

Activation of fibroblasts in collagen lattices by mast cell extract: a model of fibrosis

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Summary

Background Mast cells are resident connective tissue cells able to secrete numerous inflammatory mediators in response to tissue aggression and might be implicated in the fibrotic processes.

Objectives To study the effects of mast cell products on fibroblast activity in connective tissues.

Methods Mast cell extract was prepared by sonication of pure mast cell preparations obtained by peritoneal lavage of rats and added to the culture medium of fibroblast-populated collagen lattices.

Results Mast cell extract was able to decrease the contraction of the collagen lattices, to stimulate total protein and collagen synthesis, and to increase the expression and activation of gelatinase A/MMP-2.

Conclusion These data are consistent with the hypothesis that mast cells in connective tissue may be responsible for fibroblast activation at the early phases of tissue repair and fibrosis.

Keywords: collagen, fibroblasts, fibrosis, inflammation, mast cells, matrix metalloproteinases, tissue repair

Clinical and Experimental Allergy, Vol. 30, pp. 485–492. Submitted 22 February 1999; revised 24 April 1999; accepted 8 July 1999.

Introduction

Mast cells are resident cells of connective tissues, particularly located near blood vessels, nerves and glandular ducts. Normal human skin contains approximately 7000 mast cells/mm³ [1]. There have been many proposals to explain the role of mast cells in connective tissue. It was suggested that mast cells participate in regulating connective tissue components accumulation and connective tissue cells, such as fibroblast activity. Mast cell distribution allows the supposition of a role not only in allergic inflammatory reactions [2], but also in chronic inflammatory diseases such as scleroderma, hypertrophic scars and keloids and fibrosing alveolitis [3].

Increased number of mast cells has been observed under different physiopathological conditions. Nevertheless, only few clues support molecular interactions between mast cells and fibroblasts. Most of the reports provide data suggesting that mast cells are potent fibrogenic cells through the release of potent fibrogenic mediators. Recently, for example, it was demonstrated that mast cell tryptase was a fibrogenic factor, able to stimulate fibroblast collagen mRNA synthesis and chemotaxis [4,5]. Also, rat peritoneal mast cells have been shown to increase 3T3 fibroblast proliferation and collagen production in monolayer cultures [6].

Mast cells contain and release a large panel of substances. Prominent are histamine, neutral proteinases [6], heparin, cytokines such as interleukin-4 (IL-4), IL-5, IL-6, tumour necrosis factor- α (TNF- α) [8] and IL-13 [9] and numerous inflammation mediators, such as prostaglandin D₂, leukotriene C₄ (LTC₄) and platelet-activating factor (PAF).

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Heparin is the major glycosaminoglycan present in the connective tissue mast cells, on average 3–8 picograms per cell [10]. In granules, heparin seems to act as a stocking matrix for cationic preformed mediators.

Based on these studies, the effects were explored of mast cell products on fibroblast metabolism in the extracellular matrix. Dermal fibroblasts were embedded in collagen lattices, reproducing a tissue-like environment which provides more physiological conditions than classical monolayer cultures on plastic. We used this model to demonstrate the capacity of mast cells to activate fibroblasts and to increase matrix remodelling.

Materials and methods

Materials

Chemicals (analytical grade) were purchased from Prolabo (Paris, France), unless another source is stated. Plastic tissue culture dishes were from Nunc (Roskilde, Denmark). Tissue culture media, including fetal bovine serum (FBS), were from Gibco (Cergy-Pontoise, France). Control pro-MMP-2 and MMP-2 was from Valbiotech, Paris, France).

Cell cultures

Skin biopsies were obtained from normal subjects (age range 35–50 years) during aesthetic or reconstructive surgery. The informed consent of the donors was obtained according to the declaration of Helsinki. Fibroblasts were grown from skin explants in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, and cultured at 37 °C in a humidified incubator in a 95/5 (air/carbon dioxide) atmosphere. They were used between the 4th and 8th passages in culture.

Three-dimensional lattice cultures were performed in collagen matrices according to previously described techniques [11]. Briefly, type I collagen from rat tail tendon, at a concentration of 2 mg/mL in acetic acid 18 mM, was added to 35-mm diameter dishes containing DMEM medium with 10% FBS and NaOH 0.1 M. Fibroblasts (10^5 per lattice) were added just after the collagen solution, before fibrillation and lattice formation. Lattice diameter was measured every day after seeding by placing the dishes on a graduated rule placed on a black surface. Cells were counted with a Malassez device after lattice digestion by clostridial collagenase (2 U/mL in PBS containing 2.5 mM CaCl_2 at 37 °C), and their DNA content was measured by a fluorometric assay [12].

Mast cell extract

Mast cells were obtained by peritoneal washing from Sabra

male rats (an outbred strain from the Hebrew University). Rats were bred and maintained under exactly the same conditions and sacrificed for the peritoneal lavage when their weight reached 300–400 g. Mast cells were purified as described previously [13] by a metrizamide gradient. They represented more than 95% as assessed by metachromasia after staining with toluidine blue [13]. Three different batches of mast cells were used in this study, with very small batch-to-batch variations. Results were significant and reproducible with all the batches used. Mast cell extract was obtained by sonication on ice for 1 min at 50 Hz. After centrifugation, supernatant was collected and stored at –80 °C. Mast cell extract was added to the cultures before collagen fibrillation.

Mast cell extract was depleted from heparin by affinity chromatography on immobilized antithrombin III [14] coupled via amino groups to cyanogen bromide-activated agarose (Sigma). Samples of mast cell extracts were applied to a column containing 2 mL of antithrombin III–agarose gel, equilibrated with NaCl 0.2 M, Tris 0.1 M and pH 7.4 buffer. The column was washed with the same buffer until the effluent was free from proteins. It was then eluted with a salt gradient NaCl 0.2 M, 1 M and 3 M. Effluent fractions were analysed by optical densitometry. Quantification of heparin in mast cell extracts before and after affinity chromatography was performed by the Laboratory of Hematology (Prof. G. Potron, Dr C. Droulle, Hôpital Robert Debré, Reims, France) using a routine method (Stachrom Heparin, Diagnostica Stago, France). Heparin content before chromatography was 0.66 U/mL, whereas it was undetectable after treatment.

Zymographic analysis

Sodium dodecyl sulphate (SDS)-substrate gels were prepared as described by Herron *et al.* [15], with slight modifications [16]. Briefly, gelatin or casein were incorporated at the final concentration of 1 mg/mL into standard SDS-10% polyacrylamide gels prepared according to the method of Laemmli and Favre [17]. Culture medium was collected at various times during lattice retraction. The supernatant was mixed with sample buffer (2% SDS, 4% sucrose, 0.1% bromophenol blue in 0.25 M Tris-HCl buffer, pH 6.8) in the ratio 1/5 (v/v). An aliquot of the sample (10 μ L) was loaded onto the gel without prior boiling and submitted to electrophoresis (10 mA per gel). After migration, the gel was soaked twice in 2.5% Triton X-100 for 30 min, under gentle shaking. Gel was then incubated at 37 °C in the assay buffer specific for each substrate (gelatin: Tris 50 mM, NaCl 200 mM, CaCl_2 5 mM, Triton X-100 0.1% and 0.01% NaN_3 , pH 7.6, for 12 h; casein: Tris 100 mM, pH 7.4, 0.02% NaN_3 for 24 h). After incubation, the gel was stained for 30 min in 1% Coomassie blue G250 diluted in

10% acetic acid, methanol 30% and destained in 10% acetic acid and 20% methanol.

mRNA analysis

Collagen lattice cultures of dermal fibroblasts were exposed to varying concentrations of mast cell extract during 24 h before RNA isolation for Northern hybridization. Total RNAs were extracted by a single-step method modified from standard techniques of Chomczynski and Sacchi [18]. Their purity and integrity were checked by measurement of the $A_{260\text{nm}}/A_{280\text{nm}}$ ratio and agarose gel electrophoresis. Northern blot analysis was performed according to previously published methods [19]. Sequence-specific cDNA probes used for hybridization were the following: pro- $\alpha 1$ (I) collagen cDNA pH CAL 1 U clone, pro- $\alpha 1$ (III) collagen pH FS3 clone (generous gifts of Prof. E. Vuorio, Turku, Finland), pro-MMP2 cDNA MxI clone (generous gift of Dr W.G. Stetler-Stevenson, NCI, NIH, Bethesda, USA). Results were expressed as the ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GPDN5 clone (generous gift of Prof. P. Fort, Montpellier, France). Equal amounts of RNA were loaded onto agarose/formaldehyde gels and capillary-transferred to Pall Biotrans nylon membrane. Membranes were prehybridized for at least 2 h in a solution containing 50% formamide, $5\times$ SSC, $5\times$ Denhardt's reagent, 50 mM NaH_2PO_4 , 0.1% SDS and 250 $\mu\text{g/mL}$ denatured salmon sperm DNA, and hybridized overnight at 42 °C in the same solution containing ^{32}P -labelled cDNA probe generated using a random primer labelling kit (Appligen). After hybridization, membranes were washed twice in $2\times$ SSC, 0.1% SDS at 42 °C under gentle shaking and once more in $0.1\times$ SSC, 0.1% SDS at 42 °C, then exposed (Hyperfilm TM, Amersham). The intensity of the bands on autoradiography was quantified on a Vilbert-Lourmat image analyser, and the ratio of the different bands to GAPDH mRNA was calculated for each lane.

Protein synthesis

Total protein and collagen synthesis was measured on series of four fibroblast-populated collagen lattices. After 7 days of culture, retracted collagen lattices were exposed to varying concentrations of mast cell extract during 24 h. The culture medium was DMEM supplemented with 0.5% fetal bovine serum, 50 $\mu\text{g/mL}$ β -aminopropionitrile, 10 $\mu\text{g/mL}$ ascorbic acid, 23 $\mu\text{g/mL}$ cold proline and 5 $\mu\text{Ci/mL}$ [^3H]-proline. Culture medium and collagen lattice were collected separately at the end of the 24-h incubation period. Aliquots were taken for DNA measurements. Proteins and collagen were measured according to Peterkofsky and Diegelmann [20].

For sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the synthesized

proteins, cells in collagen lattices were cultivated during 7 days, medium was removed and replaced by DMEM without methionine and cysteine, supplemented with 50 $\mu\text{g/mL}$ β -aminopropionitrile, 10 $\mu\text{g/mL}$ ascorbic acid, 2 mM glutamine and 0.5% SVF, and various concentrations of mast cell extract. After 6 h, 200 μCi of ^{35}S -methionine were added for additional 24 h. At the end of the incubation period, 5 mM cold methionine were added to each culture medium. The samples were analysed in 7.5% acrylamide SDS-PAGE gels as described previously [21].

Western blotting

For immunoblotting, samples of conditioned medium were mixed with reducing sample buffer (62.5 mM Tris, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 0.01% bromophenol blue, pH 6.8, 5% mercaptoethanol) and subjected to electrophoresis on 8% polyacrylamide gels at 4 °C for 8 h at 200 V. The proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Immobilon, Millipore, France) by electrophoresis at 4 °C for 2 h at 80 V. The membranes were blocked with 50 mM Tris, 150 mM NaCl, pH 7.5 (TBS) containing 5% (w/v) non-fat dry milk (Bio-Rad) 0.1% Tween, at room temperature for 1 night. The transferred proteins were probed with primary antibodies diluted in TBS-Tween containing 1% milk for 2 h at room temperature. After extensive washing with TBS-Tween, the membranes were reprobed with alkaline phosphatase-conjugate goat antimouse IgG (Sigma, France) diluted 1000-fold in TBS-milk 1%-Tween for 1 h at room temperature. Additional washing in TBS allowed development with Sigma FastTM detection reagent (Sigma, France).

Results

Mast cell extract inhibits collagen gel contraction by skin fibroblasts

Fibroblasts embedded in a collagen gel contract their collagen support. Lattices were retracted to less than 50% of their initial diameter in approximately 4 days for the controls. Contraction was delayed by mast cell extract addition in the culture medium and the extent of final gel contraction was decreased (Fig. 1a). The differences between the series were maximal at days 1 and 2. The effect was dose-dependent (Fig. 1b). It was statistically significant for one mast cell/ml or more at day 1. In a control experiment, mast cell extract was replaced by fibroblast extract (1000 or 10 000 fibroblasts/ml), prepared in exactly the same way as the mast cell extract. No significant inhibition of lattice contraction was found (Fig. 1c). Depletion of heparin from the mast cell extract completely suppressed its effects on the retraction at day 3. After 7 days a slight inhibition was, however, still observed (Fig. 2).

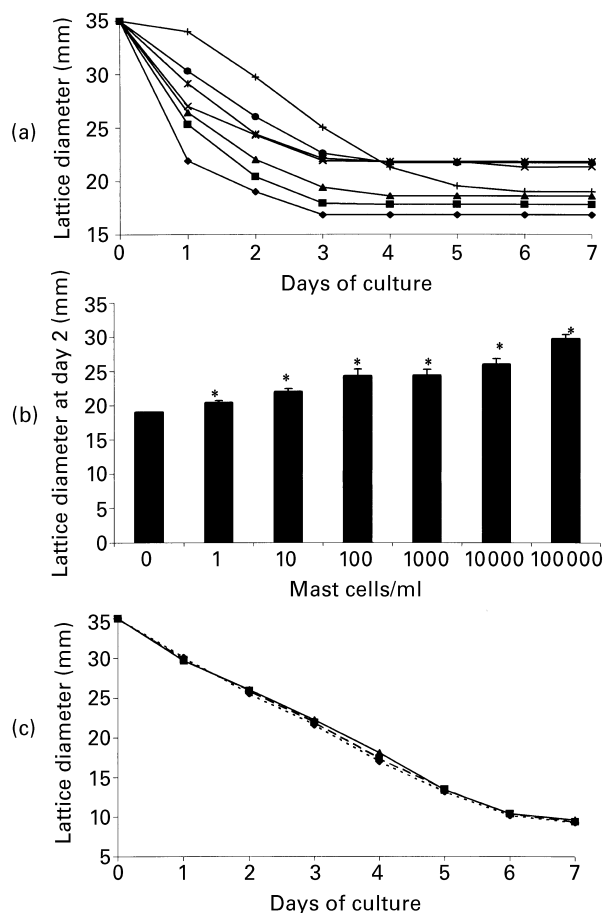


Fig. 1. Effects of mast cell (MC) extract on collagen lattice retraction by human skin fibroblasts. (a) Serial measurements of lattice diameter for the 7 days of cultures; (b) comparison of the lattice diameters at day 2; (c) lack of effect of dermal fibroblast (DF) extract on collagen lattice retraction. All data are the mean of quadruplicate experiments $\pm$ 1 SEM. *Significantly different from controls, $P < 0.001$. a: $\blacklozenge$, 0 M C/ml; $\blacksquare$, 1 M C/ml; $\blacktriangle$, 10 M C/ml; $\times$, 100 M C/ml; $*$, 1000 M C/ml; l , 10 000 M C/ml; l , 100 000 M C/ml. c: $\blacklozenge$, 0 DF/ml; $\blacksquare$, 1000 DF/ml; $\blacktriangle$, 10 000 DF/ml.

Mast cell extract stimulates fibroblast proliferation

After seeding 50 000 fibroblasts per lattice on day 0, lattices were digested with bacterial collagenase at days 1, 3 or 7 and the number of cells was counted. A significant stimulation of fibroblast proliferation was observed in cultures containing mast cell extract (Fig. 3). This effect was observed as soon as day 1 with one mast cell/ml. Trypan blue test showed no alteration of fibroblast viability.

Mast cell extract stimulates protein and collagen synthesis

Measurement of [^{3} H]proline incorporation into the total proteins and the collagens synthesized by fibroblasts cultured in collagen lattices showed that mast cell extract was

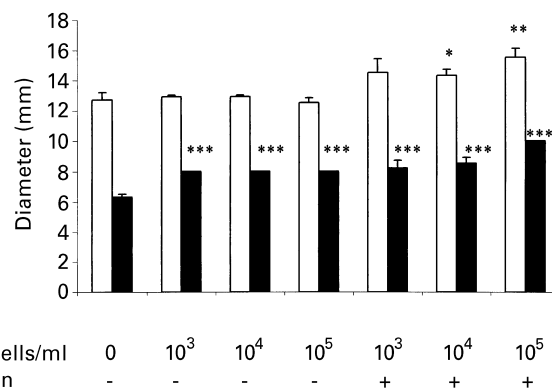


Fig. 2. Measurement of lattice diameter after 3 (open columns) or 7 (full columns) days of culture in the absence (0, controls) or presence of mast cell extract (10³, 10⁴ or 10⁵ mast cells/mL). Mast cell extract was used as a crude extract (heparin +) or after heparin depletion by affinity chromatography (heparin -). All data are the mean of quadruplicate experiments $\pm$ 1 SEM. *Significantly different from controls, $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$.

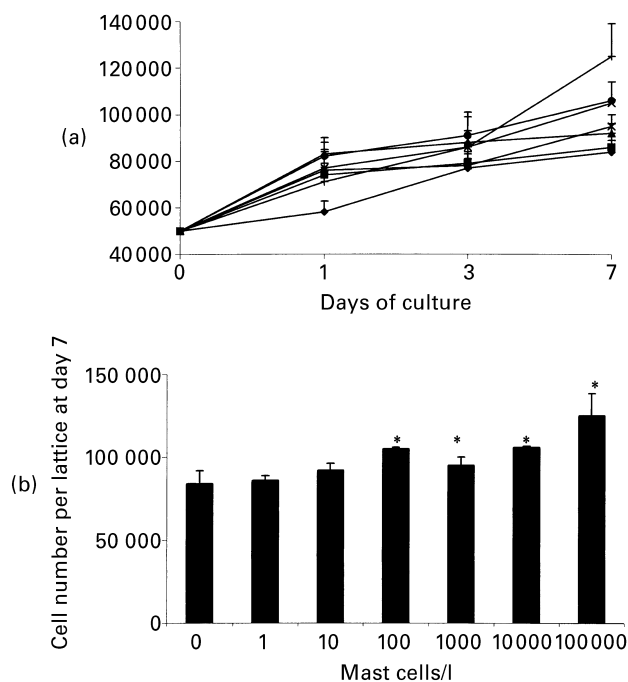


Fig. 3. (a) Kinetic measurement of fibroblast proliferation in collagen lattices incubated with increasing concentrations of mast cell extract. (b) Comparison of the cell numbers per lattice at day 7. All data are the mean of quadruplicate experiments $\pm$ 1 SEM. *Significantly different from controls, $P < 0.05$. a: $\blacklozenge$, 0 M C/ml; $\blacksquare$, 1 M C/ml; $\blacktriangle$, 10 M C/ml; $\times$, 100 M C/ml; $*$, 1000 M C/ml; l , 10 000 M C/ml; l , 100 000 M C/ml.

able to increase total protein and collagen secretion in the culture medium, compared to controls (Fig. 4a,b). The stimulation was maximal at a concentration of 100 mast cells/ml for total proteins and 10 mast cells/ml for collagen.

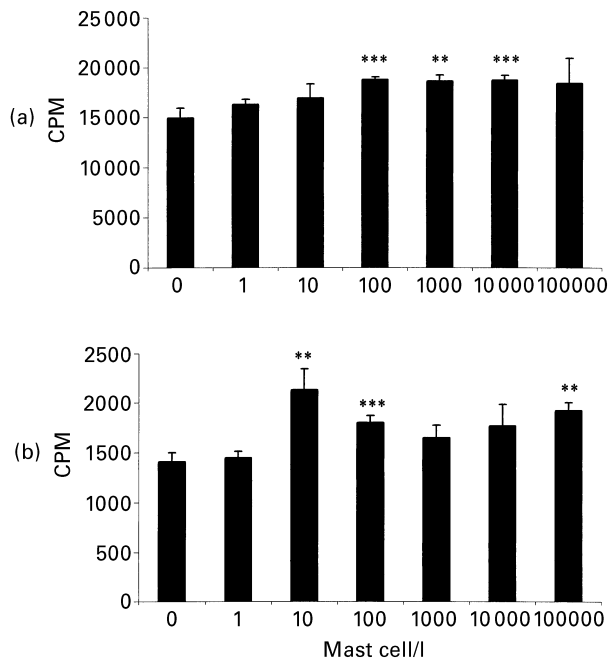


Fig. 4. Measurement of $[^3\text{H}]$ -proline incorporation in total proteins (a) and collagen (b) secreted into the culture medium by fibroblasts incubated with increasing concentrations of mast cell extracts. All data are the mean of quadruplicate experiments $\pm$ 1 SEM. **Significantly different from controls, $P < 0.02$; *** $P < 0.01$.

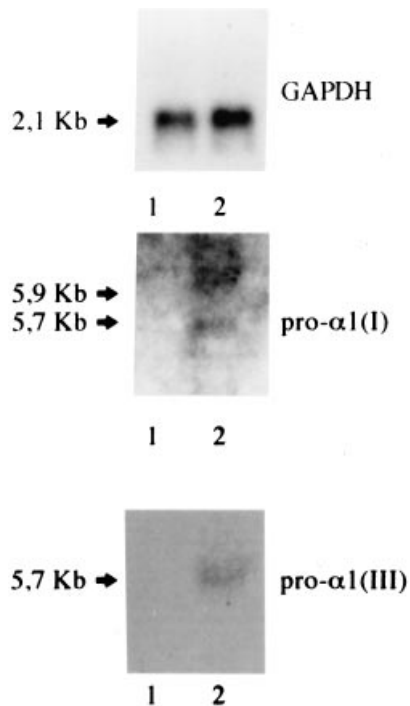
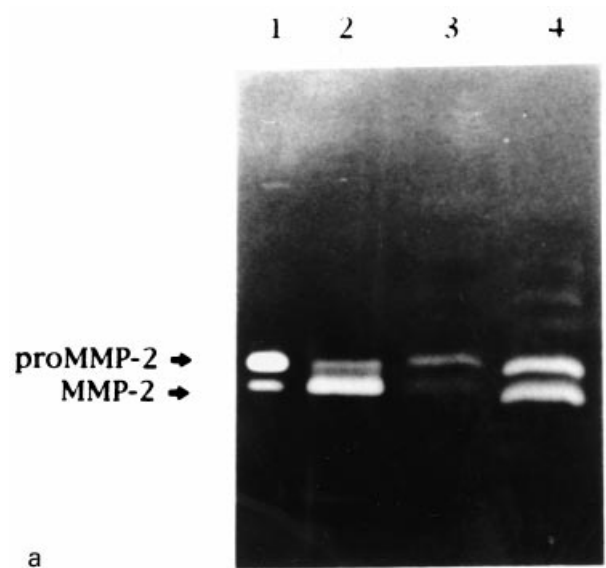
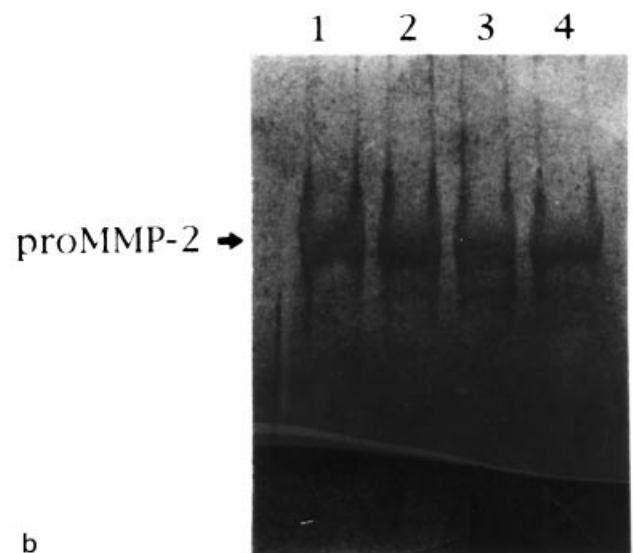


Fig. 5. Northern blot analysis of the mRNAs for pro- $\alpha 1$ (I) collagen, pro- $\alpha 1$ (III) collagen and glyceraldehyde-3-phosphate dehydrogenase. The size of the bands and the location of 28S and 18S ribosomal RNAs are indicated (1): controls; (2): after addition of mast cell extract (100 MC/mL).



a



b

Fig. 6. (a) Gelatin zymography of the culture medium. (1): Control pro-MMP-2 and MMP-2; (2): control pro-MMP-2 and MMP-2 after APMA activation; (3): culture medium of lattice cultures incubated without mast cell extract; (4): culture medium of lattice cultures incubated with mast cell extract (10^5 mast cells/mL). (b) Western blot analysis of the pro-MMP-2 protein secretion into the culture medium of lattice cultures incubated with mast cell extracts. (1): 100 000; (2): 10 000; (3): 1000 mast cells/mL; (4): controls without mast cell extract.

Northern blot analysis confirmed an increased level of pro- $\alpha 1$ (I) mRNAs (+40%) and pro- $\alpha 1$ (III) (+20%) when mast cell extract (100 mast cells/mL) was added to the culture (Fig. 5).

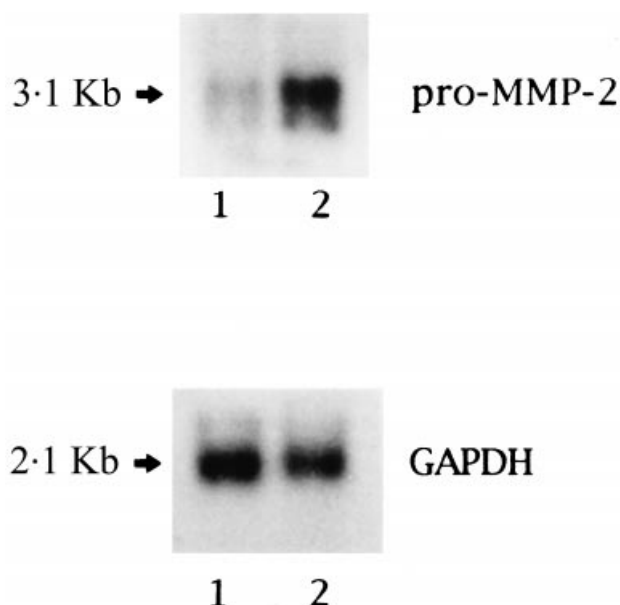


Fig. 7. Northern blot analysis of pro-MMP-2 gene expression in lattice cultures of fibroblasts incubated without (1) or with (2) mast cell extract (100 mast cells/ml).

Qualitative analysis of the proteins secreted by the dermal fibroblasts under the influence of mast cells extract was performed using SDS-PAGE. No significant difference of the repartition of the synthesized proteins was found in the presence of mast cell extract (data not shown).

Mast cell extract stimulates MMP-2 expression and activity

Gelatin zymography of the culture medium showed two major gelatinolytic bands with molecular weight 72 and 66 kDa, corresponding to pro-MMP-2 and activated MMP-2 (gelatinase A). Addition of mast cell extract induced a significant increase of both the pro-form and activated form (Fig. 6a). The increased level of the MMP-2 protein was confirmed by Western blot analysis of the culture medium (Fig. 6b). Northern blot analysis showed an increased level of pro-MMP2 mRNA (+40%) in the cultures incubated with mast cell extract (Fig. 7).

Discussion

Fibrosis is a common complication of auto-immune and chronic inflammatory diseases. In these conditions, an uncontrolled activation of connective tissue cells leads to an excessive accumulation of extracellular matrix macromolecules, mainly collagen fibres and tissue sclerosis [22]. Fibroblasts are the main cells responsible for collagen deposition in connective tissue. However, the mechanisms leading to their activation remain incompletely understood.

Several previous papers have drawn attention to the potential role of mast cells in the initial steps of connective tissue cell activation, leading to fibrosis [23].

Mast cells are resident connective tissue cells which have been found in close proximity with fibroblasts in prefibrotic areas [24]. Mast cells synthesize and accumulate in their granules several mediators able to activate fibroblasts. This is the case, for instance, for tryptase, a mast cell proteinase which stimulates fibroblast migration and/or proliferation [4,5], interleukin-4 (IL-4) and transforming growth factor- β (TGF- β), two potent activators of collagen synthesis [25,26] and many other cytokines [27]. In addition, we have demonstrated previously that rat peritoneal mast cells co-cultured with monolayers of 3T3 fibroblasts induce their proliferation and collagen synthesis [6].

It must be noted that some mast cell products might act only in a species-specific manner and, for that reason, some components of rat mast cell extract may be inactive on human fibroblasts. Many cytokines, however, such as IL-1, IL-2, IL-5, IL-6, IL-7, IL-8, IL-10, IL-11, platelet-derived growth factor (PDGF), basic fibroblast growth factor, IGF-1, TGF- β and TNF- α are as active in human as in murine systems. It is also the case for other mediators such as histamine, tryptase, chymase and heparin.

In this paper, we have studied the interactions between mast cell products and fibroblasts cultivated in three-dimensional collagen lattices. This model, initially designed by Bell and coworkers [28], provides a more physiological environment than monolayer cultures on plastic for studying the response of fibroblasts to regulatory agents. Since mast cell extract was prepared by sonication, it should be noted that the extract contained all the preformed molecules and not only those actively secreted by mast cells.

It is well established that, when embedded in a collagen gel, fibroblasts contract their support. Addition of mast cell extracts to the lattice culture medium significantly decreased the rapidity and intensity of contraction. The effect was maximal at 1000 mast cells/mL, suggesting the existence of an optimal ratio between mast cells and fibroblasts for obtaining the biological effect. It should be noted that the effect was specific, since it was not found when fibroblast extract was used instead of mast cell extract. The inhibiting effect of mast cell extract on lattice contraction was unexpected, since fibroblasts from fibrotic tissues usually contract the lattices more rapidly and more strongly than control fibroblasts [29]. The decreased contraction was not linked to a decrease of cell number or viability, as an increased cell proliferation was observed in the lattices incubated with mast cell extract. The relatively high content of heparin present in the mast cell extract might, however, be responsible for this effect: previous data from Guidry and Grinnell [30] demonstrated that heparin may inhibit collagen gel contraction by fibroblasts. Heparin depletion from

the mast cell extract by affinity chromatography only partially suppressed the inhibition, indicating that other factors, such as TGF- β [31], might be involved in this effect.

Total protein and collagen synthesis are very low in collagen lattices, compared to monolayer cultures [19]. Addition of mast cell extract to the lattice culture medium induced an increased secretion of total proteins and collagen. Northern blot analysis of collagen mRNAs showed that this effect was located at a pretranslational level. Such stimulation might be attributed to TGF- β and/or IL-4 present in the mast cell extracts [27]. Tryptase was also reported recently to stimulate collagen synthesis by lung fibroblasts [5]. The finding that the effect was maximal at 100 mast cells/mL suggests the existence of an optimal ratio between mast cells and fibroblasts within the lattices for observing the effect.

In addition to its effect on gel contraction and collagen synthesis, mast cell extract was also able to stimulate cell proliferation within the lattices and to increase the expression and activation of gelatinase A/MMP-2. Western blot analysis confirmed the increased level of the MMP-2 protein in the lattice culture medium when fibroblasts were incubated with mast cell extract. The stimulation occurred at a pretranslational level, as demonstrated by the increased level of pro-MMP-2 mRNA. MMP-2 is a product of fibroblasts. We verified in separate experiments that no MMP-2 was detectable in the mast cell extract at the concentrations used in this study. TNF- α or IL-1 released by mast cells might be responsible for the activation of the pro-MMP-2 gene expression by the fibroblasts seeded in the lattice. In addition, mast cell extract contains small amounts of tryptase, which has been shown to be able to activate pro-MMP-2 [32].

There was no direct relationship between the inhibiting effect of mast cell extract on the contraction of collagen lattices and the number of fibroblasts, protein and collagen secretion or expression/activation of gelatinase A. Different cytokines and other mediators produced by the mast cells could be involved in these different effects.

Taken together, our results demonstrate that mast cells, even at very low concentrations, are able to modulate many activities of fibroblasts seeded in a collagen matrix. Main effects concerned cell proliferation, total protein and collagen synthesis, production and activation of MMP-2. All these features are present in the initial steps of fibrosis, characterized by an increased number of fibroblasts and an acceleration of extracellular matrix synthesis and remodelling. Our data support the importance of the role of mast cell–fibroblast interactions as an early event of the fibrotic process.

Acknowledgements

This work was supported by grants from CNRS and the

University of Reims. Mrs S. Etienne and Mrs E. Deschamps are gratefully acknowledged for typing the manuscript.

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