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Research Article

Mast cell activation is a characteristic feature of chronic inflammation, a condition that may lead to fibrosis as a result of increased collagen synthesis by fibroblasts. We have investigated the potential of tryptase, the major protease of human mast cells, to stimulate collagen synthesis in the human lung fibroblast cell line MRC-5. Tryptase was isolated from human lung tissue by ion-exchange and affinity chromatography. At concentrations of 18 and 36 mU/ml, tryptase stimulated both an increase in cell numbers, and a fivefold increase in DNA synthesis as determined by methyl-[3H]thymidine incorporation. Similar concentrations of tryptase resulted in a 2.5-fold increase in collagen synthesis as determined both by incorporation of [3H]proline into collagen, and by assay of hydroxyproline concentrations in the supernatants. There was also a twofold increase in collagenolytic activity in the culture medium after tryptase treatment, indicating that the increase in collagen synthesis was not a consequence of decreased collagenase production. All of these actions of tryptase were reduced in the presence of the protease inhibitors leupeptin and benzamidine hydrochloride, indicating a requirement for an active catalytic site. SDS-PAGE and autoradiographic analysis of the [3H]collagen produced by the cells revealed it to be predominantly type I collagen. Our findings suggest that the release of tryptase from activated mast cells may provide a signal for abnormal fibrosis in inflammatory disease.

Mast Cell Tryptase Stimulates the Synthesis of Type I Collagen in Human Lung Fibroblasts

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Abstract

Mast cell activation is a characteristic feature of chronic inflammation, a condition that may lead to fibrosis as a result of increased collagen synthesis by fibroblasts. We have investigated the potential of tryptase, the major protease of human mast cells, to stimulate collagen synthesis in the human lung fibroblast cell line MRC-5. Tryptase was isolated from human lung tissue by ion-exchange and affinity chromatography. At concentrations of 18 and 36 mU/ml, tryptase stimulated both an increase in cell numbers, and a fivefold increase in DNA synthesis as determined by methyl-[^{3}H]thymidine incorporation. Similar concentrations of tryptase resulted in a 2.5-fold increase in collagen synthesis as determined both by incorporation of [^{3}H]proline into collagen, and by assay of hydroxyproline concentrations in the supernatants. There was also a twofold increase in collagenolytic activity in the culture medium after tryptase treatment, indicating that the increase in collagen synthesis was not a consequence of decreased collagenase production. All of these actions of tryptase were reduced in the presence of the protease inhibitors leupeptin and benzamidine hydrochloride, indicating a requirement for an active catalytic site. SDS-PAGE and autoradiographic analysis of the [^{3}H]collagen produced by the cells revealed it to be predominantly type I collagen. Our findings suggest that the release of tryptase from activated mast cells may provide a signal for abnormal fibrosis in inflammatory disease. (*J. Clin. Invest.* 1997. 99:1313–1321.) Key words: tryptase • proliferation • fibrosis • collagen • fibroblast

Introduction

Fibrosis, a conspicuous feature of chronically inflamed tissue, is characterized by progressive and excessive accumulation of extracellular matrix collagen (1) as a consequence of increased proliferation of fibroblasts (the major mesenchymal cell responsible for the synthesis of interstitial collagen). A feature of lung tissue from patients with fibrotic lung disease is an increased number of mast cells, many of which are in a state of partial degranulation (2) located in close proximity to proliferating fibroblasts (2, 3). Consistent with this observation are the increased concentrations of tryptase and other mast cell products in bronchoalveolar fluid collected from patients with fibrotic lung disease (4, 5). Such evidence points to a close interaction between mast cells and fibroblasts which may be pivotal in the pathogenesis not just of fibrotic lung disease, but of scleroderma, keloid formation, chronic graft versus host disease, and other fibrotic conditions (6, 7).

Long-term culture of rat peritoneal mast cells with mouse fibroblasts has been found to result in fibrogenesis (8), while the ingestion of extruded mast cell granules by fibroblasts can stimulate the secretion of collagenase (9, 10), suggesting that mast cell granule constituents may modulate connective tissue cell function. For example, the addition of histamine to cultured fibroblasts has been shown to stimulate proliferation (11) and collagen synthesis (12). Mast cells also contain substantial quantities of heparin proteoglycans within the granules. Heparin can contribute to the fibrotic process by binding to and preventing the inactivation of fibroblast growth factors (FGFs)[1] (13–15), or by facilitating binding of FGF to high-affinity receptors (13). In addition, binding to heparin sequesters FGFs into a reservoir from which they can subsequently be released by enzymatic degradation of the proteoglycan (16). Mast cells are becoming recognized also as important sources of inflammatory cytokines—including interleukins 1, 4 and 6, tumor necrosis factor, transforming growth factor β, and basic fibroblast growth factor (17)—which could confer roles in initiating and regulating processes of inflammation and fibrosis.

The most abundant product of human mast cells is the serine protease tryptase. Tryptase is a tetrameric serine protease with a molecular size of 134 kD, comprised of four monomers of 32–34 kD, each with one catalytic site (18). Its presence is restricted almost exclusively to mast cells, where tryptase exists within the secretory granules in a complex with heparin proteoglycan (19). Approximately 10 pg of tryptase per cell has been detected in mast cells derived from lung, while skin-derived mast cells contain about 35 pg tryptase per cell (20). The enzymatic activities of tryptase demonstrated include (*a*) the degradation of calcitonin gene-related peptide and vasoactive intestinal peptide (21, 22), (*b*) inactivation of fibrinogen (23), (*c*) activation of prostromelysin (24) and urinary plasminogen activator (25), and (*d*) cleavage of gelatinase and fibronectin (26). In addition, tryptase can interact with a number of cell types, and has been shown to be mitogenic for fibroblasts (27), canine smooth muscle cells (28), and epithelial cells (29), to stimulate eosinophil chemotaxis (30), to induce interleukin-8 release, and to modulate expression of intercellular adhesion molecule I (ICAM-1) on epithelial cells (29).

The ability of tryptase to stimulate fibroblast proliferation is consistent with this major mast cell product having a role in fibrosis. Despite mounting evidence that mast cells and their mediators can contribute to fibrosis by activating fibroblasts

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1. *Abbreviations used in this paper:* BAPNA, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride; FGF, fibroblast growth factor.

and stimulating collagen production, however, the actions of tryptase on collagen synthesis have not been studied. We have examined further the potential role of this protease in modulating fibroblast behavior, and have investigated in particular the ability of tryptase to stimulate collagen production from lung fibroblasts. We report that tryptase can stimulate the synthesis of collagen by fibroblasts, and that the major species produced is type I collagen.

Methods

Reagents. *N*-α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA), porcine heparin glycosaminoglycan, leupeptin, benzamidine hydrochloride, DEAE-52, DEAE-Sephacel, II heparin-agarose, and hydroxyproline were obtained from Sigma Chemical Co. (Poole, UK). L-[2,3-^{3}H]proline and *N*-[propionate-2,3-^{3}H]collagen (Rat I) were from Dupont NEN (Stevenage, UK), and collagenase (CLSPA grade) and trypsin were purchased from Worthington Biochemical Corp. (Freehold, NJ). Methyl-[^{3}H]thymidine, Hyperfilm MP, and the autoradiography enhancing reagent Amplify were from Amersham International (Little Chalfont, UK). Recombinant human transforming growth factor β (TGF-β) was purchased from R and D Systems (Abingdon, UK). A neutralizing antibody to TGF-β was purchased from Promega Ltd. (Southampton, UK), and a polyclonal antibody specific to collagen I from Euro-Path Ltd. (Bude, UK). Endotoxin in the tryptase preparations was assayed with the Toxicolor System (Seikagaku Corp., Tokyo, Japan). Protein was assayed with the Coomassie protein assay reagent (Pierce and Warriner Ltd., Chester, UK). All other reagents were of analytical grade, and were obtained from BDH (Poole, UK).

Cell culture. The MRC-5 fibroblast cell line, derived from human lung tissue, was obtained from the European Collection of Animal Cell Cultures (ECACC) (Porton Down, Salisbury, UK). The cells were grown in Minimum Essential Medium (MEM) (GIBCO BRL, Paisley, UK), supplemented with 10% fetal calf serum (GIBCO BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2, and were used routinely between passages 24 and 32 (equivalent to population doublings) as aging fibroblasts in culture have a reduced capacity for collagen synthesis (31).

Purification and characterization of tryptase. Lung tissue collected post-mortem was used as the source of mast cell tryptase. The tissue was chopped finely, and tryptase was purified from the crude extract by means of ion-exchange chromatography with DEAE-Sephacel (32) and heparin agarose chromatography, as described previously (29). The purity of the tryptase samples was determined by silver staining after SDS-PAGE on 10% reducing gels (according to the method of Laemmli) (33), and the identity of the purified protein was confirmed by immunoblotting with the AA5 anti–tryptase antibody (34). Endotoxin levels in the tryptase preparations were less than 15 pg/200 μg tryptase protein.

Assay for tryptase. Tryptase activity was assayed using the peptide substrate BAPNA. 10 μl of enzyme was added to 90 μl of 20 mM Tris buffer (pH 8.0) containing 1 M glycerol and 7.77 mM BAPNA. Absorbance was measured at 410 nm, and tryptase activity was expressed in milliunits (mU) per ml where 1 U represents that required to hydrolyze 1 μmol of substrate per min at 25°C. The specific activity of the tryptase preparations used in this study ranged from 1.8 to 2.6 U/μg.

Cell proliferation assay. Confluent fibroblasts were detached from culture flasks with a nonenzymatic cell dissociating solution (Sigma) and seeded into a 96-well microtiter plate at a density of 10^5 cells per ml in MEM containing 10% FCS. At confluence, the medium was replaced with serum-free (SF) medium consisting of MEM supplemented with 5 μg/ml bovine pancreas insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite. After 24 h of serum deprivation, purified tryptase was added following its dialysis in the presence of heparin (1:1; wt/wt; to stabilize enzymatic activity) (19) for 24 h at 4°C against phosphate-buffered saline (PBS). In experiments with protease inhibitors, tryptase with added heparin was incubated in the presence or absence of leupeptin or benzamidine hydrochloride for 1 h at 4°C, after which the BAPNA cleaving activity of tryptase was assayed to determine the percentage of inhibition achieved. Control tryptase samples to which no inhibitor was added were incubated under the same conditions. The inhibitors were used at concentrations which in preliminary experiments had been shown to be nontoxic to the fibroblasts, and without effect on thymidine incorporation. After the various additions were made, the cells were incubated at 37°C for 32 h with the addition of 1 μCi of methyl-[^{3}H]thymidine per well for the last 8 h to measure DNA synthesis. The cells were harvested and counted in scintillant. In addition, fibroblasts were seeded into 24-well plates and incubated with two optimal doses of tryptase for 72 h, and total cell number was determined by counting in a Neubauer hemocytometer (Fisher Scientific, Loughborough, UK) after staining with Trypan Blue.

Collagen assay. Collagen was assayed by measuring the incorporation of [^{3}H]proline into collagen as described previously (35). Fibroblasts were plated at a density of 10^5 per ml into 96-well microtiter plates, and grown to confluence in MEM (proline- and hydroxyproline-free) with 10% FCS and antibiotics. Confluent cells were then serum-starved for 48 h before the various additions were made. Purified tryptase with heparin (1:1, wt/wt) was dialyzed against MEM and used in the following studies. The time course of collagen production was determined by incubating the cells with 25 mU/ml of tryptase (with heparin) or with medium alone in the presence of 1 μCi of [^{3}H]proline for 3, 6, 24, and 48 h. Total protein synthesis (both collagenous and non-collagenous) was determined by precipitating all the proteins in 100 μl cell supernatant onto glass fiber filters and counting in a scintillant. Noncollagenous proteins were assayed in a second 100-μl aliquot of the same supernatant after digestion with 40 μg of purified bacterial collagenase for 2 h at 37°C, as described by Peterkofsky and Diegelman (36). The precipitated collagenase-resistant proteins were defined as noncollagenous. A preliminary experiment had shown that digestion of collagenous proteins in fibroblast supernatants with this concentration of collagenase was essentially complete after 2 h (data not shown). The difference between total and noncollagenous protein counts was taken to reflect the amount of collagen synthesized. As a control, supernatant samples were incubated for the same period with purified trypsin (Worthington) to check for specificity of digestion by collagenase (data not shown). In subsequent experiments, fibroblasts were incubated for 48 h either with medium alone or with purified tryptase (with heparin) to determine the dose response to tryptase. Levels of cell-associated collagen were assayed in the cell monolayer as described above. Cells were rinsed two times with PBS, trypsinized, and lysed in 200 μl of cold lysis buffer (20 mM Tris HCl, pH 7.4, containing 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM EGTA, 1 mM PMSF, 1 mg/ml leupeptin, 5 mM benzamidine hydrochloride, and 1% Nonidet P-40).

To investigate if the action of tryptase was mediated via its catalytic site, cells were incubated with either purified tryptase (with heparin), or with tryptase (heparin) that had been preincubated with leupeptin or benzamidine hydrochloride as described above. As controls, collagen synthesis was assayed in supernatants from fibroblasts incubated with leupeptin or benzamidine hydrochloride alone, or with various concentrations of transforming growth factor β (TGF-β) and heparin. Since TGF-β is a potent stimulator of collagen synthesis in fibroblasts (1) and fibroblasts are known to release this cytokine, it was necessary to determine if tryptase-induced collagen synthesis may be mediated via enhanced TGF-β release. An experiment was conducted in which cells were incubated with tryptase (with heparin) in the presence of either a neutralizing TGF-β antibody or an irrelevant rabbit antibody. In addition, cells were incubated with antibody alone (with heparin) or with the irrelevant rabbit antibody at the same concentrations. Towards the latter part of this study, purified preparations of the monoclonal antibody to tryptase (AA5) became available in sufficient quantities to allow immunoaffinity purification

of tryptase. An additional experiment using immunoaffinity purified tryptase was conducted to confirm the effect of tryptase on collagen synthesis.

Hydroxyproline assay. The collagen content of supernatants and cell monolayers from fibroblast cultures was assayed by spectrophotometric determination of hydroxyproline concentration according to the method of Juva and Prockop (37), incorporating some modifications described by Clark (38). Confluent fibroblast cultures were incubated with serum-free medium alone (control) or with tryptase (with heparin) in the presence or absence of inhibitors. The effect of heparin and TGF-β on the hydroxyproline content of cell supernatants was also investigated. After a 48-h incubation, the cells were trypsinized, centrifuged, and the supernatants were assayed spectrophotometrically for hydroxyproline.

Characterization of collagen type. Analysis of the collagen type synthesized by fibroblasts in response to tryptase was carried out using anion exchange chromatography, a procedure which has been shown to separate procollagen type I from type III (39), according to the method of Phan, Varani and Smith (40). The focus was on type I and type III collagens, as they represent 80% and 20%, respectively, of the total collagen synthesized by lung fibroblasts, and are the predominant connective tissue elements altered in fibrotic disorders (41). Confluent fibroblasts were incubated with either serum-free medium alone or with tryptase (with heparin) in the presence of 5 μCi/ml [^{3}H]proline for 52 h. Two flasks of cells were used for each treatment, and the supernatant from each was harvested, pooled, and retained on ice while 1 mg/ml leupeptin, 0.5 mM PMSF, 5 mM EDTA, and 2.5 mM NEM were added. The procollagens were precipitated at 4°C with 20% saturated ammonium sulphate for 24 h, and the suspension was centrifuged at 48,000 *g* for 1 h. The precipitate was resuspended in 25 mM Tris HCl (pH 7.5), 1 mM EDTA, and 2 M urea. The sample was applied onto a column of DEAE 52 that had been equilibrated in the same buffer, and procollagens were eluted according to the method of Clark (38) using a linear gradient of 0–0.22 M NaCl in 2 M urea and 25 mM Tris HCl, pH 7.5. Fractions of 5 ml were collected and counted in a scintillant. Aliquots from peak fractions with high counts were assayed for collagen as described above.

Pooled fractions from the two major peaks eluted from the DE52 column were treated as previously described (40), lyophilized, resuspended in SDS sample buffer, and analyzed by SDS-PAGE with 7.5% slab gels according to the procedure of Neville (42) (with some modifications) (43). The gel was fixed, impregnated with Amplify, dried, exposed to Hyperfilm-MP for 10 d, and developed using a commercial x-ray film developer.

Collagenase assay. Collagenase production and/or release from fibroblast cultures was assayed using ^{3}H-rat tail tendon type I collagen as substrate according to the protocol outlined in the Collagenase Assay System (New England Nuclear, Boston, MA). Supernatants from control and tryptase-treated cultures were concentrated tenfold, incubated with the radiolabeled substrate for 3 h at 21°C, centrifuged, and an aliquot was removed for counting. The collagenolytic activity was expressed as the percentage of substrate digested with reference to a standard curve using purified bacterial collagenase (0–10 μg/ml).

Results

Cell proliferation. An increase of up to fivefold in DNA synthesis was observed after incubation of quiescent cells for 36 h with tryptase (Fig. 1 *A*). The increase in DNA synthesis at 18 and 36 mU/ml tryptase was accompanied by cell division after incubating cells with tryptase for a total of 72 h (Table I). At these concentrations of tryptase, the fibroblast monolayer remained intact when examined by phase contrast microscopy, and was indistinguishable from cells which had not been exposed to tryptase, suggesting that the increase in cell growth was not a nonspecific effect of tryptase resulting from cell detachment and consequent release from contact inhibition. At the end of the labeling period, cell viability estimated by Trypan Blue exclusion was more than 90%. Stimulation of [^{3}H]thymidine incorporation by tryptase appeared to be reduced at 72 mU/ml compared to that seen at 18 and 36 mU/ml. Again, this was not due to a cytotoxic effect as viability was still more than 90%. However, when the cells were exposed to tryptase in excess of 150 mU/ml, cell viability decreased to less than 70% with the cells lifting off from the monolayer (data not shown).

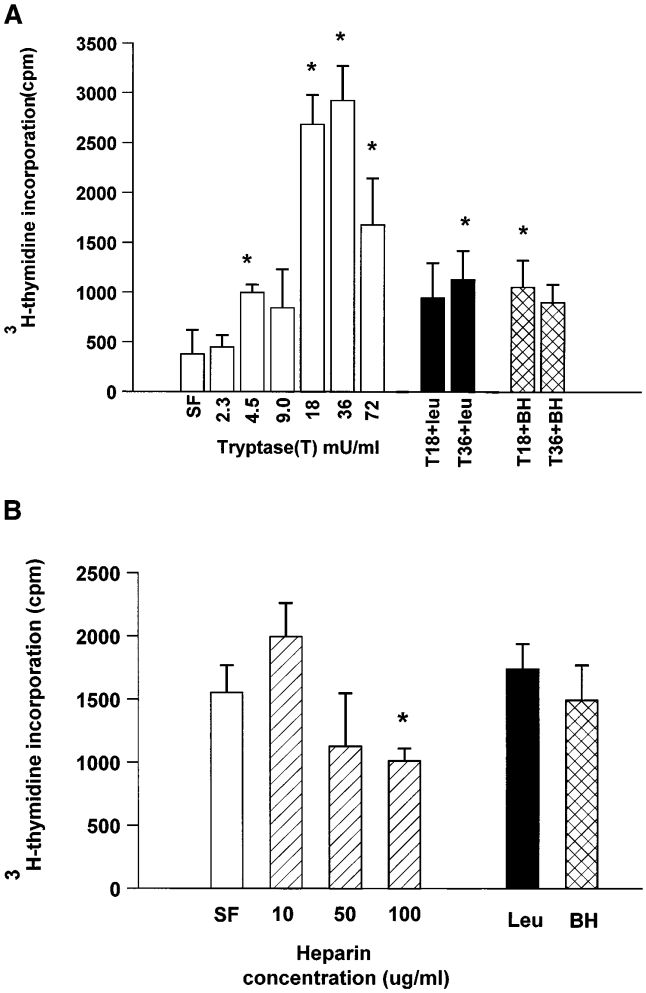

Figure 1. (*A*) Mitogenic response of fibroblasts to tryptase. Purified tryptase was incubated with quiescent cells in the presence of heparin (1:1, wt/wt). Cells were incubated also with either 18 or 36 mU/ml tryptase in the presence of 50 μg/ml leupeptin (*leu*) or 50 μM benzamidine hydrochloride (*BH*). (*B*) The effect of heparin and protease inhibitors (50 μg/ml leupeptin or 50 μM benzamidine hydrochloride) alone on cell proliferation. Values shown are means±SEM from three or four experiments. $*P < 0.05$ compared with serum-free (SF) controls.

Preincubation of tryptase with either leupeptin or benzamidine hydrochloride reduced [^{3}H]thymidine incorporation in fibroblasts (Fig. 1 *A*), suggesting dependency on an active catalytic site. Using the same concentrations of protease inhibitors, the activity of tryptase (18 and 36 mU/ml) towards the substrate BAPNA was inhibited by 90% in the case of leupeptin, and by 75% for benzamidine hydrochloride. Leupeptin and

Table I. Effect of Tryptase Treatment on Cell Numbers

Treatment	Cell number ($\times 10^5$)/well
Untreated controls	6.9±2.4
Tryptase (18 mU/ml)	7.7±0.9
Tryptase (36 mU/ml)	11.9±1.1*
Tryptase (72 mU/ml)	9.9±3.5
10% FCS	13.8±0.8*

Fibroblasts were treated with 10% FCS or with tryptase (in the presence of heparin) for 72 h. Cells were then detached and counted in a Neubauer hemocytometer. Means±SEM are shown from three individual experiments. *$P < 0.05$ compared to untreated controls.

benzamidine hydrochloride alone at the concentrations used did not significantly affect [^{3}H]thymidine incorporation in fibroblasts (Fig. 1 *B*). Since all the tryptase preparations used in this study contained heparin in a 1:1 ratio (wt/wt) in order to maintain enzymatic activity, the effect of heparin alone on cell proliferation was assessed. Heparin at the concentrations employed had no significant effect on [^{3}H]thymidine incorporation, although there was a tendency for inhibition at high concentrations of heparin (Fig. 1 *B*).

Time course of collagen secretion. In fibroblasts exposed to 25 mU/ml tryptase, there was a threefold increase in collagen synthesis after 24 h, and a fivefold increase after 48 h compared to untreated controls (Fig. 2). Cells not exposed to tryptase showed a low level of collagen synthesis with only a small increase over the same incubation period. The response was linear, and 48 h was the time point selected for further studies.

[^{3}H]proline incorporation. A 2.5-fold increase in collagen synthesis determined by the incorporation of [^{3}H]proline was detected in fibroblast supernatants following incubation with tryptase (Fig. 3 *A*). Leupeptin and benzamidine hydrochloride reduced the ability of tryptase to stimulate collagen synthesis. As was the case with the cell proliferation study, the response appeared to be reduced at 120 mU/ml tryptase, although no

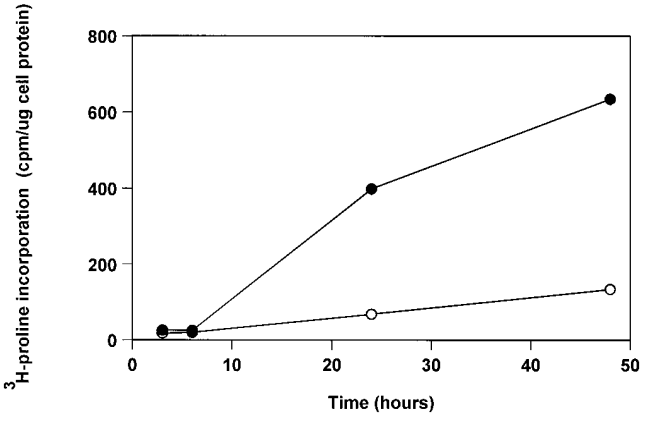

Figure 2. Time course of collagen secretion into cell culture supernatants. Fibroblasts were incubated either with medium alone (○) or with 25 mU/ml tryptase (with heparin) (●) in the presence of [^{3}H]proline. Collagen synthesis was determined from the incorporation of [^{3}H]proline from triplicate wells of two separate experiments.

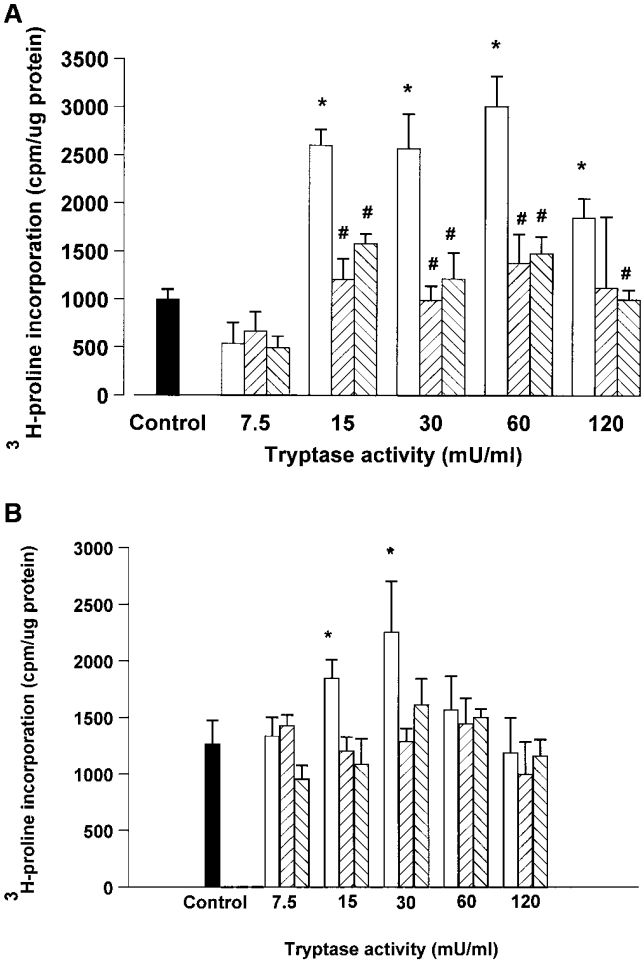

Figure 3. (*A*) Collagen synthesis (soluble) in fibroblasts incubated with medium alone (*control*) and with tryptase (with heparin) in the absence (□) or presence of 50 μg/ml leupeptin (▨) or 50 μM benzamidine hydrochloride (▧) in five separate experiments. (*B*) Collagen synthesis (cell-associated collagen) in fibroblasts treated in the same way as in (*A*) in four separate experiments. Values shown are means±SEM. *$P < 0.05$ compared with untreated controls. $^{\#}P < 0.05$ compared with non-inhibited tryptase at each concentration.

cytotoxic effects were evident, and the cell monolayer appeared intact as visualized under the microscope. Since not all the collagen synthesized is released into the supernatant, and a proportion may remain cell-associated, the cell monolayer was assayed for collagen synthesis in a similar manner, and the data was presented in Fig. 3 *B*. Very little collagen remained cell-associated, indicating that almost all the collagen synthesized by fibroblasts had been released into the supernatant. A small increase in proline incorporation was noted in lysates from cells incubated with 15 and 30 mU/ml tryptase, although the increase observed at 30 mU/ml was not reduced in the presence of benzamidine hydrochloride. It is possible that this amount may represent residual collagen associating nonspecifically with the cell surface. Leupeptin and benzamidine hydrochloride alone at the concentrations employed, and heparin at 10, 50, and 100 μg/ml, did not have any significant effects on collagen synthesis (data not shown).

Under the same conditions, 1 and 10 ng/ml TGF-β stimulated a 3.5-fold increase in collagen synthesis in fibroblasts, while

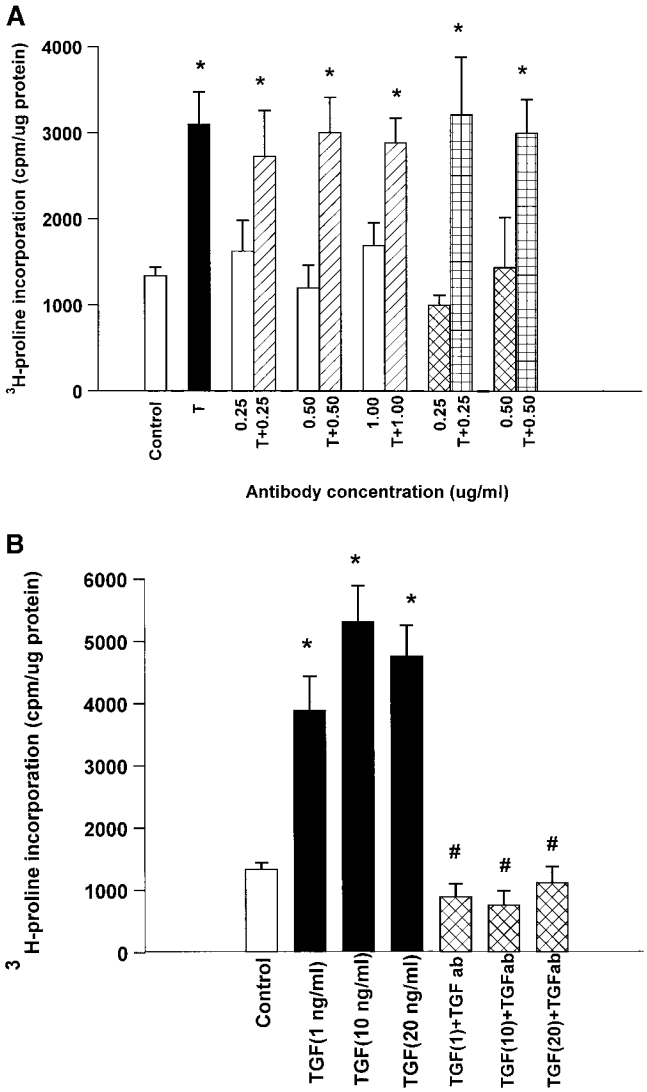

Figure 4. (*A*) Effect of a neutralizing antibody against TGF-β on tryptase-induced collagen synthesis. Fibroblasts were incubated with 25 mU/ml tryptase (*T*) with heparin (■), with 25 mU/ml tryptase (with heparin) in the presence of a neutralizing antibody (Ab) to TGF-β (▨), or with antibody (with heparin) alone at the same concentrations (□). Cells were also incubated with an irrelevant antibody (plus heparin) either alone (⊠) or in the presence of 25 mU/ml tryptase (with heparin) (⊞). (*B*) Effect of a neutralizing antibody on TGF-β-induced collagen synthesis. Cells were incubated with TGF-β alone or together with 10 μg/ml of the neutralizing antibody. Values are means±SEM from three experiments. $^*P < 0.05$ compared with untreated controls, $^\#P < 0.05$ compared with TGF-β alone.

a 5-fold increase was found at 20 ng/ml TGF-β (Fig. 4 *A*). The concentration of tryptase required to stimulate collagen synthesis by 2.5-fold was between 9.2 and 18.4 nM. When collagen assays were performed on cell supernatants after incubating cells with 25 mU/ml tryptase in the presence of a neutralizing antibody to TGF-β, no significant change in tryptase-stimulated collagen synthesis was observed, suggesting that the effect of tryptase on collagen synthesis was not mediated via increased TGF-β release (Fig. 4 *A*). There was no significant effect when cells were incubated with each of the three concentrations of TGF-β antibody in the presence of 10 μg/ml heparin. In another control experiment, the cells incubated with an irrelevant antibody in the absence or presence of 25 mU/ml tryptase showed no significant effect on collagen synthesis (Fig. 4 *B*). Collagen synthesis, however, was reduced when cells were incubated with TGF-β in the presence of the neutralizing antibody against TGF-β. Tryptase purified by an immunoaffinity procedure with antitryptase monoclonal antibody AA5 stimulated collagen synthesis to a similar extent (Fig. 5) as that found using tryptase that had been purified by DEAE- and heparin-agarose chromatography (Fig. 3 *A*).

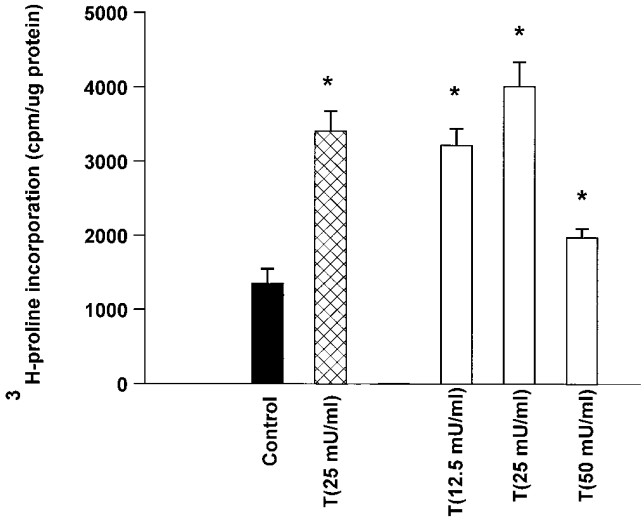

Figure 5. Effect of immunoaffinity purified tryptase on collagen synthesis. Fibroblasts were incubated with immunoaffinity purified tryptase with heparin (*open bars*), or with tryptase purified by ion-exchange and heparin agarose (*hatched bar*) chromatography. Values are means±SEM for three experiments performed in triplicate. $^*P < 0.05$ compared with controls.

Table II. Hydroxyproline Content of Supernatants from Untreated Cells or from Cells Treated With Tryptase (With Heparin) in the Presence or Absence of 50 μg/ml Leupeptin, and the Effect of TGF-β and of Heparin

Treatment	Hydroxyproline concentration
	(μmol/liter) for 10^6 cells
Untreated controls	36.9±3.3
Tryptase (T) (15 mU/ml)	52.1±5.2*
Tryptase (T) (30 mU/ml)	101.7±7.1*
Tryptase (T) (60 mU/ml)	68.2±3.6*
Tryptase (T) (120 mU/ml‡)	45.6±3.9*
T 15 + leupeptin	55.3±6.2
T 30 + leupeptin	55.0±7.3†
T 60 + leupeptin‡	58.9±4.4†
T 120 + leupeptin	49.3±6.9
TGF-β (10 ng/ml)	132.0±10.6*
Heparin (10 μg/ml)‡	45.4±5.5

All values are means±SEM of three to four experiments except where indicated (‡) for certain experiments with two experiments. $^*P < 0.05$ compared to untreated controls. $^\dagger P < 0.05$ compared with active tryptase.

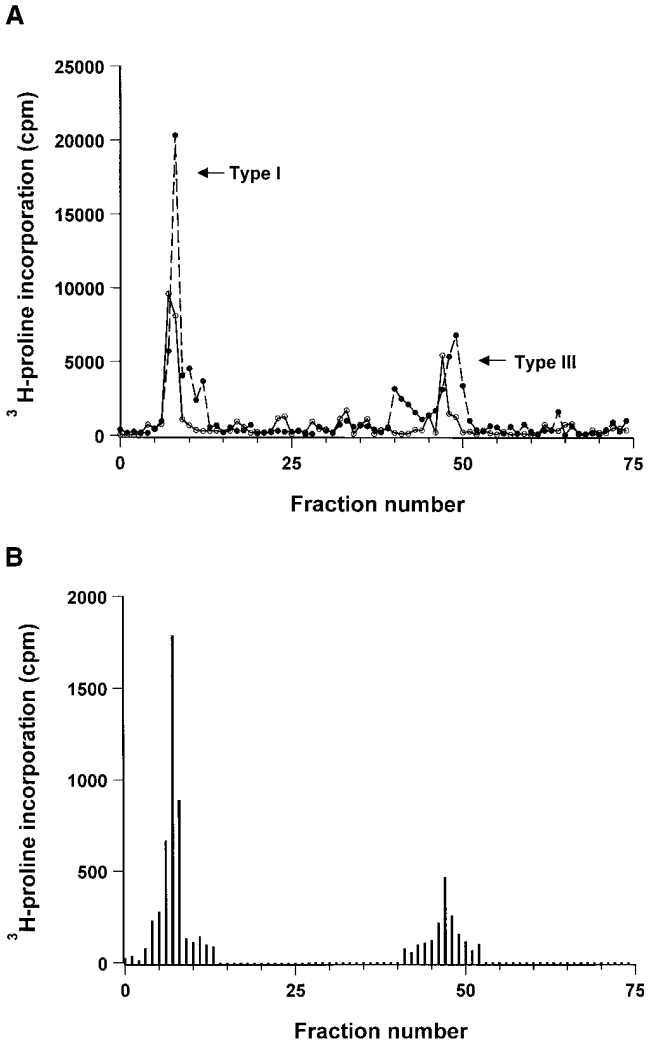

Figure 6. Separation of procollagens by DE 52 ion-exchange chromatography. (*A*) [^{3}H]proline incorporation in fractions of supernatants from control, untreated cells (○), or from cells that were treated with 25 mU/ml tryptase (containing heparin) (●) in the presence of [^{3}H]proline for 60 h. (*B*) Collagenous protein in fractions.

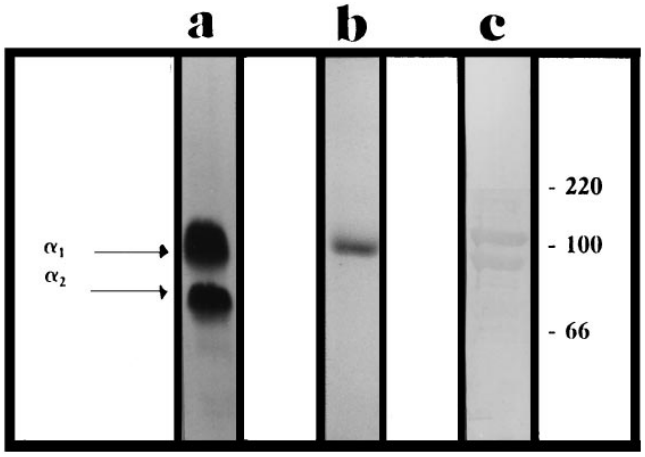

Figure 7. α-Chain composition of collagenous proteins eluted from the DE52 column. SDS-PAGE and autoradiography of pooled fractions from (*a*) peak 1 and (*b*) peak 2. (*c*) Immunoblot of peak 1 with anti-type I collagen antibody. The right margin indicates $M_r \times 10^{-3}$.

Hydroxyproline content. The hydroxyproline content of supernatants was highest following incubation of cells with 30 mU/ml tryptase, and smaller increases were observed at 60 and 120 mU/ml tryptase (Table II), consistent with the results obtained by measuring [^{3}H]proline incorporation. Exposure of cells to tryptase in the presence of leupeptin decreased the hydroxyproline content of the supernatants. A fourfold increase in hydroxyproline was measured in the supernatants of cells treated with 10 ng/ml TGF-β, while no significant effect was found with 10 μg/ml of heparin alone.

Chromatographic separation of collagens. Ion-exchange chromatography of supernatants from control or tryptase-treated cells cultured in the presence of [^{3}H]proline resolved two major peaks of radioactivity (Fig. 6 *A*), and both contained collagenous protein (Fig. 6 *B*) based on assay of the fractions for collagen as outlined above. The α-chain composition of the collagenous protein peaks was determined by SDS-PAGE and autoradiography. The protein from peak 1 consisted of pro-α1 and pro-α2 chains, which according to band intensity appeared to be present in a ratio of approximately 2:1 while peak 2 migrated as a single band (Fig. 7 *a*). On the basis of the α chain composition, peak 1 was determined to be procollagen type I, and peak 2 to be procollagen type III. Tryptase treatment of fibroblasts increased synthesis of procollagen type I by approximately twofold, while little change was observed with type III collagen (Fig. 6 *A*). The identity of type I collagen was also confirmed by immunoblotting with an antibody to type I collagen (Fig. 7 *c*).

Collagenolytic assay. The possibility was investigated that tryptase may impair collagen degradation by decreasing collagenase production, thus enhancing collagen synthesis indirectly. There was a twofold increase in total collagenolytic activity measured in supernatants of fibroblasts exposed to 25 mU/ml tryptase compared with that from untreated cells (Table III). Collagenolytic activity was abolished in the presence of the calcium chelating agent EDTA (data not shown). These findings indicate that the enhanced collagen synthesis after exposure to tryptase is not an indirect consequence of decreased collagenolytic activity.

Table III. Collagenolytic Activity in Supernatants from Untreated Cells or from Cells Treated with 25 mU/ml Tryptase in the Presence or Absence of 50 μg/ml Leupeptin

Treatment	% digestion
Untreated controls	19.2±2.9
Tryptase	28.1±2.7*
Tryptase + leupeptin‡	21.0±2.3
Bacterial collagenase‡	85.0±4.7*

Purified bacterial collagenase (10 μg/ml) was used as a positive control. Values are means±SEM of values from three experiments, except those marked ‡, which are from two experiments. *$P < 0.05$ compared with untreated controls.

Discussion

We have confirmed that tryptase can act as a growth factor for human fibroblasts, and have demonstrated for the first time that this major product of mast cell activation can stimulate the synthesis of collagen. These findings provide compelling evidence for an important association between mast cells and fibroblasts in fibrotic disease, and are in accord with observations of high levels of tryptase and other mast cell products in the biological fluids of patients with conditions that may be characterized by abnormal collagen deposition (4–6). Tryptase isolated to high purity by two different means elicited similar effects on the fibroblasts, and the identity of the stimulus as tryptase was further confirmed by the observation that the protease inhibitors leupeptin and benzamidine hydrochloride markedly reduced cell responses.

Tryptase stimulated a 2.5-fold increase in collagen synthesis in lung fibroblasts 48 h after addition to cell cultures. The mechanism did not appear to be dependent on TGF-β, as incubation of the cells with tryptase in the presence of a neutralizing antibody to TGF-β failed to alter the response to tryptase. Tryptase appears to have a differential effect on the synthesis of collagen, with its actions directed predominantly towards the synthesis of type I collagen. The synthesis of type III collagen, the other major type secreted by lung fibroblasts (45), did not appear to be affected by tryptase. The optimum concentrations of tryptase (equivalent to final concentrations of $\sim$ 1–2 μg/ml) required to elicit the increase represent concentrations of tryptase that are likely to be achieved in vivo. Tryptase concentrations as high as 13 ng/ml have been measured in BAL fluid from asthmatics (46), and since levels of tryptase may be diluted more than 100-fold in BAL fluid, these concentrations should be sufficient to trigger the responses observed.

Collagen types I, II, III, V, and XI, representing the major interstitial collagens, are composed of one to three α-chains, each of 95–100 kD molecular weight, associated to form a single triple helix structure (47). Type I collagen has an asymmetric heterotrimeric configuration composed of α1 and α2 chains in a stoichiometric proportion of 2:1, giving rise to the chain composition ($\alpha_1[I]_2$, $\alpha_2[I]$) (47) while type III collagen, a homotrimer of three α1 chains has the designation $[\alpha_1(I)]_3$. The α1 and α2 chains of type I collagen migrate at slightly different rates under reducing conditions (43). The collagen stimulated by tryptase treatment was identified as type I on the basis of the α chain composition, and by immunoblotting with a specific antibody to type I collagen. The collagens form an integral part of the extracellular matrix, and the amount of collagen deposited in the lung is tightly regulated to ensure a strict balance between biosynthesis and degradation. An inappropriate control of this balance can lead to enhanced collagen deposition resulting in fibrosis. It is now recognized that any alterations in either the amounts of collagen as reported in acute respiratory distress syndrome (48), cryptogenic fibrosing alveolitis (49), sarcoidosis (50), or indeed the type of collagens, may contribute to cellular abnormalities in the lung. The normal human lung is composed of 65% type I and 30% type III collagen. A specific increase in the amount of type I collagen, with a concomitant decrease in type III, has been reported in idiopathic chronic pulmonary fibrosis (51), and in fibrosis associated with atherosclerosis (52) and liver cirrhosis (53). In general, compliant tissues have a low ratio of type I to type III collagen, while less compliant tissues such as that found in pulmonary fibrosis, have a higher ratio. There is evidence of mast cell activation near areas of increased fibroblast activity, suggesting mast cell involvement in the fibrotic process. Stimulation of type I collagen synthesis by tryptase would be in keeping with this observation. Cells derived from animals which have been treated with bleomycin to induce experimental fibrosis show a similar increase in the synthesis of type I collagen (40). This result is in agreement with changes observed in fibroblasts treated with bleomycin in vitro (38, 40).

The preferential stimulation of type I collagen synthesis by tryptase also suggests a role for tryptase in chronic fibrosis resulting from ongoing mast cell activation since type I collagen has been reported to be found preferentially in established fibrotic lesions, while type III collagen is characteristic of early fibrosis (45). Collagen synthesis stimulated by tryptase cannot be attributed to decreased collagenase production by the cells, as there was a twofold increase in collagenase activity in the culture medium after tryptase exposure. Thus, the increase in collagen detected after tryptase treatment must reflect an increase in the net biosynthetic rate of type I collagen. Release of collagenase activity concomitant with the deposition of new collagen could be an important process in matrix remodeling. Increased collagenolytic activity has been reported in BAL fluid from patients with diffuse interstitial fibrosis (DIF) (54) where an elevation in tryptase levels has also been reported (4, 55).

An increase in the fibroblast cell population as well as an increase in collagen synthesis are key features of fibrotic disease. Our observation that tryptase can stimulate both an increase in cell numbers and a fivefold increase in DNA synthesis 32 h after addition to cultures of the MRC-5 fibroblast line is in keeping with reports from other investigators. Dog tryptase has been found to stimulate a 2- to 3-fold increase in bromodeoxyuridine uptake in cultured dog tracheal smooth muscle cells (28), while an increase of 2.5-fold in cell numbers has been reported with human lung tryptase added to human lung fibroblasts (27). The ability of tryptase to stimulate fibroblast proliferation and collagen synthesis appears to be mediated via the catalytic site. This suggests that (*a*) a proteolytic event is required to initiate the intracellular signalling pathway, or (*b*) tryptase could interact with a putative receptor at a point close to the catalytic site. The actions of another serine protease (thrombin) on cells appears to be mediated via cleavage of a tethered ligand in a specific receptor (56). A specific receptor for tryptase remains to be identified, but the action of tryptase on fibroblasts does not appear to be mediated via the thrombin receptor since it has been shown that tryptase does not affect stimulation of DNA synthesis by the synthetic thrombin receptor peptide Ser-Phe-Phe-Leu-Arg-Asn-Pro (SFFLRNP) (57). The identification of the structurally related proteinase-activated receptor-2 (PAR-2), which can be activated by trypsin (58), has reinforced the concept that proteases can bind to specific receptors and play pivotal roles as mediators of cellular events.

Tryptase has been reported to have both direct and indirect effects on connective tissue catabolism. This enzyme can degrade intact type VI collagen microfibrils (59), inactivate fibrinogen (23), and can activate latent collagenase by a process dependent on the activation of prostromelysin (24). Our data provides evidence for a new role for mast cell tryptase in fibrogenesis. Tryptase released during mast cell degranulation could provide a stimulus both for fibroblast proliferation and

collagen synthesis, resulting in increased collagen deposition in fibrotic lesions. The overall increase in collagen production stimulated by tryptase may lead to mechanical impairment in the fibrotic lung, while the selective increase in type I collagen could contribute to the overall rigidity. It has recently been reported that inhibitors of tryptase may be efficacious in controlling acute allergic reactions in sheep airways (60). The ability of tryptase to participate in processes of matrix remodelling suggests that this new class of drugs may prove beneficial in chronic conditions associated with mast cell activation.

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