The Differentiation and Function of Myofibroblasts is Regulated by Mast Cell Mediators

James Gailit,* Mary J. Marchese,† Richard R. Kew,‡ and Barry L. Gruber*†§

*Department of Veterans Affairs Medical Center, Northport, New York, U.S.A.; †Department of Medicine, School of Medicine, State University of New York at Stony Brook, New York, U.S.A.; ‡Department of Pathology, School of Medicine, State University of New York at Stony Brook, New York, U.S.A.; §Department of Dermatology, School of Medicine, State University of New York at Stony Brook, New York, U.S.A.

Myofibroblasts are fibroblasts that express certain features of smooth muscle differentiation. Increased numbers of myofibroblasts and mast cells are frequently found together in a wide variety of settings, such as normal wound repair and scleroderma skin, which suggests that mediators produced by the mast cells could play a role in the regulation of myofibroblast differentiation and function. We used a human mast cell line, HMC-1, to determine if mast cells can induce normal human dermal fibroblasts to differentiate into functional myofibroblasts *in vitro*. We monitored the differentiation process by assaying two properties of the myofibroblast phenotype: expression of α-smooth muscle actin and functional capacity to contract a collagen matrix. In both a simple coculture system and in a skin-equivalent culture system, HMC-1 cells induced α-smooth muscle actin expression by fibroblasts. HMC-1 cells also stimulated fibroblast contraction of collagen gels, and the relative amount of contraction was dependent upon the number of HMC-1 cells present. To characterize the individual contributions made by specific mast cell products, we examined the effects of histamine, tumor necrosis factor α, and tryptase. Histamine induced a clear increase in α-smooth muscle actin expression, but it did not appear to stimulate fibroblast contraction. Tumor necrosis factor α had no effect in either assay. Purified human tryptase induced α-smooth muscle actin expression, and blocking the proteolytic activity of tryptase with specific inhibitors reduced that response. Tryptase inhibitors also eliminated the ability of HMC-1 cells to stimulate fibroblast contraction, suggesting that tryptase secreted by the HMC-1 cells may be one of the active mast cell mediators. *Key words: α-actin/collagen/histamine/transforming growth factor β/tryptase. J Invest Dermatol 117:1113–1119, 2001*

Although fibroblasts are abundant in connective tissues throughout the body, not all populations of fibroblasts are homogeneous (Sappino *et al*, 1990b; Schmitt-Gräff *et al*, 1994). Some fibroblasts express features of smooth muscle differentiation. These smooth muscle-like fibroblasts are referred to as myofibroblasts (Serini and Gabbiani, 1999) and they can be identified by certain characteristic features of the cytoskeleton, particularly by the expression of α-smooth muscle actin (Sappino *et al*, 1990b). Myofibroblasts make important contributions to the growth and differentiation of tissues and organs through their interactions with epithelial cells (Gabbiani *et al*, 1971; Gabbiani and Majno, 1972; Schmitt-Gräff *et al*, 1994; Gabbiani, 1998; Powell *et al*, 1999; Serini and Gabbiani, 1999). They play a major role in inflammatory responses and in wound repair through their production of growth factors, cytokines, and other soluble mediators. Myofibroblasts also contribute to wound repair by contracting granulation tissue to reduce wound volume, and by repairing damaged extracellular matrix through the synthesis of collagen, fibronectin, and proteoglycans. When myofibroblast activity is not regulated properly, however, the result may be destructive tissue remodeling (Adler *et al*, 1989; Hebda *et al*, 1993; Weber, 1997; Powell *et al*, 1999). Myofibroblasts appear to play a fundamental role in many diseases, including scleroderma (Sappino *et al*, 1990a; Kirk *et al*, 1995), hepatic and pancreatic fibrosis (Bachem *et al*, 1998; Friedman, 2000), and pulmonary fibrosis (Gauldie *et al*, 1999; Low, 1999).

Perhaps the most critical event in the normal process of wound repair and in the evolution of fibrosis is the appearance of activated myofibroblasts (Powell *et al*, 1999). These cells can apparently originate from several different sources, and a number of different activating factors may be involved. Mast cells are a potential source of some of those factors. One remarkably consistent finding is that myofibroblasts arise in a wide variety of settings concurrently with a local increase in the number of tissue mast cells (Choi and Claman, 1987; Rothe and Kerdel, 1991; Hebda *et al*, 1993). The concept that biologically significant interactions occur between mast cells and fibroblasts is supported by the fact that numerous mediators produced by mast cells, such as histamine, tumor necrosis factor α (TNF-α), and tryptase, can influence fibroblast development, proliferation, and behavior. Tryptase, for example, is a serine protease unique to mast cells, and it can stimulate fibroblast

Manuscript received January 26, 2001; revised April 24, 2001; accepted for publication May 13, 2001.

Reprint requests to: Dr. Barry L. Gruber, Department of Medicine, SUNY at Stony Brook, Stony Brook, NY 11794-8161. Email: bgruber@mail.som.sunysb.edu

Abbreviations: AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; BABIM, bis(5-amidino-2-benzimidazolyl)methane; IMDM, Iscove's modified Dulbecco's medium; rLDTI, recombinant leech-derived tryptase inhibitor; SFA, serum-free medium containing 2 mg per ml bovine serum albumin.

proliferation (Ruoss *et al*, 1991; Hartmann *et al*, 1992; Abe *et al*, 1998) and collagen synthesis (Cairns and Walls, 1997; Gruber *et al*, 1997; Abe *et al*, 1998). As mast cells have such pleiotropic effects on fibroblasts, we explored the possibility that specific mast cell products might be able to induce normal human dermal fibroblasts to differentiate into functional myofibroblasts.

MATERIALS AND METHODS

Cell culture Several different fibroblast cultures were used over the course of this work. One culture of normal human fibroblasts isolated from foreskin, AG01523C, was obtained from the National Institute on Aging, Aging Cell Culture Repository, Coriell Institute for Medical Research (Camden, NJ). These cells were fed minimum essential medium containing Earle's salts, and 26 mM sodium bicarbonate, 25 mM HEPES, pH 7.3 (catalog #42360, Life Technologies, Gaithersburg, MD), supplemented with nonessential amino acids (#11140, Life Technologies), 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 100 U per ml penicillin and 100 μg per ml streptomycin. Three other cultures of foreskin fibroblasts were prepared in this laboratory from enzyme-digested tissue. These cultures were fed Dulbecco's modified Eagle's medium (DMEM; #12100, Life Technologies), supplemented with 44 mM sodium bicarbonate, 1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum, 100 U per ml penicillin, 100 μg per ml streptomycin, and 250 ng per ml fungizone (Life Technologies). All fibroblast cultures were maintained in an atmosphere of 5% CO_2 at 37°C.

The human mast cell line, HMC-1 (Butterfield *et al*, 1988), was obtained from J. H. Butterfield (Mayo Clinic, Rochester, MN). Cultures of HMC-1 cells were grown at 37°C, 5% CO_2, in Iscove's modified Dulbecco's medium (IMDM; catalog #12200, Life Technologies), supplemented with 36 mM sodium bicarbonate, 1.2 mM monothioglycerol (Sigma-Aldrich, St. Louis, MO), 10% heat-inactivated fetal bovine serum, 100 U per ml penicillin, 100 μg per ml streptomycin, and 250 ng per ml fungizone.

A freeze/thaw lysate of HMC-1 cells was prepared by the following procedure. HMC-1 cells were washed once with IMDM supplemented only with 36 mM sodium bicarbonate, resuspended in the same medium at a concentration of 5×10^6 cells per ml, and then sequentially frozen and thawed five times. Finally, the cell lysate was clarified by centrifugation to remove cell debris. The concentration of total protein in a typical lysate preparation was ≈0.5 μg per ml, as measured in the BCA protein assay (Pierce, Rockford, IL).

Organotypic skin cultures were prepared as described previously (Garlick and Taichman, 1994b; Gruber *et al*, 1997). The culture system used here consisted of a collagen gel matrix, containing human foreskin fibroblasts and human dermal microvascular endothelial cells, supporting a stratified layer of human foreskin keratinocytes. In some cases, HMC-1 cells were also embedded in the collagen gel. Organotypic cultures, or rafts, were grown submerged for 5 d and then raised to the air interface for another 5 d to allow for complete stratification and differentiation of surface keratinocytes. We have used this skin-equivalent culture system previously to study interactions between mast cells and dermal fibroblasts (Meng *et al*, 1995; Gruber *et al*, 1997) because it manifests epithelial stratification and other properties of living skin (Garlick and Taichman, 1994a, b).

Transforming growth factor β1 (TGF-β1) purified from human platelets, recombinant human platelet-derived growth factor BB (PDGF-BB), and recombinant human TNF-α were purchased from R&D Systems. Histamine (#H-7375) was purchased from Sigma-Aldrich.

Immunohistochemistry Normal human foreskin fibroblasts (10^5 cells) were grown for 24 h on glass coverslips (18 mm × 18 mm) in complete medium containing serum, and then HMC-1 cells (10^6 cells) or TGF-β1 (5 ng per ml) was added to some of the coverslips and cultures were continued for an additional 48 h. The coverslips were washed once gently with phosphate-buffered saline (PBS) before the cells were fixed with ice-cold acetone for 15 min. The fixed cells were washed three times with PBS containing 0.05% Tween 20 (PBS/T) and then stained with a mouse monoclonal antibody specific for α-smooth muscle actin, clone 1A4 from Sigma-Aldrich, followed by fluorescein isthiocyanate conjugated goat antimouse IgG. To identify and locate mast cells, coverslips to which HMC-1 cells had been added were stained a second time using a monoclonal antibody against human tryptase (clone B2 from L.B. Schwartz, Virginia Commonwealth University, Richmond, VA) followed by a TRITC conjugate.

Organotypic cultures containing fibroblasts and HMC-1 cells, or fibroblasts without HMC-1 cells, were prepared and grown in complete medium as described above. Paraffin-embedded sections from individual rafts were deparaffinized, rehydrated with PBS, fixed in acetone, blocked for 60 min with 1% nonfat milk in PBS, and then incubated with the 1A4 anti-α smooth muscle actin monoclonal antibody for 60 min at room temperature. After sections were washed three times with PBS, they were incubated with biotinylated horse antimouse IgG for 30 min. The slides were again washed before incubation with streptavidin–alkaline phosphatase conjugate for 30 min. After three more washes, the slides were developed with fast red substrate (BioGenex, San Ramon, CA), and then counterstained with hematoxylin. To quantitate the results of the staining, we counted the number of positive cells within a defined area (450 μm × 150 μm) on three different sections from each raft.

Collagen contraction assay This assay was adapted from a published protocol (Greiling and Clark, 1997). Fibroblast cultures at 80% confluence were harvested by treatment with 0.05% trypsin/0.02% ethylenediamine tetraacetic acid (Clonetics, Walkersville, MD). Trypsin was inactivated with trypsin neutralizing solution (Clonetics). The cells were resuspended in DMEM containing 2% bovine serum albumin (BSA) (DMEM/BSA) at a concentration of 1×10^6 cells per ml. The fibroblast suspension was mixed with neutralized collagen (Vitrogen 100, Celtrix Laboratories, Santa Clara, CA), 5 × concentrated DMEM, and sodium bicarbonate, and, in certain experiments, HMC-1 cells. The final volume was adjusted so that DMEM and sodium bicarbonate were present at normal strength and the concentration of collagen was 1.8 mg per ml. The number of fibroblasts in the final mixture was kept constant at 500,000 fibroblasts per ml. In certain experiments, the mixture also contained from 20,000 to 500,000 HMC-1 cells per ml. Aliquots (0.5 ml) of the cell mixture were added to wells of a 24-well tissue culture plate, which had been coated with 2% BSA, and the collagen was allowed to polymerize at 37°C. After a 2 h incubation, the round gels were gently detached from the plastic surface to allow contraction, 0.5 ml DMEM/BSA was added to each well, and the gels were incubated at 37°C for 1–72 h. The DMEM/BSA fed to the cells at this point, when specified, contained fetal bovine serum (10%), PDGF-BB (5 ng per ml), TGF-β1 (5 ng per ml), histamine (0.5–5 μg per ml), or TNF-α (10–100 ng per ml). It is important to emphasize that the DMEM/BSA medium used for the preparation and incubation of collagen gels did not routinely contain serum; serum was present, to stimulate contraction, only when specified. The amount of contraction was assessed by measuring collagen gel diameter after incubation for the indicated time, usually 72 h. The results presented here are expressed as a percentage of the contraction stimulated by PDGF-BB, which was used at the optimal dose established in preliminary experiments. Stimulation with PDGF-BB for 72 h typically caused the fibroblasts to contract the collagen gels to 50% or less of the size of control, unstimulated gels. Collagen gel contraction in all of these experiments was completely dependent upon the presence of fibroblasts. Gels prepared without cells, or with only HMC-1 cells, did not contract (data not shown).

Tryptase inhibitors were tested by adding the inhibitors to the HMC-1 cell suspension (500,000 cells per ml) before the HMC-1 cells were mixed with the fibroblasts and collagen solution as described above. Bis(5-amidino-2-benzimidazolyl)methane (BABIM) was kindly provided by R. Tidwell (University of North Carolina, Chapel Hill, NC). Recombinant leech-derived tryptase inhibitor (rLDTI) was a generous gift from C. P. Sommerhof (Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik und Poliklinik, München, Germany). BABIM (Caughey *et al*, 1993) and rLDTI (Sommerhoff *et al*, 1994) are both specific inhibitors of tryptase proteolytic activity. The general protocol described above was modified slightly to measure the effect of HMC-1 cells on fibroblast contraction when the HMC-1 cells were cocultured with the fibroblast, but were not incorporated into the collagen gel. In those experiments, the porous membrane of a cell culture insert (catalog #3401, Corning Costar, Cambridge, MA) was used to separate the HMC-1 cells from the collagen gel, which contained only fibroblasts. The cup-shaped inserts were carefully positioned a few millimeters above the collagen gels, and 0.5 ml aliquots of the HMC-1 cell suspension (500,000 cells per ml) were dispensed directly into the inserts. Subsequent steps followed the standard protocol.

Gel electrophoresis and immunoblotting Normal human dermal fibroblasts were seeded into six-well plates, 100,000 cells per well, and fed culture medium fully supplemented with serum and the other components listed above. After 24 h, cells were washed twice with PBS and fed serum-free medium containing 2 mg per ml BSA (SFA). After

another 24 h, cells were washed twice with PBS, and then either treated as described below or fed SFA containing the specified additions and incubated for 72 h. Finally, the wells were washed three times with PBS and the cells in each well were lysed with 0.1 ml of Tris-buffered saline containing 1% sodium dodecyl sulfate (SDS) and protease inhibitors. The protein concentration in each sample was determined with the BCA protein assay using the supplied BSA as a standard. Equal amounts of protein from each sample, typically 1–10 μg, were separated under reducing conditions on a 10% Tris-HCl Ready Gel (Bio-Rad Laboratories, Hercules, CA) in SDS running buffer, and then the proteins were electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA). The membrane blot was blocked with 1% nonfat milk in PBS/T and then incubated with the 1A4 anti-α smooth muscle actin monoclonal antibody, followed sequentially by biotinylated antimouse IgG (Vector Laboratories, Burlingame, CA) and alkaline phosphatase–streptavidin conjugate (Vector). After these incubations, the immunoblot was stained using BCIP/NBT substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and the stained immunoblot was quantitated using NIH Image.

In certain experiments, fibroblast cultures were treated with tryptase immediately before the 72 h incubation in SFA medium. Tryptase solutions of 1–10 nM were prepared in SFA adjusted to pH 6.5 (Ren *et al*, 1997, 1998). Aliquots of these solutions were incubated at ambient temperature for 60 min with no additions, with 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF Pefabloc SC; 2 mM), or with a combination of soybean trypsin inhibitor (10 μg per ml) and aprotinin (2 μg per ml). These three different tryptase solutions were then applied to fibroblast cultures prepared as described above. The cultures were incubated for 30 min at 37°C, washed twice with PBS, and finally fed SFA containing the specified additions and incubated for 72 h.

Purified human lung tryptase, soybean trypsin inhibitor (#T-9003), and aprotinin (#A-4529) were obtained from Sigma-Aldrich. AEBSF was purchased from Roche Molecular Biochemicals (Indianapolis, IN).

RESULTS

The goal of this research was to determine if HMC-1 mast cells can induce normal human dermal fibroblasts to differentiate into functional myofibroblasts. We monitored the differentiation process by assaying two important properties of the myofibroblast phenotype: α-smooth muscle actin expression and functional capacity to contract a collagen matrix. TGF-β1 was used as a positive control in many of these experiments because it potently induces α-smooth muscle actin expression (Desmoulière *et al*, 1993; Ronnov-Jessen and Peterson, 1993) and markedly stimulates collagen gel contraction (Montesano and Orci, 1988; Finesmith *et al*, 1990; Fukamizu and Grinnell, 1990).

Fibroblast α-smooth muscle actin expression In the first set of experiments normal human dermal fibroblasts were cultured on coverslips, either with or without HMC-1 cells, to determine if the presence of mast cells could induce fibroblasts to express α-smooth muscle actin. As shown in **Fig 1**, only a low level of α-smooth muscle actin was detected in unstimulated fibroblasts (**Fig 1*A***). The immunofluorescence is weak and comparatively diffuse. As expected, however, treating the fibroblasts with TGF-β1 strongly induced α-smooth muscle actin expression (**Fig 1*C***). The cytoskeleton in the spindle-shaped fibroblasts is much more brightly stained and many individual actin filaments are discernible. Notably, fibroblasts cocultured with HMC-1 cells were also strongly induced to express α-smooth muscle actin (**Fig 1*B***).

The effect of HMC-1 cells on α-smooth muscle actin expression by fibroblasts was also studied using an organotypic (Garlick and Taichman, 1994b), or skin-equivalent, culture system. Using this culture system for an earlier study (Gruber *et al*, 1997), we demonstrated that fibroblast synthesis of type α1(I) procollagen mRNA was substantially increased in the presence of mast cells. To determine if mast cells can also induce α-smooth muscle actin expression by fibroblasts, HMC-1 cells and normal dermal fibroblasts were cultured together in the collagen layer of the skin equivalent, and then α-smooth muscle actin expression in the fibroblasts was examined by immunostaining. **Figure 2** demonstrates that the presence of HMC-1 cells in the skin-equivalent

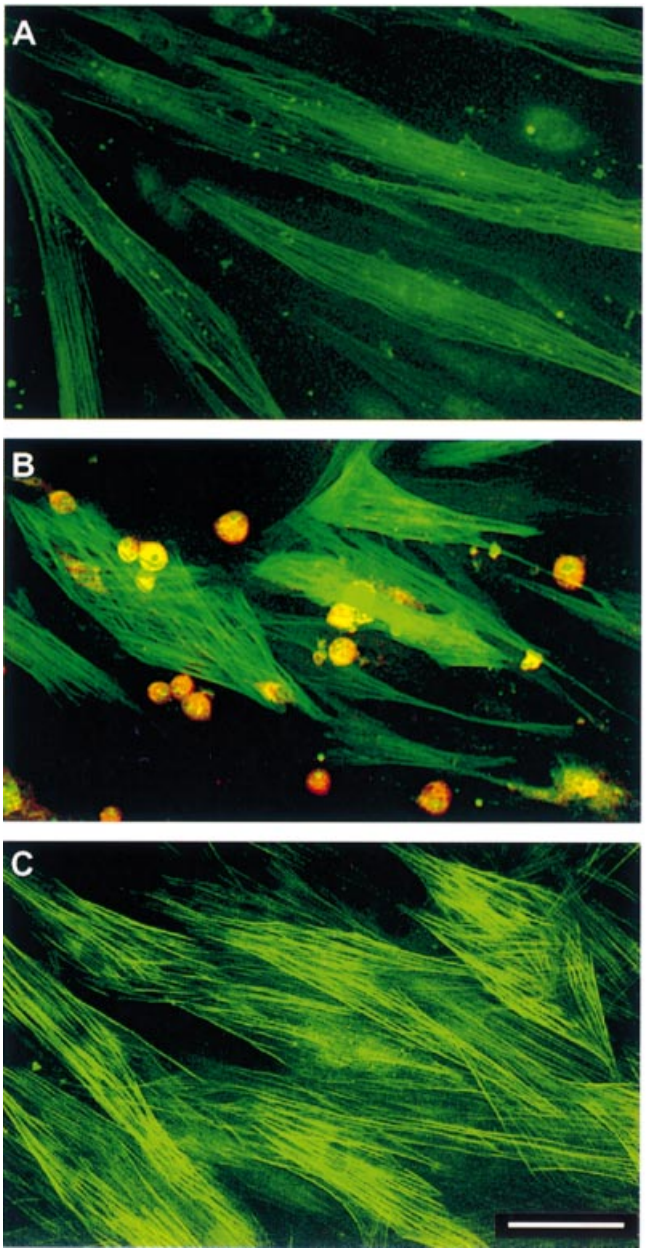

Figure 1. HMC-1 cells induce α-smooth muscle actin expression in dermal fibroblasts *in vitro*. Normal human dermal fibroblasts (10^5 cells) were grown on glass coverslips in complete medium. After 24 h, HMC-1 cells (10^6 cells) or TGF-β1 (5 ng per ml) were added, and the cells were cultured for an additional 48 h. The cultures were then examined by immunofluorescence. (*A*) Control, untreated fibroblasts stained for α-smooth muscle actin. (*B*) Fibroblasts cocultured with HMC-1 cells stained for α-smooth muscle actin (green fluorescence), and for tryptase (red fluorescence), to identify the HMC-1 cells. Areas where the green and red fluorescence overlap appear yellow. (*C*) Fibroblasts treated with TGF-β1 as a positive control and stained for α-smooth muscle actin. *Scale bar*: 40 μm.

culture system greatly increased the number of fibroblasts positive for α-smooth muscle actin. The average number of positive fibroblasts was 0.52 per mm^2 for cultures containing HMC-1 cells, but only 0.28 per mm^2 for control cultures, where the staining of individual cells was also less intense.

Fibroblast collagen contraction We next sought to determine if increased α-smooth muscle actin expression in these dermal fibroblasts was accompanied by an enhanced ability to contract a collagen matrix, an important functional property of

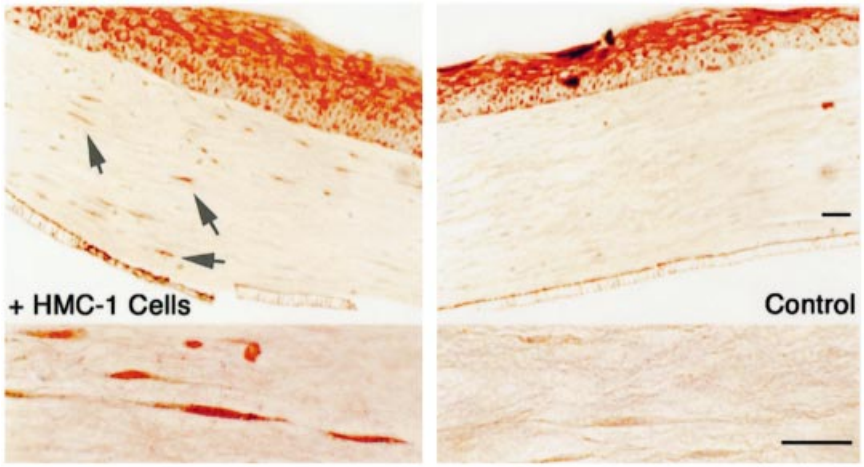

Figure 2. HMC-1 cells induce α-smooth muscle actin expression in dermal fibroblasts in an organotypic model of skin. The organotypic cultures were prepared and grown in complete medium as described in *Materials and Methods*. Shown on the left is a culture where HMC-1 cells and dermal fibroblasts were embedded in the collagen layer at a ratio of 1:4 (HMC-1 cells:fibroblasts). Shown on the right is a control culture with fibroblasts but no HMC-1 cells. The sections were stained first for α-smooth muscle actin and then counterstained with hematoxylin. The *arrows* in the *upper left panel* indicate fibroblasts stained positive for α-smooth muscle actin. A dark band of stratified keratinocytes, stained nonspecifically, is visible above the layer of collagen containing fibroblasts and HMC-1 cells. *Upper scale bar*: 30 μm; *lower scale bar*: 15 μm.

myofibroblasts (Grinnell, 1994). **Figure 3***(**A**)* presents results from experiments measuring the effects of different treatments upon the ability of normal human dermal fibroblasts to contract a collagen gel *in vitro*. Serum, PDGF-BB, and TGF-β1 all stimulated fibroblast contraction of the collagen gels, although TGF-β1 was less potent. These factors were added to the culture medium surrounding the gel. HMC-1 cells stimulated substantial fibroblast contraction when they were incorporated into the collagen gels (**Fig 3***A*, ***B***), but they had no effect on contraction when they were suspended above the gel on a porous membrane (data not shown). Interestingly, the contraction responses to HMC-1 cells and TGF-β1 displayed similar kinetics. Both stimulated appreciable contraction after 2–4 h, and nearly complete contraction after 8–10 h (**Fig 3***C*). There appeared to be a correlation between the number of HMC-1 cells in the collagen gel and their capacity to stimulate fibroblast contraction (**Fig 3***B*). Lowering the number of HMC-1 cells in the collagen gel to 40% or 20% of the number of fibroblasts produced correspondingly less contraction. When the number of HMC-1 cells was reduced to 4% of the number of fibroblasts present in the gel, no contraction was noted. One normal cell type, human microvascular endothelial cells, and two transformed cell lines, COS and HepG2, were also tested to determine if other cell types could substitute for HMC-1 mast cells. None of these cells was able to stimulate contraction (data not shown).

Effects of HMC-1 cell lysate Immunoblots were used to confirm our observation that HMC-1 cells induce α-smooth muscle actin expression in fibroblasts, and to explore further the mechanism through which this induction occurs. Normal human dermal fibroblasts were cultured alone, or with either TGF-β1 or a lysate prepared from HMC-1 cells (described in *Materials and Methods*), and then the amount of α-smooth muscle actin expressed under each condition was measured by immunoblotting. Densitometry of the immunoblot shown in **Fig 4** revealed that TGF-β1 induced a 7.9-fold increase in α-smooth muscle actin expression, and HMC-1 cell lysate induced a 2.7-fold increase. The average induction in four separate experiments was 4.4-fold for TGF-β1 and 2.1-fold for the HMC-1 lysate.

Initially we attempted to analyze cocultures of fibroblasts and HMC-1 cells in the immunoblotting assay, and we did detect increases in α-smooth muscle actin expression. The results demonstrated wide fluctuations, which we believe were probably caused by the variable but significant numbers of HMC-1 cells that attached to fibroblasts during coculture (Trautmann *et al*, 1997; 1998) and that could not be entirely removed by washing before detergent solubilization of the fibroblast monolayer. In contrast, HMC-1 cell lysate gave reproducible results in the immunoblotting assay (**Fig 4**) and was also active in the collagen contraction assay. The lysate induced 22%–36% of maximum contraction, which is comparable to the contraction induced by intermediate numbers of intact HMC-1 cells (**Fig 3***B*). Heat inactivation of the lysate completely eliminated its ability to induce α-smooth muscle actin expression and to stimulate collagen contraction (data not shown).

Effects of histamine and TNF-α As HMC-1 cell lysate appeared to be capable of inducing important features of the myofibroblast phenotype, we examined the effects of several specific mast cell products that might be responsible. A number of potential mediators, including histamine (Nilsson *et al*, 1994; Weber *et al*, 1996) and TNF-α (Grabbe *et al*, 1994; Nilsson *et al*, 1994), are produced by HMC-1 cells *in vitro* and by human mast cells *in vivo* (Metcalfe *et al*, 1997). **Figure 5** compares the effects of TGF-β1, histamine, and TNF-α on the expression of α-smooth muscle actin by human dermal fibroblasts. Although less potent than TGF-β1, histamine also stimulated an increase in α-smooth muscle actin expression. The average induction in three separate experiments was 1.9-fold after treatment with 5 μg per ml histamine. Unlike TGF-β1, however, histamine does not appear to stimulate fibroblast contraction in the collagen contraction assay. Incubation with 0.5–5 μg per ml histamine did not stimulate fibroblast contraction (data not shown). Incubation with H1 or H2 histamine receptor antagonists, to block the effects of histamine released by HMC-1 cells, did not inhibit the contraction stimulated by HMC-1 cells (data not shown). TNF-α had no significant effect on expression of α-smooth muscle actin (**Fig 5**) or on collagen contraction (data not shown).

Effects of tryptase The serine protease tryptase is another mast cell mediator produced by HMC-1 cells (Butterfield *et al*, 1990; Nilsson *et al*, 1994; Xia *et al*, 1995). We took several different approaches to determine if tryptase can modulate the myofibroblast phenotype. First, we used specific inhibitors of the enzymatic activity of tryptase to determine if tryptase released by HMC-1 cells contributes to the collagen contraction induced by HMC-1 cells. When either BABIM (50 nM) or rLDTI (25 μM) was added to collagen gels containing fibroblasts and HMC-1 cells, contraction was only 0%–14% of maximum under conditions where contraction in the absence of inhibitors was 50%–60% of maximum. Neither tryptase inhibitor affected the contraction response to PDGF-BB. Second, we tested purified human lung tryptase to determine if tryptase alone can induce α-smooth muscle actin expression by human dermal fibroblasts. As shown in **Fig 6**, a brief treatment with tryptase produced a clear increase in the expression of α-smooth muscle actin by human dermal fibroblasts. Furthermore, the induction produced by tryptase appeared to require its enzymatic activity because that effect was blocked substantially by pretreatment with AEBSF, an irreversible inhibitor of serine proteases, but not by incubation with the combination of soybean trypsin inhibitor and aprotinin, both of which inhibit most

serine proteases except tryptase (Alter *et al*, 1990; Butterfield *et al*, 1990; Schwartz, 1994).

DISCUSSION

Myofibroblasts play a central role in the normal process of wound repair and in the dysregulated processes leading to fibrosis. As the appearance of myofibroblasts often coincides with the presence of mast cells, it seems quite likely that mediators produced by mast cells are important for controlling the development and function of myofibroblasts. We used the HMC-1 human mast cell line as an experimental tool in an attempt to identify specific mast cell products capable of inducing two particular features of the myofibroblast phenotype. Histamine and tryptase, and the HMC-1 cells, induced normal human dermal fibroblasts to express α-smooth muscle actin, one distinguishing characteristic of differentiated myofibroblasts. Tryptase and HMC-1 cells also stimulated fibroblast contraction of a collagen matrix, one important function of myofibroblasts. Other myofibroblast functions, such as matrix deposition (Gruber *et al*, 1997) and matrix remodeling (Gruber *et al*, 1989), may be subject to regulation by tryptase as well.

In some situations mast cells communicate with fibroblasts through intimate physical contacts (Greenberg and Burnstock, 1983; Heard *et al*, 1992; Levi-Schaffer, 1995; Trautmann *et al*, 1997). One recent study (Yamamoto *et al*, 2000) concluded that mast cells enhance fibroblast contraction of collagen lattices partly by direct cell–cell interactions involving the c-kit receptor and the c-kit ligand, also known as stem cell factor. The collagen gels used in that study typically contained fibroblasts and HMC-1 cells at a ratio of 1:5. Although we would expect those conditions to promote more cell–cell interactions than the 1:1 ratio used in our assay, we did observe that embedding HMC-1 cells in the collagen gel stimulated contraction more effectively than simply adding any single soluble mast cell product to the culture medium, which does suggest that physical contact between the HMC-1 cells and the fibroblasts may contribute to the stimulatory signal. Close interactions do not appear to be an indispensable part of the signaling process, however. Soluble factors present in an HMC-1 cell lysate, as well as purified histamine and tryptase, had clear positive effects on α-smooth muscle expression and collagen contraction. At least some of the active factors in the cell lysate were sensitive to heat, indicating that labile proteins such as tryptase might be involved.

Mast cells produce an array of pharmacologically potent molecules affecting an astonishing number of biologic events, including the differentiation and function of myofibroblasts. Histamine (Nilsson *et al*, 1994; Weber *et al*, 1996), TNF-α (Grabbe *et al*, 1994; Nilsson *et al*, 1994), and heparin (Nilsson *et al*, 1994) are produced by HMC-1 cells *in vitro* and by human mast cells *in vivo* (Metcalfe *et al*, 1997). Histamine is a potent stimulus for smooth muscle contraction, but in our collagen gel assay it did not

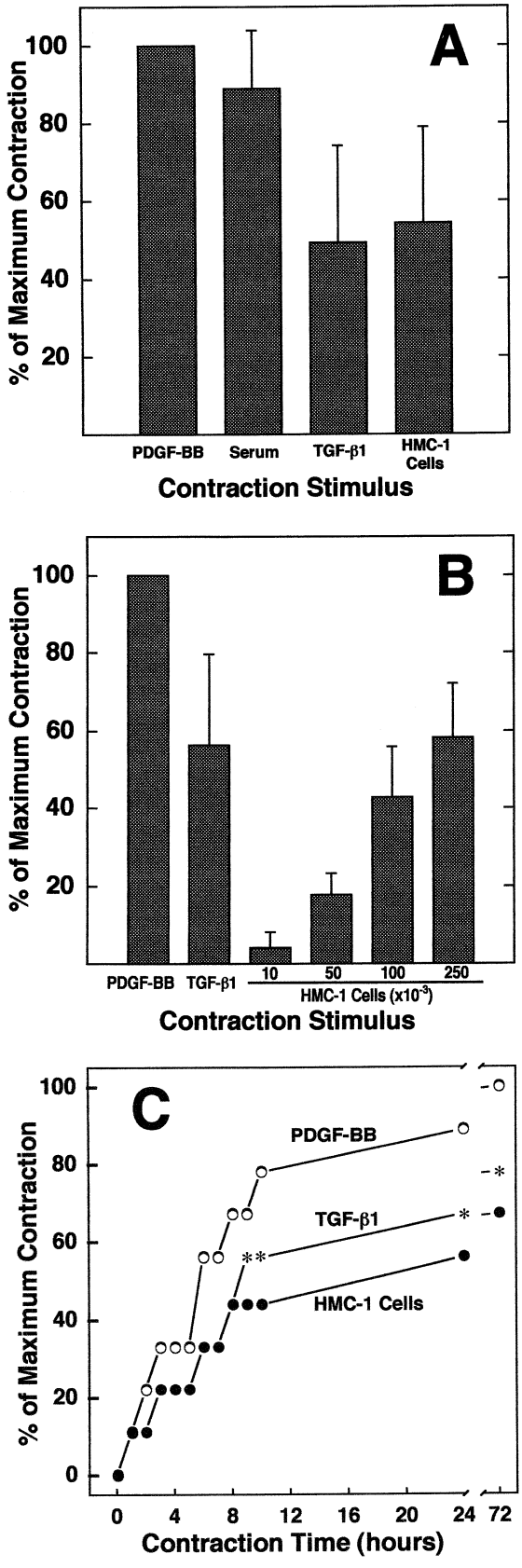

Figure 3. Collagen contraction assay. All of the collagen gels used in these experiments contained a constant number of human dermal fibroblasts (250,000 cells per gel), and some of the gels also contained the indicated number of HMC-1 cells. (*A*) Fetal bovine serum (10%), PDGF-BB (5 ng per ml), and TGF-β1 (5 ng per ml) were added separately to collagen gels containing only fibroblasts. Collagen gels containing both fibroblasts and HMC-1 cells (250,000 cells per gel) did not receive any additional stimulus. All gels were incubated for 72 h before measurement. The results are expressed as a percentage of the contraction stimulated by PDGF-BB, which was assigned the maximum value of 100%. The data shown are means and SEM from seven independent experiments with $n = 16$. Compared to control gels, $p = 0.00025$ for all four stimuli. (*B*) Contraction was stimulated, as described above, by PDGF-BB (5 ng per ml), TGF-β1 (5 ng per ml), or a varying number of HMC-1 cells (from 10,000 to 250,000 cells per gel) within the gel. Results are again expressed as a percentage of the maximum contraction stimulated by PDGF-BB. The data shown are the means and standard deviations from four independent experiments with $n = 10$. (*C*) Kinetics of contraction stimulated by PDGF-BB (5 ng per ml), TGF-β1 (5 ng per ml), or HMC-1 cells (250,000 cells per gel). The amount of contraction was measured at 1 h intervals, and the results at each time point are expressed as a percentage of the contraction stimulated by PDGF-BB after 72 h.

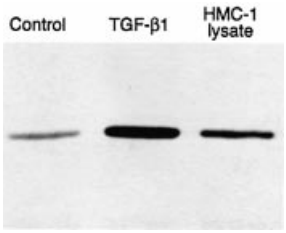

Figure 4. Immunoblot demonstrating that mast cell products induce fibroblast expression of α-smooth muscle actin. Normal human dermal fibroblasts were cultured for 72 h in serum-free medium (control) or in serum-free medium containing TGF-β1 (5 ng per ml) or HMC-1 cell lysate (diluted 1:10). Equal amounts of protein from different cultures were examined on immunoblots using the 1A4 monoclonal antibody specific for α-smooth muscle actin. TGF-β1 was used here as a positive control for induction of α-smooth muscle actin.

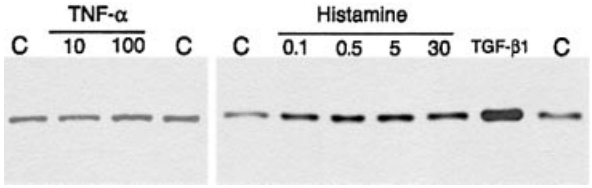

Figure 5. Immunoblot comparing effects of TGF-β1, histamine, and TNF-α on α-smooth muscle actin expression by human dermal fibroblasts. Foreskin fibroblasts were cultured for 72 h in serum-free medium (C, control) or in serum-free medium containing TNF-α (10–100 ng per ml), histamine (0.1–30.0 μg per ml), or TGF-β1 (5 ng per ml).

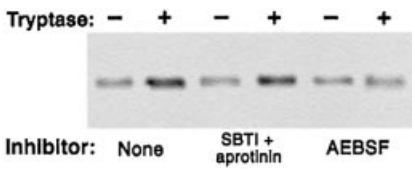

Figure 6. Immunoblot showing the effect of purified human tryptase on α-smooth muscle actin expression by human dermal fibroblasts. Fibroblast cultures were treated for 30 min with serum-free medium, pH 6.5, without (–) or with (+) the addition of 10 nM purified human tryptase and the indicated protease inhibitors as described in *Materials and Methods*. After 72 h fibroblasts were analyzed for α-smooth muscle actin expression.

reproducibly stimulate fibroblast contraction. In one earlier study using a similar assay, histamine did appear to stimulate fibroblast contraction, although it was relatively weak compared to PDGF (Clark *et al*, 1989). Consequently, it is still not clear whether or not histamine plays a direct role in regulating myofibroblast function. Nonetheless, we did observe that histamine could induce α-smooth muscle actin expression (**Fig 5**), suggesting that histamine may play a role in regulating myofibroblast differentiation. Histamine also seems to be involved in modulating several other processes intrinsic to wound repair and fibrosis, including fibroblast migration (Kupietzky and Levi-Schaffer, 1996) and production of cytokines (Zheng *et al*, 1994). TNF-α, in contrast to histamine, did not affect either myofibroblast function or differentiation. We found no indication that TNF-α by itself is a sufficient stimulus for induction of α-smooth muscle actin. It has been proposed, however, that TNF-α can facilitate the mechanism through which heparin stimulates expression of α-smooth muscle actin (Desmoulière *et al*, 1992; Schmitt-Gräff *et al*, 1994). Heparin and TNF-α are both constituents of the granules of human tissue mast cells. TGF-β is generally considered to be crucial for the regulation of myofibroblast development and behavior in tissue (Powell *et al*, 1999; Serini and Gabbiani, 1999), but we have been unable to demonstrate a critical role for TGF-β in the HMC-1 cell coculture systems.

Our last set of experiments provided evidence of a surprising new role for tryptase. Previous reports investigated the effects of tryptase on fibroblast proliferation (Ruoss *et al*, 1991; Hartmann *et al*, 1992; Abe *et al*, 1998) and collagen synthesis (Cairns and Walls, 1997; Gruber *et al*, 1997; Abe *et al*, 1998), and more recent studies focused on tryptase activation of PAR-1 and PAR-2 (Corvera *et al*, 1997; Mirza *et al*, 1997; Molino *et al*, 1997; Schechter *et al*, 1998; Akers *et al*, 2000). We are not aware of any studies published to date that specifically address tryptase regulation of myofibroblast differentiation or function. Nonetheless, there have been a number of relevant studies on thrombin stimulation of fibroblast contraction in a collagen matrix (Kolodney and Wysolmerski, 1992; Kolodney and Elson, 1993; Pilcher *et al*, 1994; 1995), and there may be some common elements in mechanisms through which tryptase and thrombin stimulate contraction. One common feature, the requirement for enzymatic activity, suggests that thrombin and tryptase may be signaling through members of the family of protease-activated receptors known as PARs (Déry *et al*, 1998). In fact, there is published evidence that thrombin stimulates fibroblast contraction through PAR-1 (Pilcher *et al*, 1994; 1995), and we have preliminary data suggesting that tryptase may be signaling through PAR-2.

This work was supported by grants from the Scleroderma Foundation to RK and BG, by grant 0548 from the Smokeless Tobacco Research Council Incorporated to RK, by a VA Merit Award to BG, and by the Research Enhancement Award Program at the Northport VA Medical Center. We wish to thank our colleagues for providing reagents, and we are especially grateful to Dr. Jonathan Garlick (Department of Oral Biology and Pathology, School of Dental Medicine, State University of New York at Stony Brook) for his assistance with the organotypic cell culture.

REFERENCES

Abe M, Kurosawa M, Ishikawa O, Miyachi Y, Kido H: Mast cell tryptase stimulates both human dermal fibroblast proliferation and type I collagen production. *Clin Exp Allergy* 28:1509–1517, 1998

Adler KB, Low RB, Leslie KO, Mitchell J, Evans JN: Contractile cells in normal and fibrotic lung. *Lab Invest* 60:473–485, 1989

Akers IA, Parsons M, Hill MR, Hollenberg MD, Sanjar S, Laurent GJ, McAnulty RJ: Mast cell tryptase stimulates human lung fibroblast proliferation via protease-activated receptor-2. *Am J Physiol Lung Cell Mol Physiol* 278:L193–L201, 2000

Alter SC, Kramps JA, Janoff A, Schwartz LB: Interactions of human mast cell tryptase with biological protease inhibitors. *Arch Biochem Biophys* 276:26–31, 1990

Bachem MG, Schneider E, Gross H, *et al:* Identification, culture, and characterization of pancreatic stellate cells in rats and humans. *Gastroenterology* 115:421–432, 1998

Butterfield JH, Weiler D, Dewald G, Gleich GJ: Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leukemia Res* 12:345–355, 1988

Butterfield JH, Weiler DA, Hunt LW, Wynn SR, Roche PC: Purification of tryptase from a human mast cell line. *J Leukoc Biol* 47:409–419, 1990

Cairns JA, Walls AF: Mast cell tryptase stimulates the synthesis of type I collagen in human lung fibroblasts. *J Clin Invest* 99:1313–1321, 1997

Caughey GH, Raymond WW, Bacci E, Lombardy RJ, Tidwell RR: Bis(5-amidino-2-benzimidazolyl)methane and related amidines are potent, reversible inhibitors of mast cell tryptases. *J Pharmacol Exp Ther* 264:676–682, 1993

Choi KL, Claman HN: Mast cells, fibroblasts, and fibrosis. New clues to the riddle of mast cells. *Immunol Res* 6:145–152, 1987

Clark RAF, Folkvord JM, Hart CE, Murray MJ, McPherson JM: Platelet isoforms of platelet-derived growth factor stimulate fibroblasts to contract collagen matrices. *J Clin Invest* 84:1036–1040, 1989

Corvera CU, Déry O, McConalogue K, *et al:* Mast cell tryptase regulates rat colonic myocytes through proteinase-activated receptor 2. *J Clin Invest* 100:1383–1393, 1997

Déry O, Corvera CU, Steinhoff M, Bunnett NW: Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *Am J Physiol Cell Physiol* 274:C1429–C1452, 1998

Desmoulière A, Rubbia-Brandt L, Grau G, Gabbiani G: Heparin induces α-smooth muscle actin expression in cultured fibroblasts and in granulation tissue myofibroblasts. *Lab Invest* 67:716–726, 1992

Desmoulière A, Geinoz A, Gabbiani F, Gabbiani G: Transforming growth factor-β1 induces α-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 122:103–111, 1993

Finesmith TH, Broadley KN, Davidson JM: Fibroblasts from wounds of different stages of repair vary in their ability to contract a collagen gel in response to growth factors. *J Cell Physiol* 144:99–107, 1990

Friedman SL: Molecular regulation of hepatic fibrosis, an integrated cellular response to injury. *J Biol Chem* 275:2247–2250, 2000

Fukamizu H, Grinnell F: Spatial organization of extracellular matrix and fibroblast activity: effects of serum, transforming growth factor β, and fibronectin. *Exp Cell Res* 190:276–282, 1990

Gabbiani G: Evolution and clinical implications of the myofibroblast concept. *Cardiovasc Res* 38:545–548, 1998

Gabbiani G, Majno G: Dupuytren's contracture: fibroblast contraction? An ultrastructural study. *Am J Pathol* 66:131–146, 1972

Gabbiani G, Ryan GB, Majno G: Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia* 27:549–550, 1971

Garlick JA, Taichman LB: Effect of TGF-β1 on re-epithelialization of human keratinocytes *in vitro*: an organotypic model. *J Invest Dermatol* 103:554–559, 1994a

Garlick JA, Taichman LB: Fate of human keratinocytes during reepithelialization in an organotypic culture model. *Lab Invest* 70:916–924, 1994b

Gauldie J, Sime PJ, Xing Z, Marr B, Tremblay GM: TGF-β gene transfer to the lung induces myofibroblast presence and pulmonary fibrosis. *Curr Top Pathol* 93:35–45, 1999

Grabbe J, Welker P, Möller A, Dippel E, Ashman LK, Czarnetzki BM: Comparative cytokine release from human monocytes, monocyte-derived immature mast cells, and a human mast cell line (HMC-1). *J Invest Dermatol* 103:504–508, 1994

Greenberg G, Burnstock G: A novel cell-to–cell interaction between mast cells and other cell types. *Exp Cell Res* 147:1–13, 1983

Greiling D, Clark RA: Fibronectin provides a conduit for fibroblast transmigration from collagenous stroma into fibrin clot provisional matrix. *J Cell Sci* 110:861–870, 1997

Grinnell F: Fibroblasts myofibroblasts, and wound contraction. *J Cell Biol* 124:401–404, 1994

Gruber BL, Marchese MJ, Suzuki K, Schwartz LB, Okada Y, Nagase H, Ramamurthy NS: Synovial procollagenase activation by human mast cell tryptase. Dependence upon matrix metalloproteinase 3 activation. *J Clin Invest* 84:1657–1662, 1989

Gruber BL, Kew RR, Jelaska A, *et al:* Human mast cells activate fibroblasts. Tryptase is a fibrogenic factor stimulating collagen messenger ribonucleic acid synthesis and fibroblast chemotaxis. *J Immunol* 158:2310–2317, 1997

Hartmann T, Ruoss SJ, Raymond WW, Seuwen K, Caughey GH: Human tryptase as a potent, cell-specific mitogen: role of signaling pathways in synergistic responses. *Am J Physiol Lung Cell Mol Physiol* 262:L528–L534, 1992

Heard BE, Dewar A, Corrin B: Apposition of fibroblasts to mast cells and lymphocytes in normal human lung and in cryptogenic fibrosing alveolitis. Ultrastructure and cell perimeter measurements. *J Pathol* 166:303–310, 1992

Hebda PA, Collins MA, Tharp MD: Mast cell and myofibroblast in wound healing. *Dermatol Clin* 11:685–696, 1993

Kirk TZ, Mark ME, Chua CC, Chua BH, Mayes MD: Myofibroblasts from scleroderma skin synthesize elevated levels of collagen and tissue inhibitor of metalloproteinase (TIMP-1) with two forms of TIMP-1. *J Biol Chem* 270:3423–3428, 1995

Kolodney MS, Elson EL: Correlation of myosin light chain phosphorylation with isometric contraction of fibroblasts. *J Biol Chem* 268:23850–23855, 1993

Kolodney MS, Wysolmerski RB: Isometric contraction by fibroblasts and endothelial cells in tissue culture: a quantitative study. *J Cell Biol* 117:73–82, 1992

Kupietzky A, Levi-Schaffer F: The role of mast cell-derived histamine in the closure of an *in vitro* wound. *Inflamm Res* 45:176–180, 1996

Levi-Schaffer F: Mast cell/fibroblast interactions in health and disease. *Chem Immunol* 61:161–185, 1995

Low RB: Modulation of myofibroblast and smooth-muscle phenotypes in the lung. *Curr Top Pathol* 93:19–26, 1999

Meng H, Marchese MJ, Garlick JA, *et al:* Mast cells induce T-cell adhesion to human fibroblasts by regulating intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression. *J Invest Dermatol* 105:789–796, 1995

Metcalfe DD, Baram D, Mekori YA: Mast cells. *Physiol Rev* 77:1033–1079, 1997

Mirza H, Schmidt VA, Derian CK, Jesty J, Bahou WF: Mitogenic responses mediated through the proteinase-activated receptor-2 are induced by expressed forms of mast cell α- or β-tryptases. *Blood* 90:3914–3922, 1997

Molino M, Barnathan ES, Numerof R, *et al:* Interactions of mast cell tryptase with thrombin receptors and PAR-2. *J Biol Chem* 272:4043–4049, 1997

Montesano R, Orci L: Transforming growth factor β stimulates collagen-matrix contraction by fibroblasts: implications for wound healing. *Proc Natl Acad Sci USA* 85:4894–4897, 1988

Nilsson G, Blom T, Kusche-Gullberg M, *et al:* Phenotypic characterization of the human mast-cell line HMC-1. *Scand J Immunol* 39:489–498, 1994

Pilcher BK, Kim DW, Carney DH, Tomasek JJ: Thrombin stimulates fibroblast-mediated collagen lattice contraction by its proteolytically activated receptor. *Exp Cell Res* 211:368–373, 1994

Pilcher BK, Levine NS, Tomasek JJ: Thrombin promotion of isometric contraction in fibroblasts: its extracellular mechanism of action. *Plast Reconstr Surg* 96:1188–1195, 1995

Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB: Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol Cell Physiol* 277:C1–C19, 1999

Ren S, Lawson AE, Carr M, Baumgarten CM, Schwartz LB: Human tryptase fibrinogenolysis is optimal at acidic pH and generates anticoagulant fragments in the presence of the anti-tryptase monoclonal antibody B12. *J Immunol* 159:3540–3548, 1997

Ren S, Sakai K, Schwartz LB: Regulation of human mast cell β-tryptase: conversion of inactive monomer to active tetramer at acid pH. *J Immunol* 160:4561–4569, 1998

Ronnov-Jessen L, Peterson OW: Induction of α-smooth muscle actin by transforming growth factor-β1 in quiescent human breast gland fibroblasts. *Lab Invest* 68:696–707, 1993

Rothe MJ, Kerdel FA: The mast cell in fibrosis. *Int J Dermatol* 30:13–16, 1991

Ruoss SJ, Hartmann T, Caughey GH: Mast cell tryptase is a mitogen for cultured fibroblasts. *J Clin Invest* 88:493–499, 1991

Sappino AP, Masouye I, Saurat JH, Gabbiani G: Smooth muscle differentiation in scleroderma fibroblastic cells. *Am J Pathol* 137:585–591, 1990a

Sappino AP, Schürch W, Gabbiani G: Differentiation repertoire of fibroblastic cells: expression of cytoskeletal proteins as marker of phenotypic modulations. *Lab Invest* 63:144–161, 1990b

Schechter NM, Brass LF, Lavker RM, Jensen PM: Reaction of mast cell proteases tryptase and chymase with protease activated receptors (PARs) on keratinocytes and fibroblasts. *J Cell Physiol* 176:365–373, 1998

Schmitt-Gräff A, Desmoulière A, Gabbiani G: Heterogeneity of myofibroblast phenotypic features: an example of fibroblastic cell plasticity. *Virchows Arch* 425:3–24, 1994

Schwartz LB: Tryptase: a mast cell serine protease. *Meth Enzymol* 244:88–100, 1994

Serini G, Gabbiani G: Mechanisms of myofibroblast activity and phenotypic modulation. *Exp Cell Res* 250:273–283, 1999

Sommerhoff CP, Söllner C, Mentele R, Piechottka GP, Auerswald EA, Fritz H: A Kazal-type inhibitor of human mast cell tryptase: isolation from the medical leech *Hirudo medicinalis*, characterization, and sequence analysis. *Biol Chem Hoppe-Seyler* 375:685–694, 1994

Trautmann A, Feuerstein B, Ernst N, Brocker EB, Klein CE: Heterotypic cell–cell adhesion of human mast cells to fibroblasts. *Arch Dermatol Res* 289:194–203, 1997

Trautmann A, Krohne G, Brocker EB, Klein CE: Human mast cells augment fibroblast proliferation by heterotypic cell–cell adhesion and action of IL-4. *J Immunol* 160:5053–5057, 1998

Weber KT: Fibrosis, a common pathway to organ failure: angiotensin II and tissue repair. *Semin Nephrol* 17:467–491, 1997

Weber S, Babina M, Krüger-Krasagakes S, Grützkau A, Henz BM: A subclone (5C6) of the human mast cell line HMC-1 represents a more differentiated phenotype than the original cell line. *Arch Dermatol Res* 288:778–782, 1996

Xia H-Z, Kepley CL, Sakai K, Chelliah J, Irani A-MA, Schwartz LB: Quantitation of tryptase, chymase, FcεRIα, FcεRIγ mRNAs in human mast cells and basophils by competitive reverse transcription-polymerase chain reaction. *J Immunol* 154:5472–5480, 1995

Yamamoto T, Hartmann K, Eckes B, Krieg T: Mast cells enhance contraction of three-dimensional collagen lattices by fibroblasts by cell–cell interaction: role of stem cell factor/c-kit. *Immunology* 99:435–439, 2000

Zheng T, Nathanson MH, Elias JA: Histamine augments cytokine-stimulated IL-11 production by human lung fibroblasts. *J Immunol* 153:4742–4752, 1994