Activated Mast Cells Are Fibrogenic for 3T3 Fibroblasts

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The extent of mast cell direct involvement in fibrosis is not defined as yet. In the present study we assessed whether long-term co-culture (up to 7 d) of functionally active rat peritoneal mast cells with 3T3 mouse fibroblasts and mast cell activation can affect fibroblast proliferation and collagen production. Co-culture of subconfluent 3T3 fibroblasts with resting mast cells or with mast cells stimulated by αIgE (1:35) or repeatedly activated by low concentrations of compound 48/80 (0.25–0.75 μg/ml) did not alter fibroblast proliferation. However, fibroblast proliferation was increased significantly (100–130%) when mast cells were repeatedly activated with higher concentrations of compound 48/80 (1–3 μg/ml). Repeated mast cell activation by compound 48/80 (0.25 μg/ml) caused a twofold increase in collagen production and this was reduced by 63% by the mast cell stabilizer nedocromil sodium (10^{-5} M). At the same time, co-culture of 3T3 fibroblasts with unstimulated or immunologically activated mast cells did not modulate their collagen production. In conclusion, we have demonstrated that mast cell activation, under certain conditions, can enhance significantly 3T3 fibroblast proliferation and collagen production, thus indicating a direct mast cell involvement in the fibrotic processes. ***Key words: mast cells/fibroblast proliferation/collagen synthesis.***
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The cardinal role of mast cells in type I hypersensitivity reactions is well established. However, mast cells have been implicated in the photogenesis of a number of chronic inflammatory conditions that result in fibrosis such as idiopathic lung fibrosis, keloids and hypertrophy scars, scleroderma, and chronic graft-versus-host disease [1]. In these conditions mast cells undergo changes in numbers and morphology that correlate with the course of fibrosis development [2,3]. These observations provide only circumstantial evidence that mast cells have a role in causing fibroblasts to express a fibrotic phenotype. However, *in vitro* studies aimed to analyze the direct effects of mast cells on fibroblast biochemical properties are still few and scattered [4–7].

We have developed in the past a mast cell/3T3 fibroblast co-culture in which viability and functional activity of rat peritoneal mast cells are maintained for more than a month [8]. Exploiting this co-culture we have demonstrated that when a linear "wound" is performed in the fibroblast monolayer and half the monolayer is scraped cell free, mast cell presence significantly enhances fibroblast migration and proliferation into the wound space [4].

In the present work, to further analyze mast cell–fibroblast interactions in fibrosis we have investigated the effects of mast cell presence and activation on 3T3 fibroblast proliferation and collagen synthesis by utilizing the mast cell/3T3 co-culture system. In these co-cultures mast cells were challenged 1) with anti-IgE antibodies in acute-single fashion, as it happens in hypersensitivity reactions, and 2) with compound 48/80 in repeated-chronic fashion. This was done both to produce an activation pattern different from the one taking place in hypersensitivity reactions and to mimic what might take place in fibrotic diseases.

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Abbreviations: αIgE, goat anti-rat IgE antibodies; 3T3, contact inhibited 3T3 Swiss albino mouse fibroblast cell line.

MATERIALS AND METHODS

Materials Dulbecco's modified Eagle's medium (DMEM), L-glutamine, streptomycin, penicillin, and fetal bovine serum were obtained from Biological Industries (Beth Haemek, Israel). Compound 48/80 was purchased from Sigma (St. Louis, MO). Nedocromil sodium was a kind gift of Fisons (UK). [^{3}H] labeled proline was from New England Nuclear (Boston, MA). Goat anti-rat IgE antibodies (αIgE) were from Bethyl Laboratories Inc. (Montgomery, TX). Sterile tissue culture plastic ware was purchased from Falcon (Oxford, CA). Contact inhibited 3T3 Swiss albino mouse fibroblast cell line was obtained from The American Type Culture Collection (Rockville, MD).

Mast-Cell Purification Mast cells were obtained from Sabra male rats (an outbred strain from the Hebrew University) and purified as previously described [8]. Purified mast cells (>95% as assessed by metachromasia after staining with toluidine blue), were seeded on 3T3 Swiss Albino mice fibroblasts. Mast cells adhere to fibroblast monolayer in 10 min and remain viable and functionally active for more than a month [8].

3T3 Fibroblast Proliferation 3T3 Swiss albino mice fibroblasts (3T3) were seeded in 96-well tissue culture plates (3×10^3 cells/well) and cultured in DMEM supplemented by 10% heat-inactivated feta! bovine serum, 100 u/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (DMEM+). After overnight culture at 37°C in 5% CO_2, to allow fibroblast adherence, rat peritoneal mast cells (3.5×10^3 mast cells/well) were seeded onto the fibroblasts. In one experiment different mast cell numbers (80–5000 mast cells/well) were seeded. One day after seeding the mast cells, culture medium was aspirated and non-adherent cells were removed by washing the monolayer with 2 ml of fresh medium. Thereafter, mast cell activation was performed by adding compound 48/80 (0.25–3 μg/ml). Every 2–3 d medium was replaced by a fresh one containing the same amount of the mast cell activator. In another series of experiments, mast

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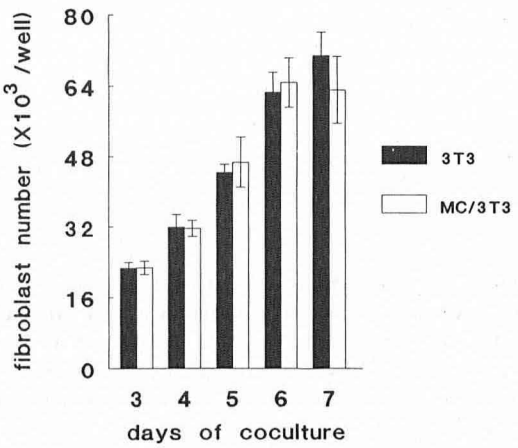

Figure 1. Co-culture of resting mast cells (MC) with 3T3 fibroblasts does not affect fibroblast proliferation. 3.5×10^3 MC were seeded on day 0 on 3T3 fibroblasts. Fibroblast numbers were determined at the indicated days. Data are mean ± SEM of seven experiments performed in triplicates.

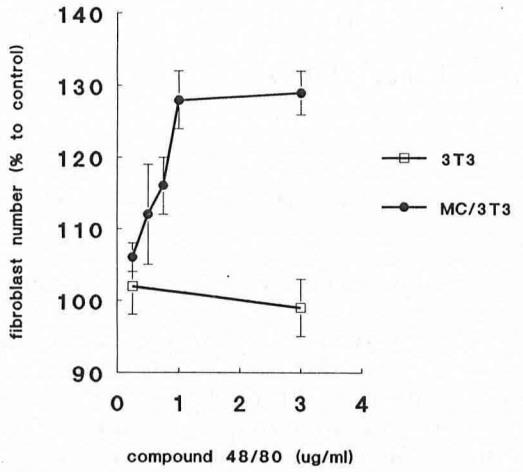

Figure 2. Mast cell (MC) activation by compound 48/80 enhances 3T3 fibroblast proliferation. 3.5×10^3 MC were seeded on 3T3 fibroblasts. MC/3T3 were activated by compound 48/80 (0.25–3 μg/ml) 1 d after MC seeding. Fibroblast numbers were evaluated on day 5 after MC activation and expressed as percent of the cell number in the control group (3T3 or MC/3T3 that were incubated with medium alone). Data are mean ± SEM of three to nine experiments performed in triplicates.

cells were single-time activated by an optimal concentration of αIgE antibodies (1:35, 1 d after seeding). Periodically (every 2–3 d) fibroblasts were obtained by trypsinization and counted by the use of a hemocytometer. Fibroblast viability was determined by Trypan Blue exclusion test.

Collagen Production by 3T3 Fibroblasts Collagen production by confluent fibroblast monolayers was assessed by [^{3}H]-proline incorporation into collagenous proteins [9]. 3T3 fibroblasts were seeded in 24-well tissue-culture plates and grown in DMEM+ medium till confluence. Mast cells (1.8×10^4) were seeded on half of the plates and 24 h after seeding the plates were washed as described above and the cultures were activated. In the case of non-immunologic activation, 3T3 and MC/3T3 co-cultures were pre-incubated for 4 d with DMEM+ (control) or DMEM+ containing compound 48/80 (0.25 μg/ml) either alone or in combination with nedocromil sodium (10^{-5} M). The medium was then removed and replaced by 0.5 ml of DMEM containing 5% fetal bovine serum, 50 μg/ml β-aminopropionitrile, 50 μg/ml ascorbic acid, [^{3}H]-proline (10 μCi/well), and compound 48/80 (alone or together with nedocromil sodium). In the case of immunologic activation, mast cells were activated once, 1 d after seeding, by addition of αIgE (1:35) along with the [^{3}H]-proline–containing mixture. Cultures were incubated for additional 24 h and [^{3}H]-proline incorporation into extracellular collagen was then determined in the supernatant [9]. To determine the cell number in each well, fibroblast were detached by trypsinization on the last day of the experiment and counted.

Histamine Release Determination To assess the degree of mast cell activation in the activation experiments, histamine release was evaluated as follows. Mast cell/3T3 were activated by compound 48/80 (0.25–3 μg/ml) or αIgE (1:35). After 20 min incubation at 37°C, supernatants were collected and mast cell/3T3 were scraped with a Teflon policeman and disrupted by continuous sonication for 1 min (50% duty cycle, Heat system Ultrasonics W380). Supernatants and cell sonicates were stored for histamine assay at −20°C. Histamine was determined in supernatants and cell sonicates by a radioenzymatic assay [10]. The percentage of histamine released from mast cells was calculated by dividing the amount of histamine in supernatants by the sum of that in supernatants and in cells.

Statistical Analysis Data are expressed as mean ± SEM. Statistical analysis was performed by the Student *t* test. p values of 0.05 or less were considered significant.

RESULTS

Mast Cell Activation Enhances 3T3 Fibroblast Proliferation First, the effect of resting, unactivated mast cells on fibroblast proliferation was assessed. As shown in **Fig 1**, the presence of unactivated mast cells (3.5×10^3 mast cells/well, 7 d of co-culture) on proliferating fibroblasts did not alter the rate of their growth, because the fibroblast numbers in cultures and in co-cultures did not differ throughout the duration of the experiment. In one experiment different mast cell numbers (80–5000 mast cells/well) were added to the fibroblast monolayer and were found to have no effect on fibroblast growth as well (data not shown).

Next the influence of a single immunologic mast cell activation on 3T3 fibroblast proliferation was examined. Mast cell activation by an optimal concentration of αIgE, which induced 25.6 ± 4.4% histamine release (mean ± SEM, n = 5), did not affect fibroblast proliferation: similar fibroblast numbers were found in resting and activated mast cell/3T3 cultures both on day 2 and 5 after mast cell activation [$(20.9 \pm 4.1) \times 10^4$ cells/well versus $(19.3 \pm 1.5) \times 10^4$; $(48.3 \pm 5.1) \times 10^4$ versus $(48.5 \pm 2.2) \times 10^4$; respectively, mean ± SEM, n = 3]. Recently we reported that mast cell/3T3 immunologic activation results in complete mast cell unresponsiveness to a second immunologic stimulus up to 7 d as evaluated by histamine release [11]. On the other hand, mast cells in co-culture with 3T3 fibroblasts can fully respond to each rechallenge with compound 48/80 by releasing percentages of histamine comparable to that of control cultures challenged for the first time [12]. We therefore evaluated whether mast cell repeated activation carried out by compound 48/80 could alter fibroblast proliferation. To find the optimal conditions, different concentrations of this mast cell activator were added to the cultures 1 d after mast cell seeding and at each medium changing (every 2–3 d) and fibroblast numbers were evaluated. As shown in **Fig 2**, mast cells that were activated by high concentrations of compound 48/80 (1–3 μg/ml) and consequently released high percentages of histamine into the culture medium (50-80%) enhanced significantly 3T3 fibroblast proliferation (100–130%, $p < 0.01$). Lower concentrations of compound 48/80, which released 15–25% histamine, did not alter significantly fibroblast proliferation. It is important to note that compound 48/80 did not influence the proliferation of 3T3 fibroblasts alone, i.e., in the absence of mast cells **(Fig 2)**. As depicted in **Fig 3**, this mast cell enhancing effect was evident and statistically significant only on days 4 and 5 after the first mast cell activation ($p < 0.05$ and $p < 0.01$, respectively).

Mast Cell Activation Enhances Collagen Production by 3T3 Fibroblasts Confluent cultures of 3T3 fibroblasts were cocultured for 5 d with resting mast cells and their collagen production was assessed by [H^3]-proline incorporation into collagenase sensitive proteins. The presence of resting mast cells on the monolayer slightly but not significantly increased collagen production (1531 ± 170 cpm/well in the absence of the mast cells and 1820 ± 240 cpm/well in their presence; mean ± SEM, n = 9, $p > 0.05$).

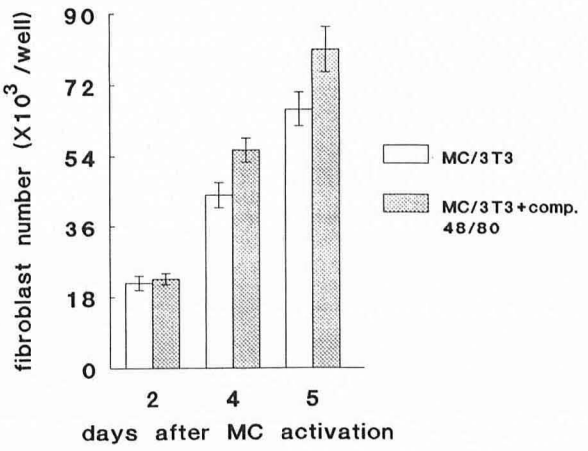

Figure 3. Enhancing effect of mast cell (MC) activation by compound 48/80 on the dynamics of fibroblast growth. 3.5×10^3 MC were seeded on 3T3 fibroblasts. MC/3T3 were activated by compound 48/80 (3 μg/ml) 1 d after MC seeding. Fibroblast numbers were determined on the indicated days. Data are mean ± SEM of five experiments performed in triplicates.

Mast cell/3T3 immunologic activation carried out by αIgE failed to modulate collagen synthesis (1950 ± 247 cpm/well for mast cell/3T3 co-cultures and 1716 ± 210 cpm/well for mast cell/3T3 activated by αIgE; mean ± SEM, n = 3, $p > 0.05$). On the other hand, mast cells repeated activation by compound 48/80, which induced 22.3 ± 4.6% histamine release (mean ± SEM, n = 5), led to a twofold increase in collagen production (1638 ± 72 versus 3527 ± 507 cpm/well, $p < 0.01$, **Fig 4**). Addition of optimal concentration [13] of nedocromil sodium (10^{-5} M) together with compound 48/80 brought about a decrease of 63% in this enhancement ($p < 0.05$, **Fig 4**). Under these experimental conditions, nedocromil sodium was found to reduce histamine release by 43 ± 12% (mean ± SEM, n = 5, $p < 0.05$). Compound 48/80 by itself or together with nedocromil sodium did not affect collagen synthesis by 3T3 cultures. Nedocromil sodium also did not influence the basal collagen production of either 3T3 or mast cell/3T3 cultures **(Fig 4)**.

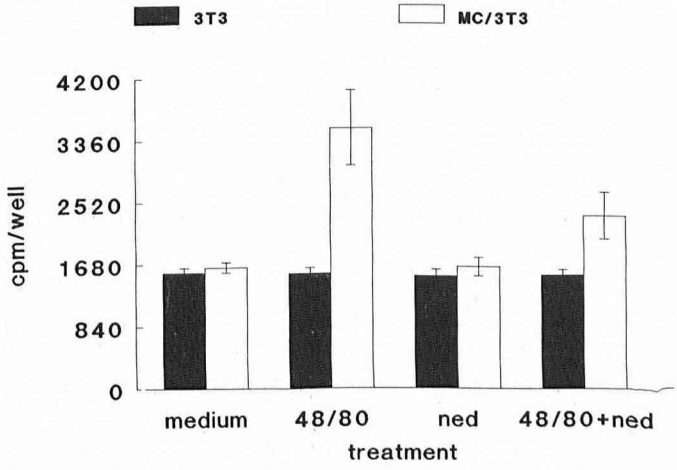

Figure 4. Mast cell (MC) activation by compound 48/80 increases 3T3 fibroblast collagen production and this effect is inhibited by nedocromil sodium. MC/3T3 cocultures were incubated with compound 48/80 (0.25 μg/ml) alone or in combination with nedocromil sodium and collagen synthesis was evaluated as described in *Materials and Methods*. In these quiescent-confluent fibroblast cultures, in which collagen synthesis was measured, activation of MC did not influence fibroblast proliferation. In fact, fibroblast numbers were similar in co-cultures stimulated by compound 48/80 [$(32.3 \pm 4.7) \times 10^4$ cells/well] and in those just incubated with medium [$(29.7 \pm 2.7) \times 10^4$ cells/well]. Data are mean ± SEM of five experiments performed in triplicates.

DISCUSSION

Mast cells have been postulated to have an important role in fibrosis in which the two major hallmarks are enhanced collagen synthesis by fibroblasts and/or their increased proliferation. There is general agreement that increased numbers of mast cells are associated with several fibrotic conditions in which there is enhanced fibroblast proliferation and/or enhanced synthesis of collagen and other extracellular matrix products. The suggestion that mast cells participate in fibrotic diseases stems primarily from morphologic data demonstrating, together with dynamic changes in their numbers, a change in their ultrastructure indicative of activation [1,2,14–16]. Only few *in vitro* studies would also indicate the existence of a direct and unique role of mast cells in fibrosis [4–7].

In the present work our aim was to determine whether alive and functionally active connective tissue-type mast cells, and not just mast cell–derived mediators, can directly affect fibroblast properties. In consequence we have utilized an *in vitro* defined system that we have developed in the past [8], in which rat peritoneal mast cells (of the connective tissue phenotype) are co-cultured with mouse 3T3 fibroblasts for up to 7 d. In this study we have also investigated the effects of different activation modalities of mast cells on fibroblast proliferation and collagen synthesis. Co-cultured mast cells were activated either by an IgE-dependent activator (αIgE antibodies) or by an IgE-independent one (compound 48/80). In fact, we aimed to compare the effects of 1) a mast cell immunologic activation that takes place in type I hypersensitivity reactions, which causes an acute anaphylactic type of histamine release and usually does not result in fibrosis, and 2) a mast cell chronic-repeated activation that might be the one taking place in fibrotic conditions in which mast cell stimulation is doubtfully carried out by IgE-mediated mechanisms. There is in fact some evidence that mast cells might be chronically activated during fibrosis development and that this activation differs from the anaphylactic one [3,17–20]. For example, mast cells in the skin of chronic graft-versus-host disease mice show a slowly developing (over period of weeks) granule depletion and cellular activation without evidence of granule exocytosis to cell membrane as typically takes place during an acute immunologic activation [3,21]. Dvorak and Kissell reported that also in wound healing the morphology of human skin mast cells suggested that they underwent a piecemeal type of activation rather than an anaphylactic, IgE-dependent one [19]. This activation pattern has been seen in other fibrotic conditions such as idiopathic interstitial fibrosis of the lung [20]. In fibrosis, mast cell chronic activation is probably caused by the constant presence of endogenous stimuli in contrast to mast cell activation in allergic diseases, which is caused by exogenous antigens through IgE-mediated mechanisms.

Recently we have reported that compound 48/80 can activate rat mast cells to release repeatedly the same percentage of histamine over a course of 3–4 activation cycles [12]. Therefore, in the present work, to imitate a chronic-repetitive type of mast cell activation, we have exposed mast cells to compound 48/80 for 5–7 d. It is worth mentioning that mast cell activation carried out by compound 48/80 also differ from IgE-dependent stimulation in that compound 48/80 induces histamine secretion in a receptor-independent manner, does not require extracellular calcium, and induces different intracellular biochemical events [22,23].

In the present study, we first assessed whether mast cells can affect fibroblast proliferation. The most suitable experimental system is the one in which subconfluent and hence still exponentially growing fibroblast cultures are used. Mast cells were seeded on these subconfluent cultures and fibroblast numbers were assessed at

various time points. Resting, unactivated mast cells were found not to affect fibroblast proliferation. It was hypothesized that even unactivated mast cells might affect fibroblast properties because mast cells have been shown to be able to transfer their granules to fibroblasts through a process termed "transgranulation" [24]. Transgranulation takes place without any sign of mast cell activation by developing specialized cell-to-cell contracts between mast cells and fibroblasts. Nevertheless in our study even mast cell immunologic activation and mast-cell stimulation with low concentrations of compound 48/80 did not affect fibroblast proliferation. However, fibroblast proliferation was increased significantly (100–130%) when mast cells were repeatedly activated with higher concentrations of compound 48/80. Dayton *et al* demonstrated that co-culture of mouse bone-marrow–derived mast cells (mucosal-type mast cells) with confluent 3T3 fibroblasts led to a drastic increase in fibroblast proliferation [7]. In their model the fibroblast monolayer was damaged and fenestrated due to the effects of the exponentially growing mast cells caused by the essential presence of interleukin-3 in the culture medium. Also the presence of activated rat peritoneal mast cells on "wounded" 3T3 fibroblast monolayers significantly enhanced the proliferation and migration of fibroblasts into the "wounded" area [4]. It has been reported that the addition of sonicated dog mastocytoma cells to cultures of human skin fibroblasts caused a threefold increase in cell proliferation [5]. In another study mastocytoma cell lines were found to secrete proliferative activity for human, hamster, and rabbit fibroblasts [6]. We have furthermore recently noted that incubation of sonicated rat peritoneal MC with dermal fibroblasts obtained from chronic graft-versus-host disease patients and from normal individuals enhanced their proliferation by 21–97%. Similarly, the mast cell sonicate induced a 20% increase in the proliferation of normal rat fibroblasts (Garbuzenko E, Nagler A, Rubinchik E, Levi-Schaffer F, unpublished observations). These observations show that the effect of mast cells on fibroblast proliferation is not restricted to the 3T3 murine cell line but occurs also with primary normal (human and rat) and diseased (human) fibroblasts.

Collagen synthesis was determined in confluent 3T3 fibroblasts co-cultured with mast cells. Co-culture with resting mast cells or immunologically activated mast cells did not significantly alter collagen production. However, repeated mast cell activation with low concentrations of compound 48/80 significantly increased collagen production. In contrast it has been reported that mast cell products can stimulate the production of active collagenase and hence participate in collagenolysis as indicated by experiments on gingival [25] and synovial [26] fibroblasts. Interestingly we found that addition of an optimal concentration of the mast cell stabilizer nedocromil sodium to our co-cultures partly abrogated the mast cell–induced enhancement of collagen synthesis. This effect is probably achieved through the reduction of mediator release from mast cells rather than by a direct effect on fibroblasts. In fact, fibroblast basal production of collagen was not affected by the drug alone.

In view of the effects of mast cell activation on fibroblast biochemical properties, it is now important to define which are the mast cell–derived mediators responsible for both the increase in fibroblast proliferation and collagen synthesis that we observed in our system. Mast cells release a wide spectrum of biologically active compounds that can exhibit both fibrogenic and fibrolytic activities. For example, histamine was found to stimulate in a dose-dependent manner the proliferation of human and guinea-pig fibroblasts [27,28] and to enhance collagen synthesis by guinea-pig skin fibroblasts [29]. On the other hand, the mast cell–specific proteases, tryptase and chymase, can degrade connective tissue elements [30] by modulating collagenase activity [31]. Mast cell proteases were found to increase fibroblast growth as well [32]. In addition, it is important to point out that it has been recently shown that mast cell stimulation results in mRNA synthesis and/or production of several cytokines [33], some of which, such as interleukin-1, -4, and -6, tumor necrosis factor-α, and transforming growth factor-β, may have an important role together with the classic mast cell mediators in mast cell/fibroblast interactions [34].

In summary, utilizing a long-term mast cell/3T3 fibroblast co-culture system we found that repeated rat peritoneal mast cell activation by compound 48/80 enhances significantly 3T3 fibroblast proliferation and collagen production. Therefore this is the first report that mast cells kept alive and functionally reactive in a defined *in vitro* microenvironment can directly cause biochemical changes in fibroblasts that are related to fibrosis development.

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