgE-activated mast cells in the absence or presence of 10⁻⁴ mol/L ketotifen, which significantly inhibited histamine release from mast cells. Values shown are the mean ± SEM of 5 separate experiments. ***P* < .01 versus the untreated control; #*P* < .05 for the difference between the indicated groups.

ketotifen significantly inhibited histamine release from mast cells stimulated by anti-IgE.

The effect of ketotifen on fibroblast proliferation was examined with another series of fibroblasts. At a concentration of 10⁻⁴ mol/L, ketotifen did not alter fibroblast proliferation in the absence or presence of IgE-sensitized mast cells (3 × 10⁵ cells/mL). As shown in Fig 2, the enhancement of fibroblast proliferation by IgE-mediated activation of mast cells was significantly inhibited by ketotifen at this concentration (*P* < .05).

Finally, the effect of histamine on human dermal fibroblast proliferation was examined. Preliminary studies showed that histamine enhanced [³H]thymidine incorporation by human dermal fibroblasts and that a histamine concentration of 10⁻³ mol/L significantly increased incorporation. As shown in Fig 3, this effect of histamine was inhibited by ketotifen in a dose-dependent manner, and a ketotifen concentration of 10⁻⁶ mol/L significantly inhibited the promotion of fibroblast proliferation by histamine (*P* < .05).

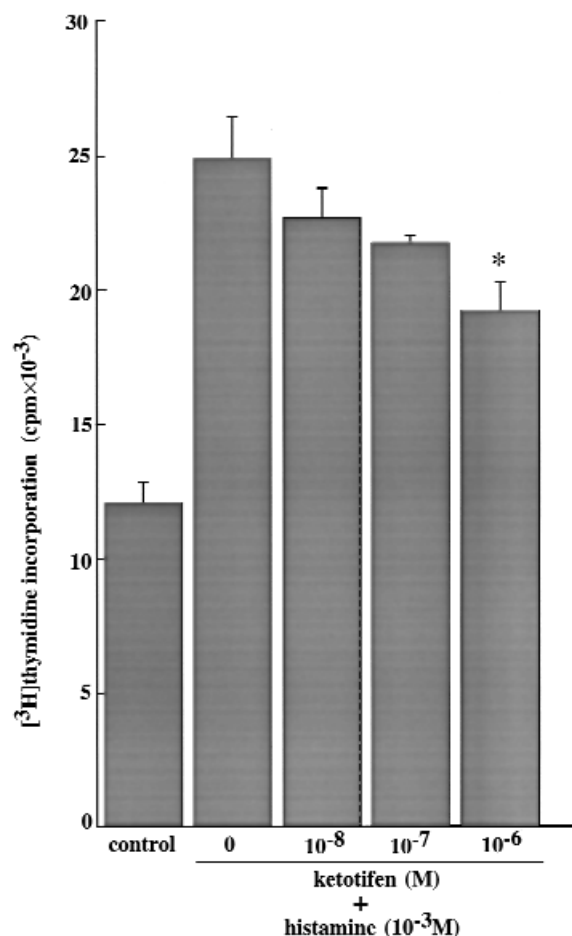


FIG 3. Effect of ketotifen on the proliferative response of human dermal fibroblasts to histamine. Incorporation of [³H]thymidine was assessed for fibroblasts incubated with medium alone (*control*) and with medium containing 10⁻³ mol/L histamine in the absence or presence of different concentrations of ketotifen. Values shown are the mean ± SEM of 5 separate experiments. **P* < .05 versus cells incubated with medium containing histamine in the absence of ketotifen.

Effect of IgE-mediated activation of mast cells on type I collagen production

The addition of IgE-sensitized mast cells did not alter the procollagen type I C-peptide concentration in fibroblast culture supernatant when compared with control cultures (Fig 4). Adding different numbers of mast cells that were activated with anti-IgE also failed to modulate collagen synthesis.

DISCUSSION

Mast cells have been postulated to have a role in the process of fibrosis, in which collagen synthesis by fibroblasts and fibroblast proliferation are enhanced. The suggestion that mast cells participate in fibrotic disease stems primarily from morphologic investigations of the involved skin of patients with scleroderma and studies of

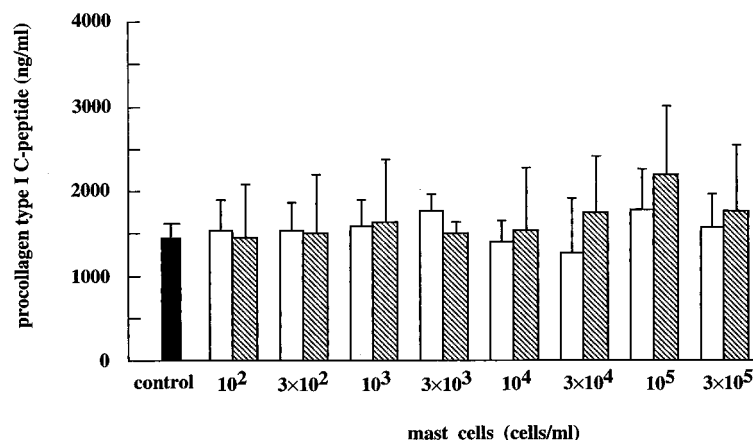


FIG 4. Type I collagen production by human fibroblasts cocultured with human mast cells. Mast cells derived from umbilical cord blood cells were sensitized with IgE and seeded onto confluent monolayers of fibroblasts from normal dermis. Type I collagen production by fibroblasts was assessed by measuring the procollagen type I C-peptide concentration in the culture supernatants of fibroblasts incubated with medium alone (*control*) and with IgE-sensitized mast cells in the absence (*open bars*) or presence (*hatched bars*) of anti-IgE. Values shown are the mean $\pm$ SEM of 5 separate experiments.

fibrotic lung tissue.² However, most of the previous *in vitro* studies that indicated a direct and unique role for mast cells in fibrosis either used rodent mast cells or only assessed the effect of specific mast cell secretory products on fibroblasts, as reviewed by Gruber.¹⁹

Recent advances have allowed us to obtain cultured human mast cells from umbilical cord blood cells.¹²⁻¹⁴ These are a mixture of tryptase-, chymase-positive mast cells and tryptase-positive mast cells. These cells are functionally mature, histamine-containing, IgE receptor-positive, metachromatic mast cells, which can be activated with IgE.^{13,14} Although the HMC-1 mast cell line represents a reasonable alternative experimental approach, it does not express the high-affinity surface receptor for IgE membrane receptor, and the morphologic condition of these cells reflects an immature stage of differentiation.⁹ Thus experimental findings obtained with HMC-1 cells require further confirmation by studies on mature human mast cells.

Our aim of this study was to determine whether functionally active human mast cells could directly affect human fibroblasts through a process involving IgE. We used a system in which cultured human mast cells were cocultured with human dermal fibroblasts and investigated the effects of IgE-mediated mast cell activation on proliferation and type I collagen synthesis by the fibroblasts. The most suitable experimental system is subconfluent and still exponentially growing fibroblast cultures. In the present study, mast cells in different numbers were seeded onto subconfluent cultures of human dermal fibroblasts, and fibroblast proliferation was assessed with the use of [³H]thymidine incorporation. Because the mast cells were cultured in the presence of stem cell factor and IL-6, it was possible that these cells might also affect fibroblasts even without anti-IgE stimulation. However, we showed that IgE-sensitized mast cells without anti-IgE

stimulation did not affect fibroblast proliferation and that anti-IgE did not influence the proliferation of fibroblasts in the absence of sensitized mast cells. On the other hand, fibroblast proliferation in the presence of anti-IgE-activated mast cells was significantly higher than in the presence of sensitized mast cells without anti-IgE stimulation.

Because the development of skin fibrosis in tight skin (TSK) mouse has been shown to be prevented by the administration of ketotifen, a mast cell inhibitor,²⁰ the effect of ketotifen on fibroblast proliferation was investigated to confirm that the increased proliferation was due to mast cell activation by anti-IgE. At a concentration of 10⁻⁴ mol/L, which showed a significant inhibitory effect on IgE-mediated histamine release from cultured human mast cells, ketotifen inhibited the increase of fibroblast proliferation. However, the inhibitory effect of ketotifen on the mast cell-dependent promotion of fibroblast proliferation was only modest, and cultured human mast cells can release a variety of mediators,^{13,21} including histamine, which has been shown to stimulate the proliferation of human lung fibroblasts.²² Therefore further studies were performed. It was shown that 10⁻³ mol/L histamine significantly increased the proliferation of human dermal fibroblasts and that this increase was inhibited by ketotifen in a concentration-dependent manner. At a concentration of 10⁻⁶ mol/L, ketotifen significantly inhibited the histamine-induced increase of fibroblast proliferation. Therefore it seems clear that IgE-mediated activation of cultured human mast cells can enhance human dermal fibroblast proliferation. The promotion of fibroblast proliferation by mast cells (3 $\times$ 10⁵/mL) was higher than by 10⁻³ mol/L histamine, which indicated that activated mast cells may release a variety of fibrogenic mediators including histamine. In fact, recent studies from our laboratory showed that mast cell tryptase stimulates human dermal fibroblast proliferation.²³

In contrast, when type I collagen synthesis was assessed with confluent human dermal fibroblasts cocultured with IgE-sensitized mast cells, the procollagen type I C-peptide concentration in the culture supernatant was not affected by anti-IgE stimulation. This indicated that IgE-mediated activation of human mast cells did not alter type I collagen production by human dermal fibroblasts.

Our results suggest that IgE-mediated mast cell activation might promote fibroblast activity *in vivo*. However, IgE-mediated activation of mast cells during immediate hypersensitivity reactions does not usually result in fibrosis. Because there are several lines of evidence,^{1,2} which suggests the occurrence of chronic mast cell activation during the development of fibrosis, it seems that mast cell activation in fibrotic conditions differs from the anaphylactic response and that mast cells may release a wide spectrum of biologically active compounds with fibrogenic and fibrolytic activities. For example, both histamine²² and tryptase^{23,24} have been shown to be mitogenic for cultured fibroblasts. Recently, Cairns and Walls²⁵ showed that tryptase stimulated the synthesis of type I collagen messenger RNA in confluent human lung fibroblasts, although Kofford et al²⁶ reported that human mast cell chymase directly cleaved type I procollagen. In the light of these data, our present finding that the activation of IgE-sensitized human mast cell enhances human dermal fibroblast proliferation, but not type I collagen production, may be reasonable because our cultured mast cells included both tryptase-, chymase-positive cells and tryptase-positive cells. On the other hand, very recent studies from our laboratory showed that cord blood-derived human cultured mast cells produce transforming growth factor β_1 ,²⁷ which has been shown to increase type I collagen production by human dermal fibroblasts.¹⁶ However, the influence of mast cells on the fibroblast proliferative response is complicated, and when mast cells are activated by IgE-mediated mechanisms *in vivo*, mediators and cytokines released from these cells may influence collagen production by fibroblasts either directly or indirectly by effects on other cells. These complexities of the *in vivo* situation are difficult to reproduce *in vitro*, so further studies are required.

In conclusion, we found that IgE-mediated activation of IgE-sensitized cultured human mast cells could significantly increase the proliferation of subconfluent human dermal fibroblasts but did not promote type I collagen production by confluent fibroblasts under the conditions tested.

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