

Human mast cells stimulate fibroblast proliferation, collagen synthesis and lattice contraction: a direct role for mast cells in skin fibrosis

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Summary

Background Mast cells, the key cells of immediate hypersensitivity type reactions, have also been postulated to have a central role in influencing tissue remodelling and fibrosis occurring in the skin.

Objective Our aim was to investigate the direct role of human mast cells (HMC) in skin fibrotic processes, by assessing the effects of the addition of the human mast cell line HMC-1 to human skin fibroblasts, and to identify the responsible mediators.

Methods HMC-1 sonicates were added to human skin fibroblasts and the following parameters were evaluated: proliferation ($[^3\text{H}]$ -thymidine), collagen synthesis ($[^3\text{H}]$ proline), activity of matrix metalloproteinases (MMPs) (zymography) and tissue inhibitors of metalloproteinases (TIMPs) (reverse zymography), and collagen gel contraction.

Results HMC-1 sonicate increased significantly both proliferation and collagen production in the human skin fibroblasts and these properties were not affected by heating of the sonicate (56°C, 30 min, or 100°C, 3 min). Two main mast cell mediators, histamine and tryptase, were found to be responsible for the increase in fibroblast proliferation and collagen production. HMC-1 sonicate did not display any pre-formed gelatinase activity, and its addition to the fibroblasts did not change their pro-MMP-2 and MMP-2 activity. On the other hand, HMC-1 were found to possess TIMP-1 and TIMP-2. Addition of HMC-1 had no effect on fibroblasts TIMP-1 but induced a dose-dependent increase of TIMP-2 activity. In addition, HMC-1 sonicate seeded together with the fibroblasts in tri-dimensional collagen gel significantly enhanced their contraction.

Conclusion We have shown that human mast cells, by granule-stored and therefore quickly releasable mediators, increase human skin fibroblast proliferation, collagen synthesis, TIMP-2 and collagen gel contraction. Therefore, mast cells have a direct and potentiating role in skin remodelling and fibrosis.

Keywords collagen gel, collagen, fibroblasts, fibrosis, human, mast cells, MMPs, proliferation, skin, TIMPs

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Introduction

Mast cells, the recognized key cells of allergic inflammatory responses, are tissue dwelling cells found in connective tissues and on mucosal surfaces. In the skin they reside mostly in the dermis, associated with blood vessels and appendages [1].

In allergy, mast cell activation carried out by IgE-dependent mechanisms, results in the prompt release of granule-stored mediators such as histamine, proteoglycans, tryptase and/or chymase, nerve growth factor (NGF) and tumor necrosis factor- α (TNF- α), together with the newly synthesized lipid mediators, such as prostaglandins, leukotrienes and platelet-activating factor [2]. Mast cells also produce and release at

later times an array of cytokines with inflammatory and pro-inflammatory properties [3].

Mast cells can also be stimulated by immunoglobulin (Ig)E-independent activators and by pro-inflammatory cytokines [4,5]. This would point to a role of mast cells not only in allergic inflammation but also in several inflammatory diseases with non-allergic etiopathology in which mast cell hyperplasia and activation take place. Indeed, mast cells have for a long time been postulated to contribute not only to inflammatory responses but also to the consequent physiological tissue remodeling or to overt fibrotic processes occurring in many tissues [6]. In the skin, dynamic changes in number, ultrastructure and function of mast cells have been reported in scleroderma and scleroderma-like diseases [7,8], in chronic graft-vs.-host disease (cGVHD) [9], and in hypertrophic scars and keloids [10]. We have recently obtained evidence for a mast cell role in skin fibrosis, both in a murine model of cGVHD and in patients with cGVHD [11,12].

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In vitro studies have now accumulated on the fibrogenic effects of either rodent mast cell products or selected mast cell mediators [6]. We had previously shown that activation of rat peritoneal mast cells cocultured with 3T3 murine fibroblasts increased their migration, proliferation and collagen synthesis [13,14]. Recently, we have found that rat peritoneal mast cell sonicates added to skin fibroblasts seeded in tridimensional collagen lattices augmented collagen production and fibroblast proliferation while inhibiting gel contraction [15]. In addition, in this model, rat mast cells increased fibroblast MMP-2 and pro-MMP-2 activity. Recently a human mast cell line (HMC-1) derived from a patient with mast cell leukaemia has become available for *in vitro* human studies [16–18]. HMC-1 exhibits a phenotype that in several aspects is similar to normal human tissue tryptase-containing mast cells. However, it lacks surface expression of the high affinity IgE receptor [18] and therefore cannot be stimulated by IgE-independent mechanisms but only by non-IgE-mediated ones such as the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) or the calcium ionophore ionomycin [17]. In our studies we opted to use the cellular sonicate of these cells as a source of all the pre-formed mediators, and not supernatants of HMC-1 activated by these IgE-dependent activators, as these activators can also directly stimulate the fibroblasts. Utilizing this line, Gruber et al. [19] have demonstrated, in an organotypic skin-equivalent culture system, that HMC-1 activate fibroblast type I collagen mRNA production and chemotaxis, mainly through tryptase.

In the present study, we have investigated the effect of the addition of human HMC-1 sonicate, as a source of mast cell pre-formed mediators, to human skin fibroblasts cultured in monolayers and in collagen lattices. The main hallmarks of fibrosis, such as proliferation, collagen production, MMPs and TIMPs activity and gel contraction, were evaluated. Our data provide evidence for mast cell stimulation of several fibroblast fibrogenic properties and hence for their direct contribution to increasing skin fibrosis.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), Iscove's medium, L-glutamine, streptomycin, penicillin, fetal calf serum (FCS) and Hanks's balanced salt solution were obtained from Biological Industries (Beth Haemek, Israel). Trichloroacetic acid was purchased from Merck (Darmstadt, Germany). Metrizamide, ascorbic acid, β -aminopropionitrile, collagenase, N-ethylmaleimide, histamine, heparin, pyrillamine, cimetidine, morpholinoethanesulphonic acid monohydrate (MES), monothioglycerol and reagents for molecular biology and zymography were obtained from Sigma Chemicals (St Louis, MO, USA). Neutralizing mouse anti-human TGF- β_1 - β_2 - β_3 monoclonal antibodies and human recombinant TNF- α were purchased from Genzyme Diagnostics (Cambridge, MA, USA). Neutralizing mouse anti-human TNF- α monoclonal antibodies were purchased from R & D (Minneapolis, MN, USA).

Human tryptase, purified from HMC-1, was a kind gift from Dr R. Numerof (Axys Pharmaceuticals, San Francisco, CA, USA). Tryptase inhibitor GW-585361A was a kind gift from Dr Keith Ray (Glaxo-Wellcome, Stevenage, UK). [^{3} H]-labelled proline and [^{3} H]-labelled thymidine were purchased from

DuPont (NEN®, Boston, MA, USA). Control MMPs were obtained from Valbiotech (Paris, France); TIMP-1 was from Calbiochem (France Biochem, Meridon, France); TIMP-2 was a generous gift of Dr H. Nagase (University of Kansas, KS, USA). Type I collagen from rat tail tendon was prepared in our laboratory and its purity was checked by SDS-polyacrylamide gel electrophoresis. Other reagents and chemicals were obtained from Frutarom Chemicals (Haifa, Israel) and were of analytical grade or of the best grade available. Sterile tissue culture plastic ware was purchased from Falcon (Oxford, CA, USA) and from Nunc (Roskilde, Denmark).

Mast cells and preparation of mast cell sonicates

The human mast cell line HMC-1 was a kind gift from Dr J. Butterfield (Mayo Clinic, Rochester, MN, USA). Cells were cultured as previously described [16].

HMC-1 sonicates were obtained by bath sonication (1 min, 0°C, Heat Systems Ultrasonics W 380, duty cycle 5s, output power 50%) of a cell suspension, containing 5×10^6 HMC-1/mL DMEM. Sonicates were microcentrifuged for 5 min at 14000g at 4°C and debris-free supernatants ('sonicate') were collected into aliquotes and stored until used at -80°C.

Fibroblasts

The human foreskin fibroblast cell line HSG8 (ATCC No. CRL 1635) was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Fibroblasts were cultured in 25cm² tissue culture flasks in DMEM, containing 10% heat-inactivated FCS, 2mm-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin (supplemented DMEM) at 37°C in 5% CO₂. Fibroblasts were subcultured by trypsinization (0.5% trypsin-EDTA in Hank's balanced salt solution without Ca²⁺ and Mg²⁺).

Adult dermal fibroblasts were obtained from biopsies of normal skin from informed consent volunteers who underwent minor skin surgery. The biopsies were placed as explants and fibroblasts obtained and cultured as described [20]. In pilot experiments no differences were found between the foreskin and adult skin fibroblasts. All the data presented, with the exception of some of the zymography and reverse zymography, were obtained with the human foreskin cell line.

Fibroblast proliferation and collagen production

Proliferation of subconfluent fibroblast monolayers incubated with HMC-1 sonicates (1.5×10^3 – 300×10^3 HMC-1/well) in 96-well plates for 24h was assessed using [^{3} H]-thymidine incorporation assay essentially as previously described [20]. Data from [^{3} H]-thymidine incorporation assay are expressed as cpm/well.

Collagen production by confluent fibroblast monolayers incubated with HMC-1 sonicates (10.5×10^3 – 10.5×10^4 HMC-1/well) in 24-well tissue culture plates for 24h was assessed by [^{3} H]-proline incorporation into collagenous proteins as previously described [20]. Data are expressed as cpm of [^{3} H]-proline incorporation in 10^4 fibroblasts.

The different HMC-1 sonicate cell numbers in the proliferation and collagen production assays are the result of calculations aimed to keep the fibroblast/mast cell ratio (*c.* 30:1) similar in both the assays and to take into account that proliferation is performed on subconfluent, while collagen production is carried out on confluent, monolayers.

Heat treatment

HMC-1 sonicate was subjected to heating at either 56°C for 30 min, or 100°C for 3 min. The sonicate was then centrifuged (5 min, 120 × g) and supernatants were collected and kept at -80°C until used. Fibroblast proliferation or collagen synthesis was assessed as described above for the untreated sonicate.

Addition of mast cell mediators, or of antihistamine drugs, or of anti-TNF- α neutralizing antibodies or of a tryptase inhibitor to fibroblasts and assessment of their proliferation and collagen synthesis

Stock solutions of histamine and heparin (10^{-3} M) were prepared in DMEM. Working concentrations of histamine (10^{-7} – 10^{-4} M), heparin (10^{-9} – 10^{-5} M) and TNF- α (0.2–20 ng/mL) were prepared by diluting stock solutions either in DMEM, containing 2% FCS for proliferation, or in modified DMEM for collagen synthesis. Tryptase was dissolved in MES buffer (20 mM MES, 2 mM CaCl₂, 20% glycerol, 5 mg/mL heparin, 0.3 M NaCl). Equimolar concentrations of heparin were dissolved in the same buffer and used as controls for tryptase experiments. Tryptase and heparin stock solutions (41.37 μ M and 10^{-4} M, respectively) were diluted in DMEM (12.5–50 nM).

In the experiments in which the effects of antihistamine drugs were assessed, fibroblasts were pre-incubated with either pyrilamine (anti-H-1) or cimetidine (anti-H-2) (10^{-7} – 10^{-6} M) for 30 min at 37°C before addition of HMC-1 sonicate. In the experiments in which the effects of anti-TNF- α and of the tryptase inhibitor were evaluated, HMC-1 sonicate was pre-incubated with either anti-TNF- α neutralizing antibodies (0.15–1.5 μ g/mL) or with the tryptase inhibitor (10 μ M) for 30 min at 37°C before their addition to the fibroblast cultures. TGF- β has not been reported to be found stored in HMC-1 [17,21]. Consequently, anti-TGF- β neutralizing antibodies (0.18–1.8 μ g/mL) were used as non-relevant control antibodies in the experiments in which anti-TNF- α was used. In some experiments the concomitant addition (under the same pre-incubation conditions described above) of the HMC-1 sonicate with combinations of anti-TNF- α , cimetidine and the tryptase inhibitor to the skin fibroblasts was evaluated on their proliferative capacity. Fibroblast proliferation and collagen synthesis were assessed in the differently treated cultures as described above.

Gelatin zymography and reverse zymography

SDS-substrate/PAGE was performed as described [22]. Gels for zymography comprised 0.1% gelatin, 10% polyacrylamide or, for reverse zymography, 0.1% gelatin, 15% polyacrylamide and 20 ng/mL purified activated MMP-2. Gelatinase and inhibitory activities were evaluated as previously described [19]. The intensity of the bands was measured by automated image analysis (NIH Image 1.62 program). HMC-1 sonicate was added to fibroblast cultures grown either in monolayer (10^5 cells/well) or in tridimensional collagen lattices (10^2 – 10^6 /lattice) in DMEM containing 0.1% BSA for 24 or 48 h.

Samples (10–25 μ L) were collected and applied onto the SDS-PAGE gel and run until the marker dye reached the bottom of the gel. The gel was stained with 0.5% Coomassie brilliant blue G 250. Molecular masses were derived from the molecular weight markers and standard pro-MMP2 run on the same gel.

Tridimensional collagen lattice contraction

Tridimensional lattice cultures were performed in collagen matrices as described [23]. Briefly, type I collagen from rat tail tendon (2 mg/mL in 18 mM acetic acid, prepared as previously described [24]) was added to 35-mm bacteriological dishes containing DMEM (10% FCS) and 0.1 M NaOH. Fibroblasts (10^5) were added just after collagen solution, alone or with different concentrations of HMC-1 sonicate, before fibrillation and lattice formation. Lattice diameter was then measured for 7 days by placing the dishes on a graduated ruler placed on a black surface. Cells were counted with a Malassez device after lattice digestion by clostridial collagenase (2 mg/mL in phosphate buffered saline (PBS) containing 2.5 mM CaCl₂ at 37°C).

Statistical analysis

All the experiments were carried out in triplicate or quadruplicate for the stated number of experiments. Data are expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed by Student's paired *t*-test with *P*-values of <0.05 being considered significant.

Results

Human mast cell sonicate increases skin fibroblast proliferation and collagen production

To evaluate the effect of mast cells on skin fibroblast proliferation, fibroblasts were incubated with increasing concentrations of HMC-1 sonicate. Proliferation response was monitored by [³H]-thymidine incorporation into fibroblast DNA. As shown in Fig. 1(a), mast cell sonicate at lower mast cell contents ($<10.5 \times 10^3$) did not significantly affect fibroblast proliferation, while sonicate from higher mast cell contents (35×10^3) significantly stimulated skin fibroblast proliferation ($74.4 \pm 5.7\%$ increase, $n=4$, $P<0.0009$). Two higher concentrations of the sonicate, namely 100×10^3 and 300×10^3 , were added to the fibroblasts in additional experiments ($n=2$). While 100×10^3 HMC-1 sonicate still induced a slight increment in proliferation (915.3 ± 50.6 cpm/well, i.e. $22.0 \pm 7.1\%$ increase vs. the increase induced by 35×10^3 HMC-1), 300×10^3 HMC-1 sonicate caused a small decrease in proliferation (832.6 ± 25.0 cpm/well, i.e. $9.0 \pm 2.4\%$ decrease vs. the increase induced by 100×10^3 HMC-1). The addition of increasing concentrations of HMC-1 sonicate (10.5×10^3 – 10.5×10^4) to confluent skin fibroblast monolayers increased their collagen production in a concentration-dependent fashion, with a maximum increase of $156.4 \pm 10.8\%$ ($P<0.01$, $n=3$) at the highest concentration of sonicate added (Fig. 1b).

Partial characterization of the mediators involved in the fibrogenic effects

To partially characterize the fibrogenic mediator(s) contained in the mast cell sonicate, HMC-1 sonicate was subjected to heating at either 56°C for 30 min or 100°C for 3 min. The heating did not significantly influence the sonicate-mediated induction of either fibroblast proliferation or collagen synthesis. In fact, in the proliferation assay fibroblasts cultured in medium alone exhibited 590.9 ± 163.3 cpm/well (mean $\pm$ SEM, $n=5$). Addition of untreated HMC-1 sonicate (35×10^3 cells/well) or of sonicate heated at 56°C for 30 min increased this

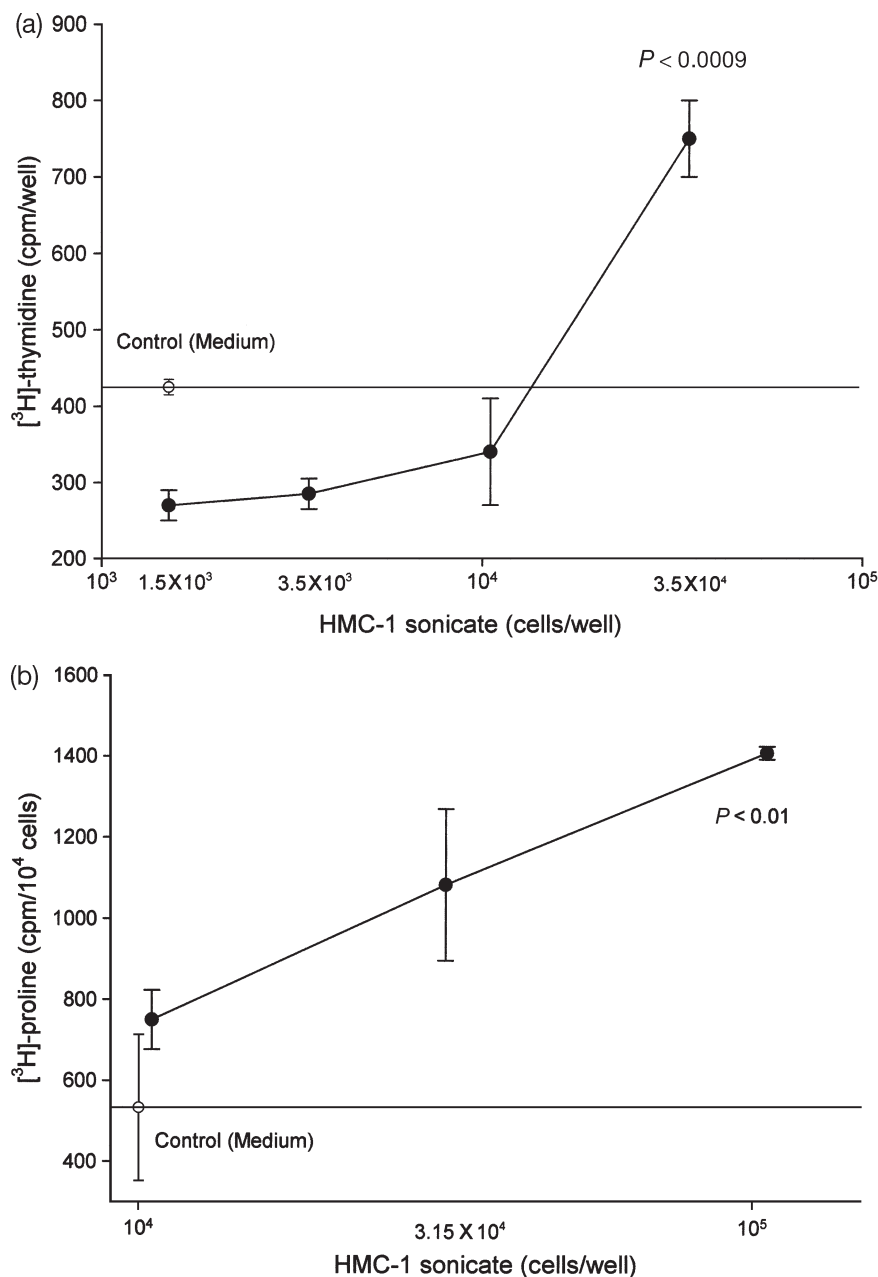


Fig. 1. Human mast cell sonicate stimulates human skin fibroblast proliferation and collagen synthesis. (a) Subconfluent cultures of human skin fibroblasts were incubated with different concentrations of HMC-1 sonicate or medium alone (control) for 24 h and the proliferation of fibroblasts was assessed by [³H]-thymidine incorporation. Data are the mean $\pm$ SEM of four experiments performed in triplicate. (b) Confluent monolayers of human skin fibroblasts were incubated with different concentrations of HMC-1 sonicate or medium alone (control) for 24 h and collagen production was evaluated by [³H]-proline incorporation into collagenase sensitive proteins. Data are the mean $\pm$ SEM of three experiments performed in triplicate.

value, respectively, to 1499.4 ± 183.3 cpm/well and to 1814.3 ± 230.3 cpm/well. In the collagen synthesis assay fibroblasts cultured in medium alone exhibited 557.8 ± 84.1 cpm/10⁶ cells (mean $\pm$ SEM, $n=3$). Addition of untreated HMC-1 sonicate (10.5×10^4 cells/well) or of sonicate heated at 56°C for 30 min increased this value, respectively, to 864.2 ± 21.1 cpm/10⁴ cells and to 876.2 ± 30.2 cpm/10⁴ cells and 952.1 ± 63.0 . Similar data were obtained with sonicate heated at 100°C for 3 min (not shown). This would indicate that the fibrogenic mast cell mediators, at least in the sonicate, are not heat labile.

We next evaluated the role of histamine, tryptase, and TNF- α , three possible fibrogenic pre-formed mast cell mediators on skin fibroblast proliferation and collagen synthesis. These mediators were added at concentrations comparable with the ones contained in the number of HMC-1 that we had used in the

previous experiments [18]. As shown in Figs 2 (a and b) histamine (10^{-4} M/10⁻⁵ M), significantly increased skin fibroblast proliferation ($58.4 \pm 3.7\%$, $P < 0.002$, $n=4$) and collagen synthesis ($89.5 \pm 9.2\%$, $n=3$, $P < 0.03$) in comparison with cultures incubated with medium alone. The fibrogenic effect of the HMC-1-derived sonicate on skin fibroblasts could not be completely abrogated by pre-incubation with antihistamine drugs. In fact, pyrilamine did not affect the enhancement of fibroblast proliferation by HMC-1, while cimetidine (10^{-7} and 10^{-6} M) decreased it, although not significantly ($43.8 \pm 8.1\%$ and $43.6 \pm 6.8\%$ decrease, respectively, $n=6$, $P < 0.13$ and $P < 0.1$). Neither pyrilamine nor cimetidine affected the HMC-1 mediated enhancement of skin fibroblast collagen synthesis.

This would indicate that histamine is not the only factor that mediates the mast cell-mediated fibrogenic effects. We therefore

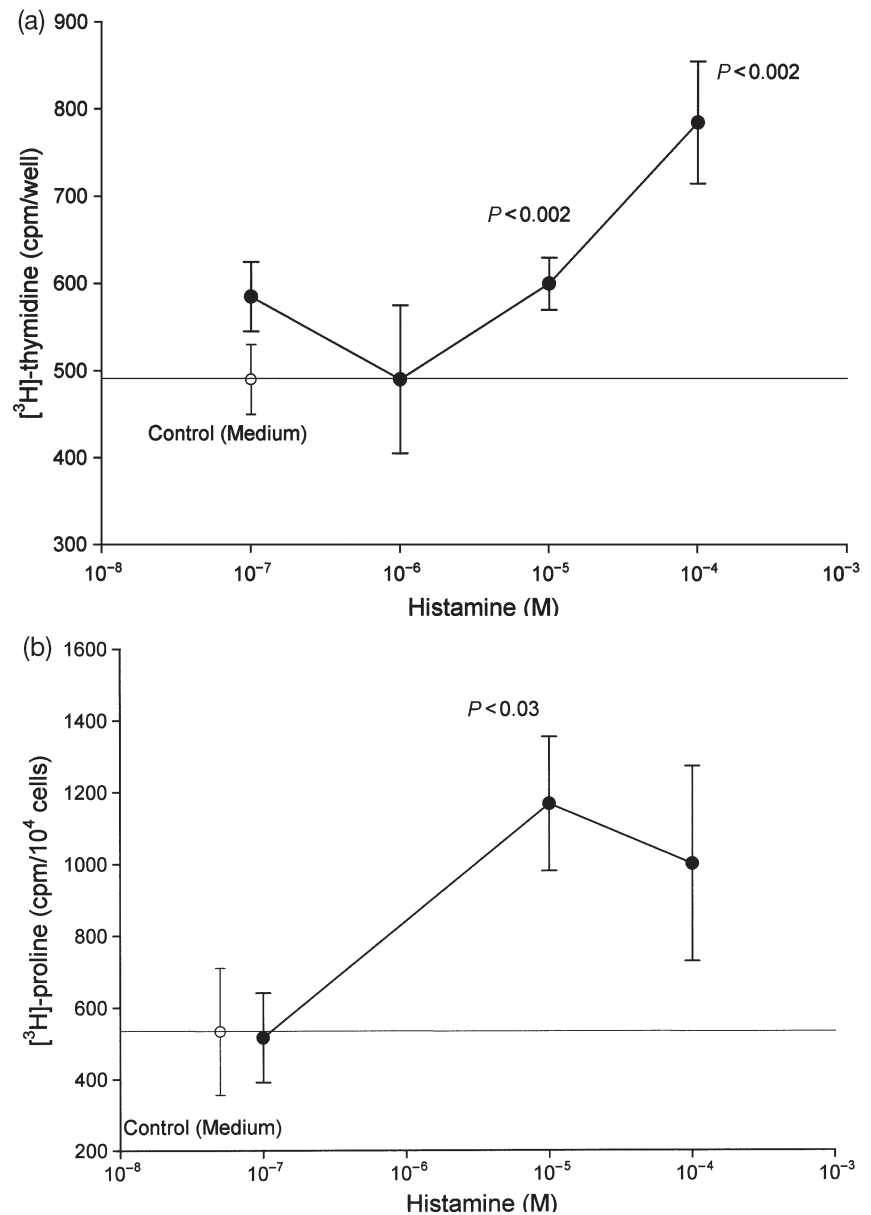


Fig. 2. Histamine stimulates human skin fibroblast proliferation and collagen synthesis. Histamine was added to fibroblast cultures and their proliferation (a) and collagen synthesis (b) were evaluated as described in Fig. 1. Data are the mean $\pm$ SEM of four (a) and three (b) experiments performed in triplicate.

looked for the activity of other mast cell pre-formed fibrogenic mediators such as tryptase, heparin and TNF- α . Tryptase significantly enhanced both fibroblast proliferation (12.5 nm , $46.7 \pm 5.3\%$ increase, $P < 0.001$, $n = 4$) (Fig. 3a) and collagen synthesis ($191.4 \pm 17.8\%$ increase, $n = 3$, $P < 0.008$) (Fig. 3b). Pre-incubation of HMC-1 sonicate with the tryptase inhibitor GW-585361 A ($10 \mu\text{M}$) decreased by $38.8 \pm 5.9\%$ its proliferatory activity ($n = 2$). Heparin, another mast cell pre-formed mediator, that was added together with tryptase to the fibroblast culture to preserve its enzymatic activity [25], when added alone (10^{-9} – 10^{-6} M), did not significantly influence either proliferation or collagen synthesis. Similarly, the addition of TNF- α to skin fibroblasts did not significantly alter their proliferation or their collagen synthesis (not shown). However, pre-incubation of HMC-1 sonicate with neutralizing antibodies to TNF- α (0.15 and $1.5 \mu\text{g/mL}$), decreased slightly but significantly its property to enhance fibroblast proliferation ($30.4 \pm 5.0\%$ decrease,

$P < 0.02$, $n = 4$ and $58.7 \pm 7.6\%$ decrease, $P < 0.05$, $n = 4$, respectively). At the same time, anti-TNF- α antibodies did not affect HMC-1-mediated increase in collagen production (not shown).

In the next series of experiments we aimed to evaluate the effect of the addition of optimal concentrations of cimetidine (10^{-6} M), anti-TNF- α antibodies ($1.5 \mu\text{g/mL}$) and the tryptase inhibitor ($10 \mu\text{M}$) on HMC-1-induced fibroblast proliferation. The addition of each one of them alone inhibited fibroblast proliferation, respectively, by $40.5 \pm 1.2\%$, $36.9 \pm 3.1\%$ and $33.5 \pm 4.9\%$ ($n = 3$). Cimetidine and anti-TNF- α together inhibited by $39.8 \pm 4.8\%$, while a decrease in inhibition was found when cimetidine and the tryptase inhibitor were added together ($7.7 \pm 10.0\%$ inhibition, $n = 3$). Consequently, addition of the three reagents together resulted in a slight decrease in inhibition of proliferation ($29.9 \pm 2.6\%$, $n = 3$), probably due to some interference between cimetidine and the tryptase inhibitor or other undetermined factor(s).

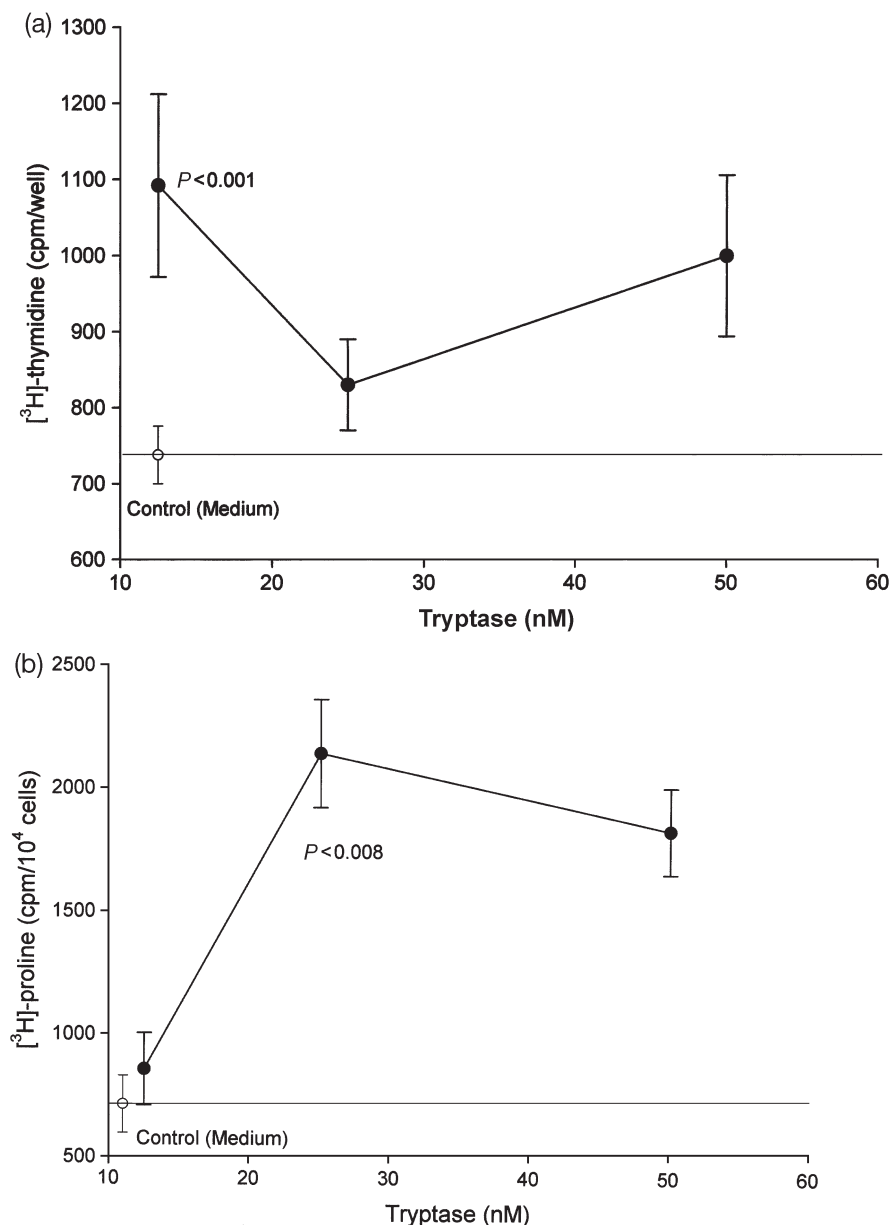


Fig. 3. Tryptase stimulates human skin fibroblast proliferation and collagen synthesis. Tryptase was added to fibroblast cultures and their proliferation (a) and collagen synthesis (b) were evaluated as described in Fig. 1. Data are the mean $\pm$ SEM of four (a) and three (b) experiments performed in triplicate.

Human mast cell sonicate contains TIMP-1 and TIMP-2, increases fibroblast TIMP-2 activity, but does not influence their MMPs activity

Skin fibroblasts cultured in monolayers or in tridimensional collagen lattices for 48 h were found by gelatin zymography to display mainly the latent form of MMP-2 (pro-MMP-2) and a minimal amount of the activated form of MMP-2 (not shown).

HMC-1 sonicates analysed by gelatin zymography were found to be devoid of any intrinsic gelatinase activity and their addition to skin fibroblasts did not alter their MMPs activity (not shown). On the other hand, HMC-1 sonicates were found to express some TIMP-1 and TIMP-2 activity (reverse zymography) (Fig. 4). When added to the skin fibroblasts in monolayers or grown in lattices, the HMC-1 sonicates did not alter their expression of TIMP-1 but induced a dose-dependent increase of TIMP-2 activity in the fibroblasts (up to 2.7 fold at 10^6 HMC-1 sonicate) (Fig. 4b).

Human mast cell sonicate increases tridimensional lattice contraction by skin fibroblasts

HMC-1 sonicate was added to collagen lattices (10^2 – 10^6 per lattice) seeded with 10^5 fibroblasts. Measurements of lattice diameters showed that HMC-1 sonicate increased lattice contraction (Fig. 5). The increase in lattice contraction was evident and significant on day 1 after seeding. Fibroblast cell numbers did not vary in the different lattices when evaluated on the last day of the experiment (not shown).

Discussion

In the present study we have shown that the human mast cell line HMC-1 contains pre-formed mediators that enhance human skin fibroblast proliferation, collagen synthesis and collagen gel contraction.

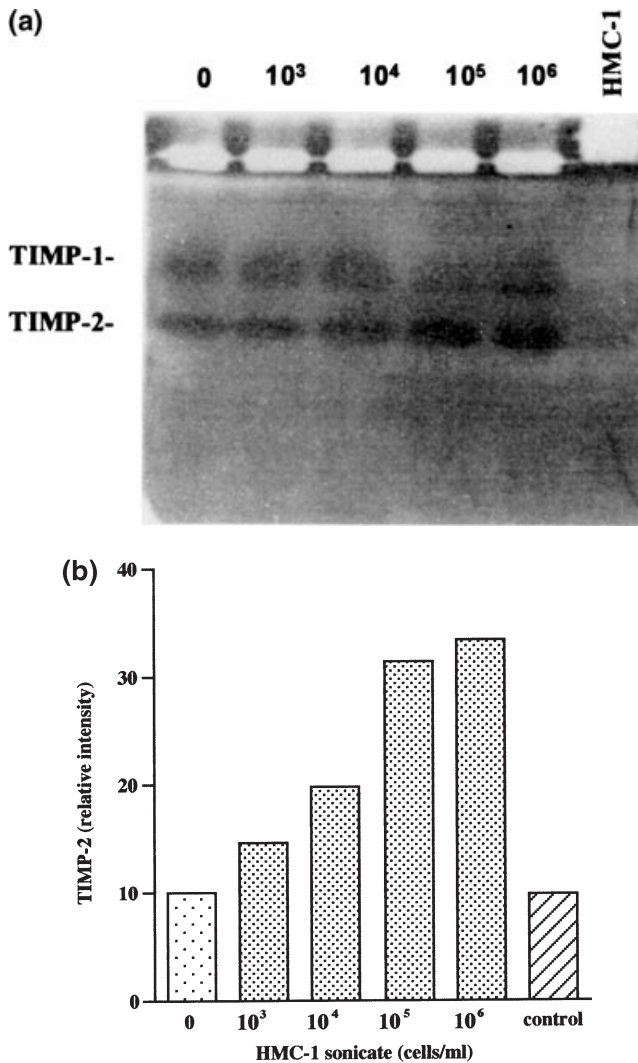


Fig. 4. Human mast cell sonicate contains TIMP-1 and TIMP-2 and enhances TIMP-2 activity of human skin fibroblasts. Skin fibroblasts were seeded in collagen lattices (10^5 /lattice) and incubated with HMC-1 sonicate ($0, 10^3, 10^4, 10^5, 10^6$ cells/mL). Culture medium was collected at day 7 and analysed by reverse zymography. The positions of migration of TIMP-1 and TIMP-2 are indicated. (a) zymogram; (b) densitometric analysis of (a); (c) control: HMC-1 (10^5) alone.

The proximity of mast cells, the cardinal cells of allergic inflammation, in the early stages of fibrosis and areas of tissue remodelling was first noticed in 1878 by Paul Ehrlich, who also postulated a role for mast cells in these processes [26]. In the skin, mast cells have been suggested to have an important role in scleroderma and in scleroderma-like conditions [7,8,12], hypertrophic scars and keloids [10]. We have previously shown that rat peritoneal mast cells enhance migration, proliferation and collagen production of 3T3 murine fibroblasts [13,14]. In addition, more recently, we have demonstrated that rat mast cell sonicate affected human skin fibroblast properties when the fibroblasts were seeded in tridimensional collagen gels [15].

However, studies aimed at defining the effects of human mast cells on biochemical characteristics of human skin fibroblasts are lacking partly because of the scarcity of a good source of human tissue mast cells. Butterfield et al. [16] have produced the human mast cell line HMC-1, which has characteristics of the

tryptase-positive mast cell subset found mostly at mucosal surfaces and during inflammatory and early fibrotic events of the skin, such as in scleroderma [27].

Consequently, in the present study, to analyse the direct contribution of human mast cells to skin fibrosis, we have studied the effects of HMC-1 on some human skin fibroblast properties. We found that the addition of HMC-1 cell sonicate to skin fibroblast monolayers increased significantly both proliferation and collagen production. Similarly, Wooley and coworkers [28] have shown that sonicated mouse mastocytoma cells added to cultures of human skin fibroblasts increased their proliferation. More recently Gruber et al. [19] also proved that the presence of HMC-1 cells in a dermal equivalent increased skin fibroblast collagen type I mRNA synthesis. On the other hand, Abe et al. [29] showed that IgE-mediated activation of human cord blood-derived mast cells increased proliferation of adult human dermal fibroblasts but did not promote type I collagen production. The lack of activity of these types of mast cells on collagen production might be due to sources of both mast cells and fibroblasts that differ from the one used in our study.

In order to partially characterize the mediator responsible for the HMC-1 fibrogenic effects, the mast cell sonicate was subjected to heating. This treatment did not alter its biological activity, indicating that in the cellular sonicate the fibrogenic mediator(s) is heat stable. Histamine and some cytokines, such as TNF- α [30], are known to be heat stable. However, the heat stability of mediators when in association with a cell sonicate rather than in a purified, crystalline or recombinant form, can also be greatly increased. Histamine is one of the potential fibrogenic factors stored and released by mast cells upon activation within a few seconds. Indeed, we observed that histamine significantly increased fibroblast proliferation and collagen synthesis. Cimetidine, an anti-H-2 drug, but not pyrilamine, an anti-H-1 drug, partially blocked HMC-1 enhancing effect on skin fibroblast proliferation. Jordana et al. [31] and Hatamochi et al. [32] also found that histamine stimulated in a dose-dependent manner the proliferation and collagen production of fibroblasts from normal human lung and from human and guinea-pig skin fibroblasts, respectively. Interestingly, in an *in vitro* model of a wound performed in 3T3 fibroblast monolayers, we had previously shown that the proliferative and chemotactic effect of mast cells was partially mediated by histamine through H-2 receptors [33]. We also tested the effects of tryptase, heparin and TNF- α , three main pre-formed mast cell mediators, on skin fibroblast proliferation and collagen synthesis. Tryptase, a relatively specific mast cell neutral protease, has also been reported to induce direct proliferative activity towards Chinese hamster lung Rat-1 fibroblasts [34] and direct stimulation of mRNA for collagen production in human skin and lung fibroblasts [19,35]. Data concerning heparin and fibrosis are still controversial [36,37]. Tryptase in our system stimulated both proliferation and collagen synthesis in the skin fibroblast cultures, in agreement with previous published works, and a tryptase specific inhibitor (GW-585361A) inhibited HMC-1-induced fibroblast proliferation. Heparin had no effect in our system. TNF- α is an attractive mediator with pleiotropic functions on fibroblasts that is stored in the cytoplasmic granules of rat peritoneal, HMC-1, and human skin mast cells [38]. On the one hand it stimulates the growth of human diploid FS-4 fibroblasts even under contact inhibition

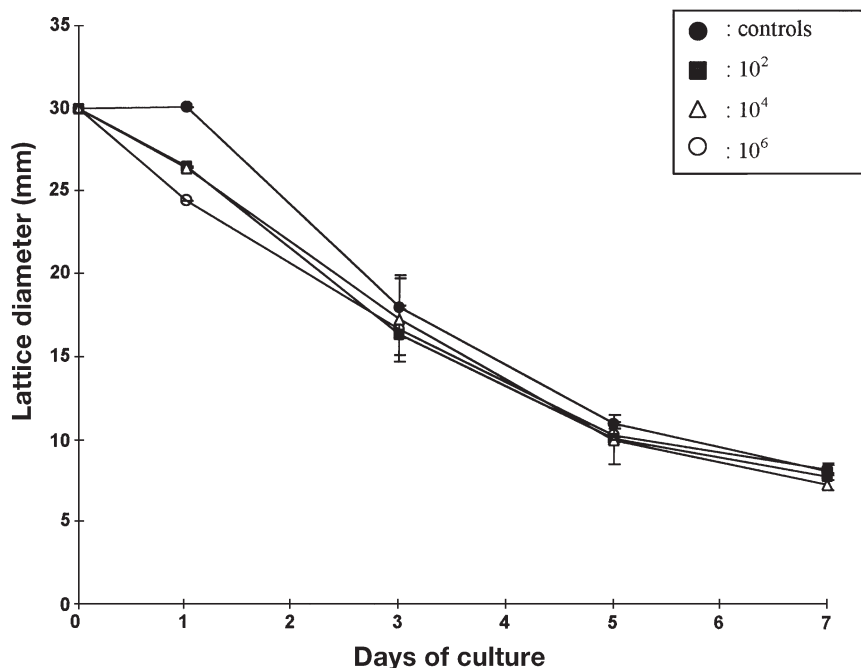


Fig. 5. Human mast cell sonicate stimulates collagen lattice contraction by human skin fibroblasts. Collagen lattices were seeded on day 0 with 10^5 fibroblasts/lattice in the presence of increasing concentrations of HMC-1 sonicate. Data are the mean $\pm$ SEM of four experiments carried out in quadruplicate. Full circles = controls; full squares = HMC-1 sonicate/mL (10^2); open triangles = HMC-1 sonicate/mL (10^4); open circle = 10^6 HMC-1 sonicate/mL (10^6).

conditions [39], while on the other hand, it inhibits collagen synthesis and at high concentrations it stimulates collagenase synthesis by human gingival fibroblasts [40]. Although when added alone to human skin fibroblasts $\text{TNF-}\alpha$ had no influence on their proliferation or collagen synthesis, we were able to show that HMC-1-mediated enhancement of skin fibroblast proliferation was partially blocked by pre-incubation with $\text{TNF-}\alpha$ neutralizing antibodies.

In a recent study it was found that other mast cell mediators such as prostaglandin D_2 and carboxypeptidase A, in addition to tryptase, increased variably fibroblast proliferation and collagen synthesis [41]. Taken together these results may indicate that the mast cell-mediated fibrinogenic effect towards skin fibroblasts is a combined effect of several mediators including histamine, tryptase and $\text{TNF-}\alpha$. We have recently found that human peripheral blood eosinophils also induce human skin fibroblast proliferation and collagen synthesis, an effect partly mediated by pre-stored $\text{TGF-}\beta$ [20].

As collagen production results from a fine balance between its synthesis and breakdown, we assessed in parallel assays the effect of mast cells in modulating fibroblast matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Skin fibroblasts grown in monolayer or tridimensional cultures were shown to secrete pro-MMP-2 and MMP-2. These two forms of the enzyme were not affected by the addition of HMC-1 sonicate that by itself did not exhibit any pre-formed gelatinolytic activity. On the other hand, Lohi et al. [42] demonstrated that lung-purified tryptase added to conditioned medium of fibroblast containing MMP-2 could activate it. However, in our system, the addition of tryptase to the skin fibroblasts did not alter their pro-MMP-2 expression (unpublished observation). Interestingly, although rat peritoneal mast cell sonicate did not show any pre-formed MMPs activity on its own, when added to human skin fibroblast it increased fibroblast pro-MMP-2 production and induced the formation of MMP-2. Rat mast cells also increased the mRNA for MMP-2

in human skin fibroblasts [15]. This difference between rat and human mast cells might result from the presence of species-specific mediators.

More recently, the constitutive expression of MMP-9 mRNA and the secretion of active MMP-9 from PMA-activated HMC-1 has been shown [43]. It is conceivable therefore that activation is mandatory to cause matrix metalloproteinase production by these types of mast cells. We were able to identify the presence of small amounts of TIMP-1 and TIMP-2 in the HMC-1 sonicate. HMC-1 sonicate addition to the skin fibroblasts did not influence their TIMP-1 expression but increased TIMP-2 in a dose-dependent fashion. On the other hand, we have recently shown that human peripheral blood eosinophils contain TIMP-1 and TIMP-2 but do not influence fibroblasts TIMPs [20]. Therefore mast cells regulate fibroblasts TIMPs in a different way than eosinophils, probably because they contain different mediators.

An important parameter of tissue repair and fibrosis is wound contraction, which can be mimicked *in vitro* by seeding fibroblasts in collagen lattices. HMC-1 sonicate, even at low cell concentrations, stimulated skin fibroblast collagen lattice contraction, a finding that indicates a possible role for mast cells also in the latest stages of repair. Interestingly, in our previous work in which we included in the skin fibroblast collagen lattice, rat peritoneal mast cell sonicate, an inhibition of contraction rather than a stimulation was detected [15]. In the case of the rat sonicate it was found that heparin, which is contained in high amounts in rat mast cell granule, was partially responsible for this inhibition. Incidentally, in contrast to published information [18] the HMC-1 sonicate was found to contain mostly chondroitin sulphate and only traces of heparin (Y. Wegrowski, F. Maquart, L. Levi-Schaffer; unpublished observation). Recently, Yamamoto et al. [44] also found that in cocultures of HMC-1 and skin fibroblasts in three-dimensional lattices, HMC-1 enhanced contraction in a direct cell-cell contact, mediated in part by SCF/cKit interactions. As in our

studies we have been using a cellular sonicate and not intact cells, other factors might be relevant in the gel contraction.

In summary, we have demonstrated that human mast cells have the potential to directly modulate human skin fibroblast properties towards a fibrotic phenotype. It is important to point out that the pro-fibrogenic activity of mast cells towards skin fibroblasts is similar to that exhibited by human peripheral blood eosinophils [20], although carried out by different specific mediators. It is therefore possible to speculate that in dermal fibrosis, which is the end result of some inflammatory processes, depending on the disease, mast cell profibrogenic activity alone or together with the eosinophil profibrogenic activity is of primary pathophysiological importance.

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