

Effect of mast cell–derived mediators and mast cell–related neutral proteases on human dermal fibroblast proliferation and type I collagen production

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Background: Possible involvement of mast cells in various fibrotic conditions has been suggested, but the relative contribution of each mast cell mediator and neutral protease to fibroproliferative activity remains to be elucidated.

Objective: We investigated the effect of mast cell–derived mediators and mast cell–related neutral proteases on type I collagen production and proliferation by human dermal fibroblasts.

Methods: Mast cell–derived mediators or neutral proteases were added to cultured fibroblasts from normal dermis, and cell proliferation and type I collagen synthesis were assayed.

Results: Fibroblast proliferation was increased by 2.8×10^{-9} mol/L prostaglandin D₂ and 10 µg/mL carboxypeptidase A, but not by leukotriene D₄ or cathepsin G at the concentrations studied. Proliferation was increased by tryptase in a concentration-dependent manner, and a significant increase was observed at concentrations of 1 and 10 µg/mL. Production of type I collagen by fibroblasts was increased in the presence of 2.0×10^{-9} mol/L leukotriene D₄ and 10 µg/mL tryptase.

Conclusion: Mast cell–derived mediators prostaglandin D₂ and leukotriene D₄ and mast cell–related neutral proteases carboxypeptidase A and tryptase increase the proliferation and type I collagen production of human dermal fibroblasts in various manners. (*J Allergy Clin Immunol* 2000;106:S78–84.)

Key words: Mast cell, mediator, neutral protease, dermal fibroblast, proliferation, type I collagen

Mast cells frequently accumulate at sites of fibrosis, such as the involved skin of patients with scleroderma¹ and in the pulmonary tissues of patients with fibrotic lung disorders.² In these conditions, mast cells undergo changes of numbers and morphology that are correlated with the course of fibrosis. These observations provide circumstantial evidence that mast cells may have a role in causing fibroblasts to express a fibrotic phenotype. However, in vitro studies of the direct effect of mast cell–derived mediators on fibroblasts have produced con-

Abbreviation used

DMEM: Dulbecco's modified Eagle's medium

flicting results. Concerning histamine, a major mast cell product, Boucek and Noble³ reported that exposure of mouse 3T6 fibroblasts to histamine for 3 days resulted in an increase of cell numbers, and Jordana et al⁴ reported that histamine enhanced human lung fibroblast proliferation in a dose-dependent manner. There have also been reports that histamine stimulates the proliferation of human skin fibroblasts.^{5,6} On the other hand, Johnson and Johnson⁷ reported the inhibition of human skin fibroblast proliferation by histamine.

Mast cell neutral proteases are enzymes that are stored in mast cell secretory granules and released by activation. Our knowledge of human mast cell proteases has recently increased tremendously, and it is not surprising that current concepts of their potential biologic role have greatly expanded. The ability of tryptase,⁸ a serine protease selectively concentrated in the secretory granules of human mast cells,⁹ to stimulate fibroblast proliferation is consistent with a major role of mast cells in fibrosis. Despite mounting evidence that mast cells can contribute to fibrosis by activating fibroblasts^{10–12} and stimulating collagen mRNA synthesis,^{13,14} the influence of mast cell–derived mediators and neutral proteases on collagen production by fibroblasts has not yet been fully determined. Moreover, the relative amounts of each mediator or neutral protease released from mast cells after activation and their contributions to the fibroproliferative effect of mast cells have not been explored.

In the present study, we examined whether a variety of mast cell–derived mediators and mast cell–related neutral proteases could influence human dermal fibroblast proliferation and type I collagen production in the absence of comitogenic serum components.

METHODS

Reagents

Histamine, porcine heparin, bovine pancreas carboxypeptidase A, and human leukocyte cathepsin G were purchased from Sigma Chemical Co (St Louis, Mo). Prostaglandin D₂ and leukotriene D₄ were provided by Ono Pharmaceutical Co (Osaka, Japan). Purified

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human lung trypsinase was obtained from Vital Products (St Louis, Mo). The purity of the trypsinase was determined by SDS-PAGE on 10% reducing gel, according to the method of Laemmli.¹⁵ Proteins were silver-stained with a kit from Bio-Rad Laboratories (Richmond, Calif). The activity of trypsinase was measured with *n*-benzyl-DL-arginine-pNA activity (milliunits), where 1 U of enzyme activity was defined as the amount degrading 1 μ mol of substrate per minute at 25°C. The specific activity of trypsinase was 5.27 mU/ μ g.

Culture of human dermal fibroblasts

After informed consent was given, normal skin tissues were obtained from surgical specimens of the forearm. The tissues from each subject were cut into small pieces, and the adherent outgrowth cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nihon Seiyaku, Tokyo, Japan) containing 10% FCS (Cytosystems, Castle Hill, Australia). Incubation was done at 37°C in a 5% carbon dioxide-humidified incubator; the culture medium was changed every 3 days. The cells were passaged with trypsin and were maintained in a subconfluent state, being expanded for 3 passages before the experiments. Nine different normal skin surgical specimens were used in the this study, which complied with the requirements of the Review Board of Gunma University School of Medicine.

Fibroblast proliferation

Subconfluent monolayers of fibroblasts were trypsinized, seeded into 96-well tissue culture plates at a density of 2.5×10^4 cells/mL in DMEM containing 10% heat-inactivated FCS, and cultured at 37°C in a 5% carbon dioxide-humidified incubator overnight to allow adherence. The medium was then replaced with FCS-free DMEM. After 24 hours of serum deprivation, a mast cell-derived mediator or neutral protease was added. Unless otherwise indicated, porcine heparin was added to trypsinase at a weight ratio of 1:1 to stabilize enzymatic activity.¹⁶ As a control for trypsinase, heparin at concentrations from 0.01 to 10 μ g/mL was used in the proliferation assay. Unless otherwise indicated, serum-free medium was used as the control for mast cell mediators and the rest of the proteases. After 24 hours of culture at 37°C in the incubator, the medium was aspirated, and the culture plates were washed twice with PBS.

The proliferative response of fibroblasts was measured with [³H]thymidine incorporation. In brief, fibroblasts were incubated with [³H]thymidine (specific activity of 230 Bq/mmol; American Radiolabeled Chemicals, St Louis, Mo) at a final concentration of 10 μ Ci/mL. After 6 hours of incubation at 37°C, the cells were washed 3 times with cold PBS and 4 times with cold 0.1 N perchloric acid solution. After addition of 0.2 mL of 0.1 N NaOH/0.1% SDS per well, the radioactivity was measured with a liquid scintillation counter (Beckman LS7000; Beckman Instruments, Fullerton, Calif) after cells were dissolved in 4 mL of Aquasol-2 (New England Nuclear, Boston, Mass). For each experiment, the counts per minute were averaged from 3 determinations.

Measurement of type I collagen synthesis

Procollagen type I C-peptide was measured as a marker of type I collagen synthesis.^{17,18} Therefore type I collagen production by fibroblasts was assessed by measuring the procollagen type I C-peptide concentration by ELISA with the use of a procollagen type I C-peptide kit (Takara Shuzo, Kyoto, Japan).¹⁹ In brief, fibroblasts were seeded into 96-well tissue culture plates at a density of 2.5×10^4 cells/mL and cultured at 37°C in a 5% carbon dioxide-humidified incubator in DMEM containing 10% heat-inactivated FCS until confluence. The medium was then replaced with FCS-free DMEM and a mast cell-derived mediator or neutral protease was added after 24 hours of serum deprivation. The control for trypsinase was heparin at concentrations from 0.01 to 10 μ g/mL, although serum-

free medium was used for the other mediators. After culture for 24 hours at 37°C, the procollagen type I C-peptide concentration in the culture supernatant was measured.

Statistical analysis

Data were expressed as the mean $\pm$ SD. Statistical analysis was performed with the Statview software program (version 4.0; Abacus Concepts, Berkeley, Calif). Group data were subjected to analysis of variance to determine the overall impact of treatments within an experiment, with additional post hoc testing with Fisher's protected least significant difference test to determine the statistical significance of individual treatments on the parameter in question. Differences were only defined as significant if both analysis of variance and Fisher's test yielded a probability value of .05 or less.

RESULTS

General description

Preliminary studies showed that when human dermal fibroblast proliferation was assayed in the absence or presence of mast cell-derived mediators or mast cell-related neutral proteases, the peak of [³H]thymidine incorporation was at 24 to 48 hours, so fibroblast proliferation was measured 24 hours after the addition of each mediator or neutral protease. In the presence of each mediator or neutral protease (except cathepsin G) at the concentrations used in this study, the fibroblast monolayer remained intact when examined by phase contrast microscopy, and cell viability was more than 90% at the end of [³H]thymidine labeling. In contrast, the fibroblast monolayer showed damage and defects in the presence of cathepsin G.

Effect of mast cell-derived mediators on fibroblast proliferation and type I collagen production

At concentrations from 10^{-7} to 10^{-4} mol/L, histamine did not affect [³H]thymidine incorporations by fibroblasts and the procollagen type I C-peptide level in fibroblast culture supernatant (data not shown).

At a concentration of 2.8×10^{-9} mol/L, prostaglandin D₂ significantly enhanced [³H]thymidine incorporation by fibroblasts compared with the control ($P < .05$). However, prostaglandin D₂ at concentrations from 2.8×10^{-11} to 2.8×10^{-8} mol/L did not affect the procollagen type I C-peptide concentration in the culture supernatant (Fig 1).

At concentrations from 2.0×10^{-11} to 2.0×10^{-8} mol/L, leukotriene D₄ did not affect [³H]thymidine incorporation by fibroblasts. On the other hand, a leukotriene D₄ concentration of 2.0×10^{-9} mol/L significantly increased procollagen type I C-peptide in the culture supernatant compared with the control ($P < .05$; Fig 2).

Effect of mast cell-derived neutral proteases on fibroblast proliferation and type I collagen production

At a concentration of 10 μ g/mL, carboxypeptidase A significantly increased [³H]thymidine incorporation by

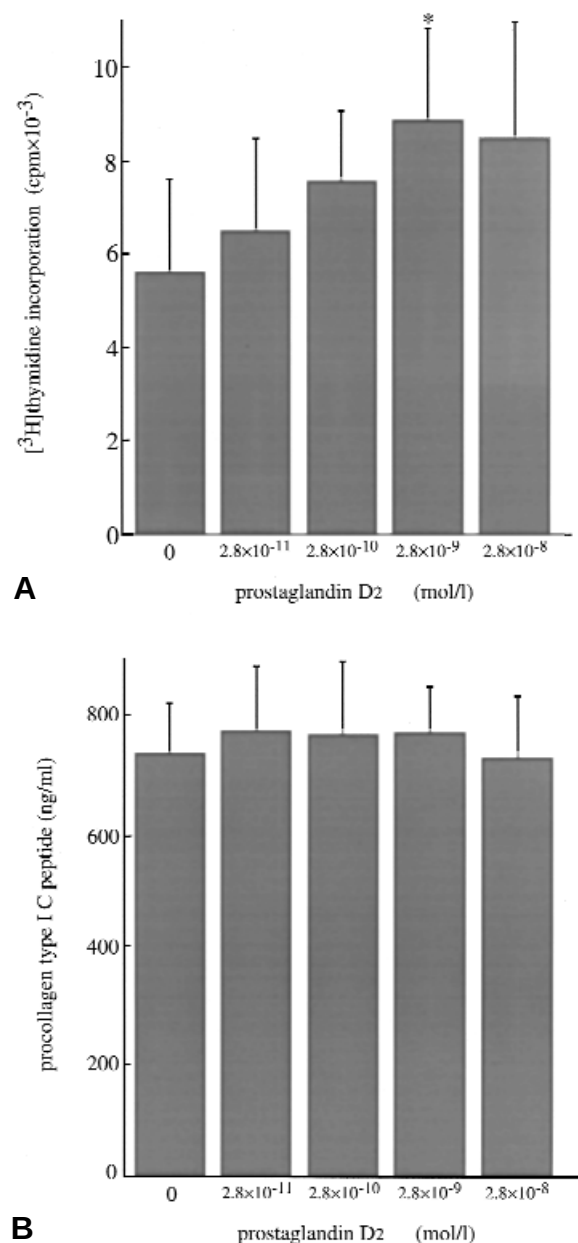


FIG 1 Proliferative response of human dermal fibroblasts and type I collagen production in the presence of prostaglandin D₂. **A**, Subconfluent human dermal fibroblasts were incubated for 24 hours with serum-free medium alone (control) or prostaglandin D₂, and the proliferative response was measured with [³H]thymidine incorporation. Each column represents the mean $\pm$ SD from 5 separate experiments. * $P < .05$ versus the serum-free control. **B**, Confluent human dermal fibroblasts were incubated for 24 hours with serum-free medium alone (control) or prostaglandin D₂, and type I collagen production was assessed by the measurement of the procollagen type I C-peptide concentration in the supernatant. Each column represents the mean $\pm$ SD from 5 separate experiments.

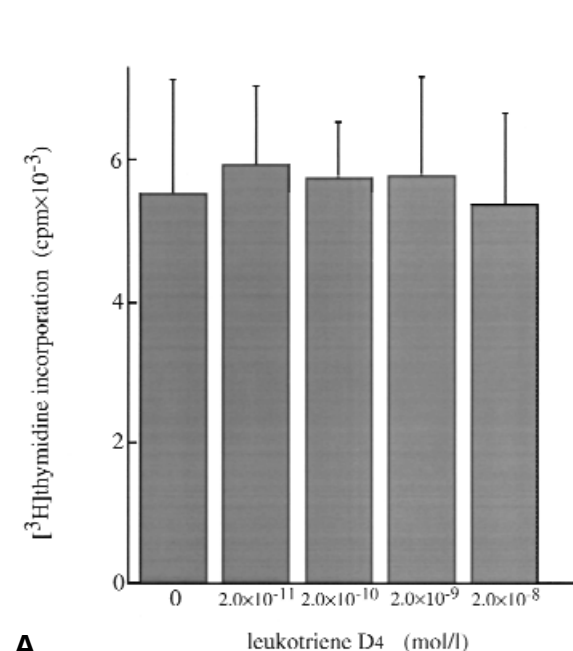
fibroblasts compared with the control ($P < .05$). However, carboxypeptidase A concentrations from 0.01 to 10 ng/mL did not affect the procollagen type I C-peptide level in the culture supernatant (Fig 3).

At concentrations from 0.01 to 1 ng/mL, cathepsin G did not affect [³H]thymidine incorporation into fibroblasts. However, these cathepsin G concentrations significantly decreased procollagen type I C-peptide levels in the culture supernatant compared with the control ($P < .01$; Fig 4). After 24 hours of incubation with cathepsin G at the concentrations studied, the fibroblast monolayer was damaged and showed defects when examined by phase contrast microscopy, which suggested that the decrease of type I C-peptide might be due to a nonspecific effect of cathepsin G.

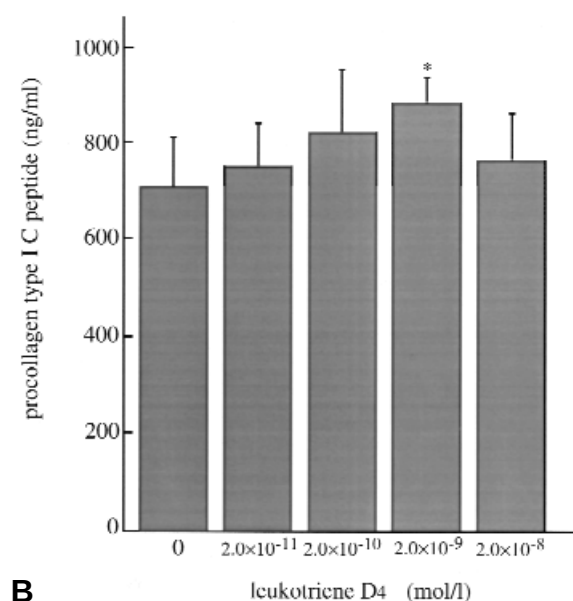
Fibroblast proliferation was significantly increased by tryptase at concentrations of 1 and 10 μ g/mL, and the effect was concentration dependent ($P < .05$ at 1 μ g/mL, and $P < .01$ at 10 μ g/mL; Fig 5). In all of the experiments on tryptase, heparin was added to the enzyme at a weight ratio of 1:1, because tryptase rapidly lost its activity at 37°C in the absence of heparin. Therefore the effect of heparin on fibroblast proliferation was also investigated. It was found that heparin alone at concentrations from 0.01 to 10 μ g/mL did not significantly influence fibroblast proliferation (data not shown). After 24 hours of exposure to tryptase at the concentrations used in this study, the fibroblast monolayer remained intact when examined by phase contrast microscopy and was indistinguishable from cells that had not been exposed to tryptase, which suggests that the increase in cell growth was not a nonspecific effect of tryptase resulting from cell detachment and subsequent loss of contact inhibition. A significant increase of the procollagen type I C-peptide concentration in the culture supernatant was also detected after incubation with 10 μ g/mL tryptase ($P < .01$; Fig 5). In contrast, heparin alone at concentrations from 0.01 to 10 μ g/mL did not increase procollagen type I C-peptide in the culture supernatant (data not shown).

DISCUSSION

Among mast cell-derived mediators, histamine has been studied most extensively with respect to its role in wound healing.²⁰⁻²² However, the role of histamine in regulating the growth and metabolism of interstitial cells, particularly fibroblasts, has not been extensively investigated. An early study by Boucek and Noble³ indicated that histamine could increase mouse 3T6 fibroblast proliferation. They also noted that histamine inhibited the proliferation of a primary culture of mouse embryo skin, which suggests that the effect of histamine on the growth of fibroblasts might depend on various factors such as cell type and culture conditions. For example, histamine was reported to stimulate the proliferation of human lung fibroblasts⁴ and skin fibroblasts in a dose-dependent manner,^{5,6} but Johnson and Johnson⁷ found that histamine inhibited human skin fibroblast proliferation. Thus some authors⁴⁻⁶ have reported an increase and others⁷ a decrease in fibroblast proliferation. Histamine was also shown to enhance collagen synthesis by guinea pig skin fibroblasts,²³ and a recent study by Kikuchi et al²⁴

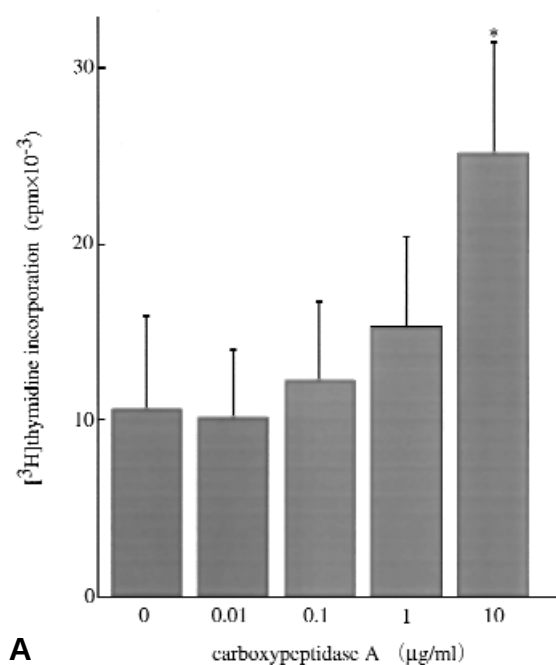


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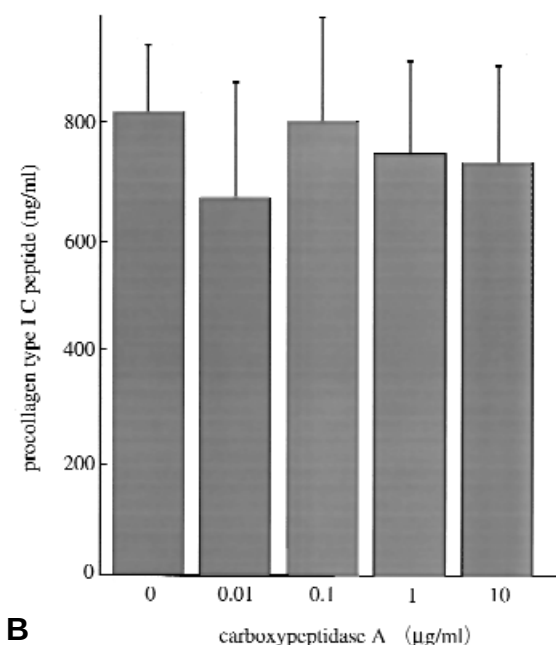


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FIG 2. Proliferative response of human dermal fibroblasts and type I collagen production in the presence of leukotriene D₄. **A**, Subconfluent human dermal fibroblasts were incubated for 24 hours with serum-free medium alone (control) or leukotriene D₄, and the proliferative response was measured with [³H]thymidine incorporation. Each column represents the mean ± SD from 5 separate experiments. **B**, Confluent human dermal fibroblasts were incubated for 24 hours with serum-free medium alone (control) or leukotriene D₄, and type I collagen production was assessed by the measurement of the procollagen type I C-peptide concentration in the supernatant. Each column represents the mean ± SD from five separate experiments. **P* < .05 versus the serum-free control.



A



B

FIG 3. Proliferative response of human dermal fibroblasts and type I collagen production in the presence of carboxypeptidase A. **A**, Subconfluent human dermal fibroblasts were incubated for 24 hours with serum-free medium alone (control) or carboxypeptidase A, and the proliferative response was measured with [³H]thymidine incorporation. Each column represents the mean ± SD from 5 separate experiments. **P* < .05 versus the serum-free control. **B**, Confluent human dermal fibroblasts were incubated for 24 hours with serum-free medium alone (control) or carboxypeptidase A, and type I collagen production was assessed by the measurement of the procollagen type I C-peptide concentration in the supernatant. Each column represents the mean ± SD from 5 separate experiments.

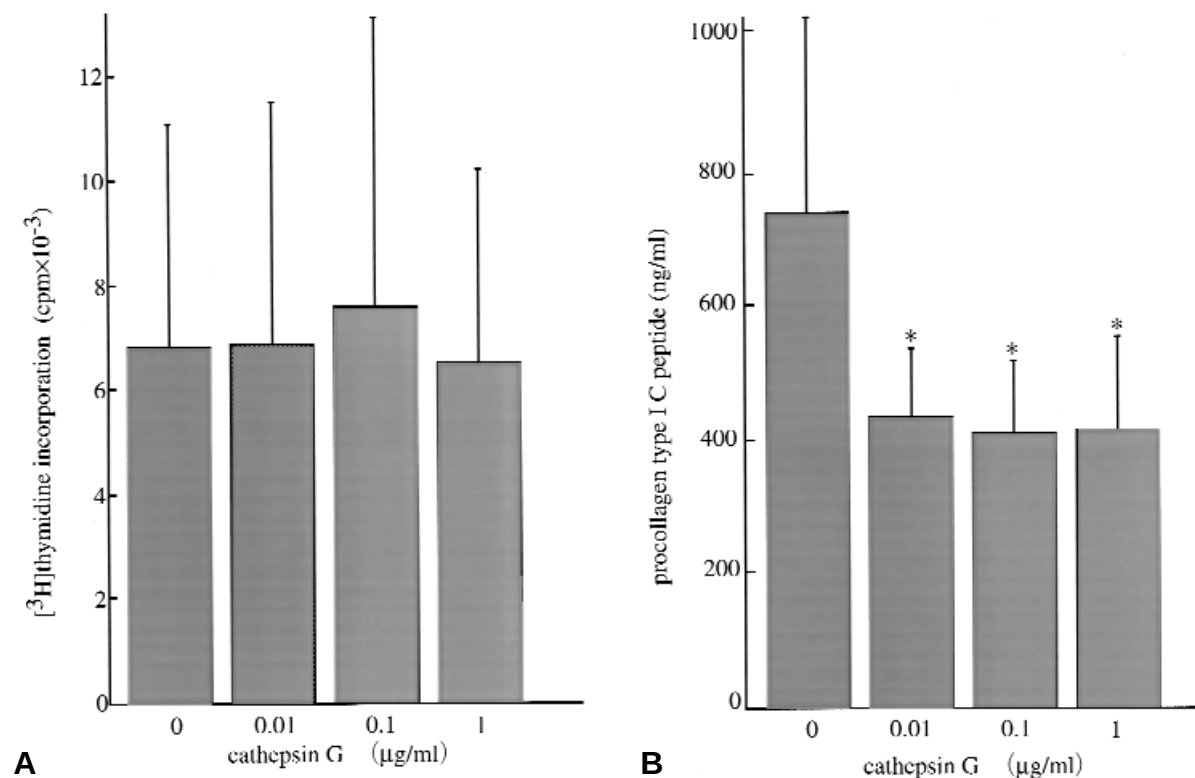


FIG 4. Proliferative response of human dermal fibroblasts and type I collagen production in the presence of cathepsin G. **A**, Subconfluent human dermal fibroblasts were incubated for 24 hours with serum-free medium alone (control) or cathepsin G, and the proliferative response was measured with [³H]thymidine incorporation. Each column represents the mean $\pm$ SD from 6 separate experiments. **B**, Confluent human dermal fibroblasts were incubated for 24 hours with serum-free medium alone (control) or cathepsin G, and type I collagen production was assessed by the measurement of the procollagen type I C-peptide concentration in the supernatant. Each column represents the mean $\pm$ SD from 5 separate experiments. * $P < .01$ versus the serum-free control.

revealed that histamine increased procollagen type I carboxyterminal propeptide production by keloid fibroblasts, but not normal human skin fibroblasts.

Increased proliferation and collagen synthesis by fibroblasts are key features of fibrotic diseases. The aim of the present study was to determine whether mast cell-derived mediators and mast cell-related neutral proteases could directly affect human dermal fibroblasts with respect to proliferation and type I collagen synthesis in the absence of comitogenic serum components. First, we assessed whether mast cell-derived mediators could affect fibroblast proliferation. The most suitable experimental system is one that uses subconfluent and hence still exponentially growing fibroblasts. Therefore each mast cell-derived mediator was added to subconfluent cultures, and fibroblast proliferation was examined with [³H]thymidine incorporation.

Prostaglandin D₂ is a major cyclooxygenase product of mast cells.²⁵ At a concentration of 2.8×10^{-9} mol/L, prostaglandin D₂ stimulated a 1.6-fold increase of [³H]thymidine uptake but did not alter the procollagen type I C-peptide concentration in the culture supernatant. Interestingly, a prostaglandin D₂ concentration of 10^{-8}

mol/L, similar to that which enhanced fibroblast proliferation in the present study, has been shown to stimulate thymidine incorporation by growth plate chondrocytes.²⁶

Human mast cells generate leukotriene C₄ in response to stimulation,²⁷ and leukotriene C₄ is then converted to leukotriene D₄ and leukotriene E₄. In the present study, leukotriene D₄ did not affect [³H]thymidine incorporation into fibroblasts. On the other hand, a leukotriene D₄ concentration of 2.0×10^{-9} mol/L stimulated a 1.3-fold increase of the procollagen type I C-peptide concentration in the culture supernatant. Phan et al²⁸ reported that leukotriene C₄ and D₄ concentrations from 10^{-11} to 10^{-8} mol/L stimulated fibroblast collagen synthesis in a concentration-dependent manner. Overall, the effect of mast cell-derived mediators on fibroblast proliferative responses seems to be complex.

The present study also assessed whether mast cell-related neutral proteases could affect fibroblast proliferation. A carboxypeptidase has been localized to the secretory granules of human mast cells from skin and lung.²⁹ Human mast cell carboxypeptidase is a novel member of the family of zinc-containing carboxypeptidases and is functionally similar to bovine carboxypepti-

dase A.³⁰ In the present study, bovine pancreas carboxypeptidase A was used as a substitute for human mast cell carboxypeptidase, and its effect on fibroblast proliferation was investigated. A carboxypeptidase A concentration of 10 $\mu\text{g/mL}$ stimulated a 2.4-fold increase in [^3H]thymidine uptake, but concentrations from 0.01 to 10 $\mu\text{g/mL}$ did not affect the type I collagen level in the culture supernatant. Mast cells from adult foreskin contain 16 pg of carboxypeptidase per cell,³⁰ so the concentration range of carboxypeptidase A used in the present study could conceivably be achieved in vivo. Although some potential substrates for this enzyme have been investigated, its biologic role still remains unknown. This is the first demonstration that carboxypeptidase A stimulates human dermal fibroblast proliferation.

Cathepsin G is a neutral protease present in neutrophils and monocytes, and a cathepsin G-like protease has been demonstrated in human mast cells by immunocytochemical studies, although a protein with a slightly higher molecular weight than the predominant form of neutrophil cathepsin G has been identified in extracts of human skin mast cells by immunoblotting.³¹ However, it has not yet been purified from human mast cells, so cathepsin G from human leukocytes was used for this study. At concentrations from 0.01 to 1 ng/mL, cathepsin G decreased procollagen type I C-peptide in the fibroblast culture supernatant without affecting [^3H]thymidine incorporation.

The most abundant neutral protease in human mast cells is tryptase. This is a tetrameric serine protease that is almost exclusively found in mast cells, where it exists within the secretory granules as a complex with heparin proteoglycan that may serve to stabilize the enzyme and to regulate its actions on release.^{16,32} In addition to its enzymatic activity, tryptase can interact with a number of cells and regulate cellular events. Ruoss et al⁸ first demonstrated a potent mitogenic effect of tryptase on lung fibroblasts. Tryptase has also been shown to stimulate the proliferation of other cells, such as smooth muscle cells³³ and epithelial cells.³⁴ In the present study, we used tryptase concentrations that could conceivably be achieved in vivo, assuming that there are approximately 10,000 mast cells/mm³ and that each cell contains approximately 35 pg of tryptase.^{35,36} Fibroblast proliferation was significantly increased in the presence of tryptase at concentrations of 1 and 10 $\mu\text{g/mL}$ compared with that in the absence of tryptase, and a tryptase concentration of 10 $\mu\text{g/mL}$ stimulated an 8.1-fold increase of [^3H]thymidine uptake. In the proliferation assay, porcine heparin was added to tryptase at a weight ratio 1:1 to stabilize enzymatic activity.¹⁶ Because heparin has been shown to induce proliferation when added to the cultures of pulmonary fibroblasts,³⁷ the effect of heparin at concentrations from 0.01 to 10 $\mu\text{g/mL}$ was also investigated. However, no significant effect of heparin on [^3H]thymidine incorporation by fibroblasts was observed, which suggests that the increased proliferation is likely to be tryptase-specific and not related to heparin. When type I collagen synthesis by confluent human dermal fibro-

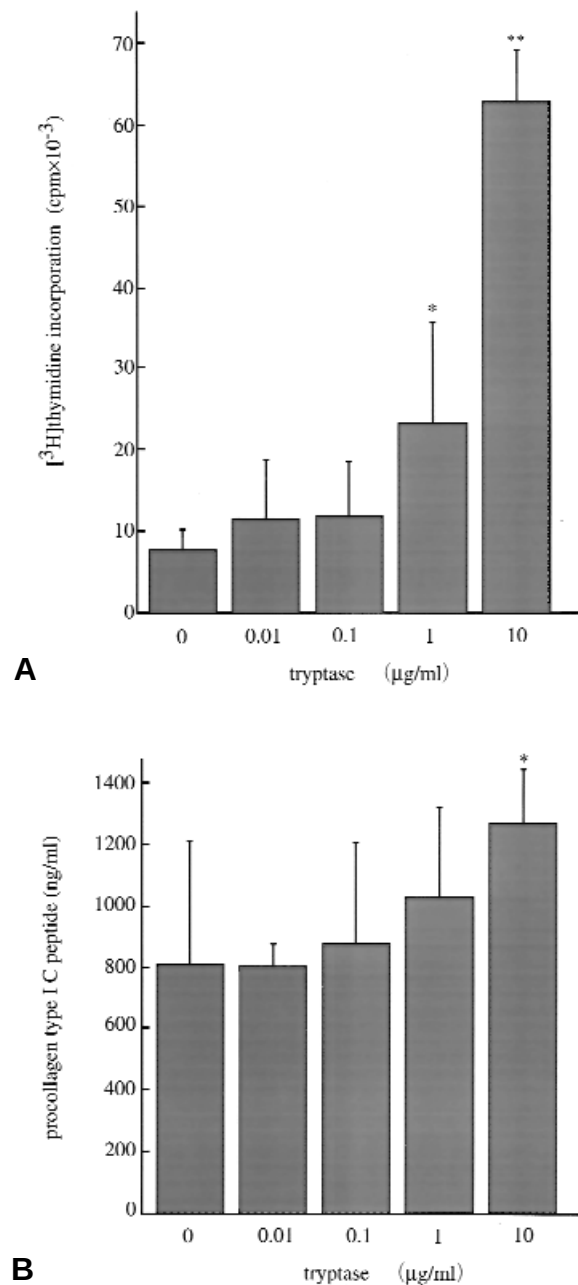


FIG 5. Proliferative response of human dermal fibroblasts and type I collagen production in the presence of tryptase. **A**, Subconfluent human dermal fibroblasts were incubated for 24 hours with serum-free medium alone (control) or purified human lung tryptase plus heparin, and the proliferative response was measured with [^3H]thymidine incorporation. Each column represents the mean $\pm$ SD from 4 separate experiments. * $P < .05$ and ** $P < .01$ versus the serum-free control. **B**, Confluent human dermal fibroblasts were incubated for 24 hours with serum-free medium alone (control) or purified human lung tryptase plus heparin, and type I collagen production was assessed by the measurement of the procollagen type I C-peptide concentration in the supernatant. Each column represents the mean $\pm$ SD from 4 separate experiments. ** $P < .01$ versus the serum-free control.

blasts was determined in the presence of tryptase, the procollagen type I C-peptide concentration in the culture supernatant was increased in a concentration-dependent manner, and tryptase stimulated a 1.6-fold increase at a concentration of 10 µg/mL. As was the case in the proliferation assay, heparin concentrations from 0.01 to 10 µg/mL had no significant effect on the procollagen type I C-peptide concentration. Thus the present study confirmed that tryptase can act as a growth factor for human dermal fibroblasts and demonstrated that this major product of human mast cells can stimulate type I collagen production by human dermal fibroblasts.

In conclusion, our observation that mast cell-derived mediators and mast cell-related neutral proteases can stimulate both fibroblast proliferation and type I collagen synthesis by human dermal fibroblasts is in agreement with other reports and suggests that the *in vivo* effects of mast cells on fibrotic conditions may be complex. It also appears likely that tryptase and carboxypeptidase are 2 potent fibrogenic factors that are capable of inducing fibroblast proliferation and type I collagen synthesis.

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