Tryptase Increases Proliferative Activity of Human Conjunctival Fibroblasts through Protease-Activated Receptor-2

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Purpose. Tryptase that is released by mast cell degranulation has recently been thought to play a key role in wound healing in allergic bronchitis. Conjunctival fibroblasts secrete mediators and extracellular matrices that could exacerbate inflammation and papillary formation in allergic conjunctivitis. This study was conducted to investigate the effect of tryptase on the proliferation of conjunctival fibroblasts and studied whether this effect was mediated by protease-activated receptor (PAR)-2.

Methods. Conjunctival fibroblasts were cultured with or without tryptase (0.1 ng/mL to 1.0 μg/mL), and the proliferation rate was assessed after 48 hours. The effects of tryptase inhibitors (leupeptin, benzamidine) and a PAR-2 agonist (SLIGKV) were examined. The existence of PAR-2 mRNA and protein in conjunctival fibroblasts was examined by RT-PCR and Western blot analysis, respectively. The existence of PAR-2 in cultured conjunctival fibroblasts and conjunctival papillae from patients with vernal keratoconjunctivitis, as well as conjunctival tissue from normal subjects was examined by immunohistochemistry.

Results. Conjunctival fibroblast proliferation was upregulated by tryptase in a dose-dependent manner ($P < 0.001$). Leupeptin and benzamidine inhibited tryptase-induced fibroblast proliferation ($P < 0.05$), and SLIGKV mimicked tryptase's effect. PAR-2 mRNA and protein were detected in cultured conjunctival fibroblasts using RT-PCR and Western blot analysis. PAR-2 immunoreactivity in both the cultured conjunctival fibroblasts and in stromal cells in excised conjunctival tissues was observed.

Conclusions. Tryptase increased conjunctival fibroblast proliferation and this response appeared to be mediated by PAR-2. Mast cells are the most likely source of tryptase in the conjunctiva and may play an important role in chronic exacerbations with conjunctival papillary formation in allergic conjunctivitis. (*Invest Ophthalmol Vis Sci.* 2005;46:4622–4626) DOI:10.1167/iovs.05-0388

Giant papillary formation is a major characteristic of severe chronic allergic conjunctivitis, like atopic keratoconjunctivitis (AKC) or vernal keratoconjunctivitis (VKC). Histopathologically, excised papillae consist of the conjunctival epithelium containing goblet cells, a cluster of inflammatory leukocytes (lymphocytes, plasma cells, eosinophils, mast cells, and neutrophils), and newly formed vessels among excessive fibrosis. Under these conditions, conjunctival fibroblasts are suspected to increase in number and to be activated, secreting soluble inflammatory mediators and extracellular matrix molecules.

An increased number of mast cells[1–5] and elevated tryptase concentrations[6–10] have been reported to exist in the tears of patients with allergic conjunctivitis, including AKC and VKC. These facts may indicate that mast-cell–derived tryptase contributes to not only the IgE-mediated early-phase reaction, but also the exacerbation of late-phase inflammation and papillary formation in chronic allergic conjunctivitis.

Tryptase is a trypsin-like serine protease that is released by mast cell degranulation at inflammatory sites. Tryptase has several in vitro functions that may be important in the pathogenesis of asthmatic inflammation: the inactivation of fibrinogen and the inhibition of fibrinogenesis, the activation of tissue matrix metalloproteinases (MMPs) including MMP-3, the inactivation of neuropeptides (including bronchodilatory vasoactive intestinal peptide), the stimulation of lung fibroblast proliferation and collagen synthesis, eosinophil chemotaxis, and the upregulation of intercellular adhesion molecule (ICAM)-1 expression and of IL-8 synthesis by bronchial epithelial cells.[11]

The mechanism by which tryptase exerts these effects is not fully understood, although recent evidence has suggested that tryptase may activate protease-activated receptor (PAR)-2. PAR-2 is a member of the PAR family, which includes PAR-1, -3, and -4. The distribution of PAR-2 in human tissue is not fully known, although previous investigations have detected mRNA expression in the stomach, intestine, glandular epithelial cells of salivary glands and the pancreas, vascular endothelial cells, tracheal epithelial and smooth muscle cells, liver, kidney, and central and peripheral nervous systems.[12–14]

The endogenous activation of PAR-2 by trypsin or tryptase requires the cleavage of the extracellular NH_2-terminal domain, revealing a new peptide sequence (tethered ligand) that is able to bind to sites within the second extracellular loop of the seven-transmembrane receptor.[14,15] The ability of tryptase to activate PAR-2 appears to differ among tissues or cells expressing PAR-2.[15] Akers et al.[16] reported that the proliferation of lung fibroblasts was mediated by PAR-2 activation by tryptase from human mast cells. Frungieri et al.[17] also reported that both recombinant and human mast cells-derived tryptase increased the proliferation of fibroblasts from human foreskin by PAR-2 activation.

In the present study, we assessed the tryptase-induced proliferation of primary cultured conjunctival fibroblasts to investigate the pathogenesis of giant papillary formation. We also investigated the existence of PAR-2 on conjunctival fibroblasts, because this receptor is thought to mediate tryptase-related effects.

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TABLE 1. Sequences of PAR-2 and GADPH Primers

PAR-2	
Forward primer	GTT GAT GGC ACA TCC CAC GTC
Reverse primer	GTA CAG GGC ATA GAC ATG GC
GAPDH	
Forward primer	GTC TTC ACC ACC ATG GAG AAG GCT
Reverse primer	CAT GCC AGT GAG CTT CCC GTT CA

MATERIALS AND METHODS

Human skin β-tryptase was obtained from Promega (Madison, WI). Anti-PAR-2 antibodies (SAM-11) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Leupeptin and benzamidine were obtained from Biogenesis (Poole, UK) and Research Organics (Cleveland, OH). The PAR-2 agonist peptide, SLIGKV (H-Ser-Leu-Ile-Gly-Lys-Val-OH), was obtained from Bachem (Bubendorf, Switzerland). The anti-human mast cell tryptase (MCA1438) was obtained from UK-Serotec, Ltd. (Oxford, UK).

Conjunctival Fibroblast Cultures

Human conjunctival tissue was excised from normal volunteers after obtaining their informed consent. Human conjunctival fibroblasts were established in culture, as previously described.[18] The cells were cultured in 35-mm culture dishes (Iwaki Co., Tokyo, Japan) and were studied from the passages 3 to 9. The purity of each cell type was assessed by cell morphology and immunostaining for vimentin.

Cell Proliferation Assay

Conjunctival fibroblasts were removed from the culture dishes by diluting the cultures 1:10 with 0.05% trypsin-0.53 mM EDTA (Invitrogen-Gibco, Grand Island, NY) in PBS and incubating for 5 minutes. Cells were resuspended in Dulbecco's modified Eagle's medium (DMEM/F12; Invitrogen-Gibco BRL) supplemented with 10% fetal calf serum (FCS). The cells were cultured in 96-well culture plates (5000 cells per well) for 24 hours in DMEM/F12 supplemented with FCS. Then, the medium was replaced with serum-free DMEM/F12 containing tryptase, tryptase with leupeptin or benzamidine, or SLIGKV. A proliferative assay was then performed with a cell-counting kit (Dojindo Laboratories, Kumamoto, Japan) after the cultures were incubated at 37°C for 42 hours. Ten microliters of a mixture of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid, and 1-methoxy-5-methylphenazinium methylsulfate was added to each well according to the attached protocols. The cells were then incubated at 37°C for 2 hours, and their absorbance was measured at a wavelength of 415 nm. Each experiment was performed in triplicate using fibroblasts from three different donors.

RT-PCR for PAR-2 mRNA

The conjunctival fibroblasts were cultured in six-well culture plates (2.0×10^5 cells per well) for 72 hours in DMEM/F12 supplemented with FCS. Then, the culture medium was replaced with FCS-free DMEM/F12, and the cells were cultured for 24 hours. The cells were then washed with PBS, and the total RNA was extracted (RNeasy Mini Kit; Qiagen, Valencia, CA). cDNA was obtained (Super Script II; Invitrogen, Carlsbad, CA). We performed reverse transcription followed by PCR amplification. Briefly, 1 ng of cDNA was added to a 24-μL reaction volume containing 1 μL of random primers and 23 μL of buffer (Platinum PCR Super Mix; Invitrogen). The amplification conditions were 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute for 38 cycles. Electrophoresis was conducted on 2% (wt/vol) analytical grade agarose gels that were subsequently stained with ethidium bromide. The sequences of the forward and reverse primers for PAR-2 and GAPDH are shown in Table 1.

Western Blot Analysis for PAR-2

The conjunctival fibroblasts were cultured in six-well culture plates (2.0×10^5 cells per well) for 72 hours in DMEM/F12 supplemented with FCS. Then, the culture medium was replaced with FCS-free DMEM/F12, and the cells were cultured for 24 hours. The cells were washed twice with ice-cooled phosphate-buffered saline and extracted with 100 μL of NP-40 (Sigma-Aldrich, St. Louis, MO) containing protease inhibitors. Protein extracts were obtained from the lysed tissues using a commercially available protein isolation solution (NP-40 with protein inhibitor cocktails). Ten microliters of protein extract and 10 μL of sample buffer with bromophenol blue were mixed and reacted at 100°C for 5 minutes, then electrophoresed (NuPAGE 10% BT; Invitrogen) and transferred onto a nitrocellulose membrane (Hybond-ECL; AP BioTech). The membrane was then blocked in a solution consisting of 5% nonfat dry milk and tris-buffered saline (TBS)-Tween 20 (pH 7.4). Immunoreactions were performed using an anti-PAR-2 monoclonal antibody (SAM-11) in 1% nonfat dry milk and TBS-Tween 20 (dilution 1: 500). This step was followed by the addition of a horseradish peroxidase–conjugated goat-anti mouse IgG1 (1:20,000; Upstate Biotechnology, Lake Placid, NY). The immune complexes were placed in chemiluminescent reagent (ECL Western Blot detection reagents; GE Healthcare, Piscataway, NJ) and exposed to film.

Immunohistochemistry

For immunohistochemistry, conjunctival fibroblasts from three different donors were seeded in four-well chamber slides at a density of 20,000 cells/well in DMEM/F12 containing 10% FCS. The cells were incubated at 37°C for 1 week. The cells were fixed with 4% formalin for 10 minutes at room temperature. Endogenous peroxidase was blocked by 3% H_2O_2 in methanol for 3 minutes, followed by incubation with normal donkey serum to block nonspecific staining for 10 minutes. After washing in TBS (0.1 M, pH 7.4), the cells were incubated with a mouse anti-PAR-2 monoclonal antibody (SAM-11; 10 μg/mL) for 1 hour at room temperature. For negative control experiments, the primary antibody was replaced with preimmune mouse IgG2a. After washing in TBS for 10 minutes, the samples were processed (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol (3,3′-diaminobenzidine [DAB]-peroxidase staining). The cells were then counterstained with hematoxylin and examined by light microscopy.

PAR-2 or tryptase immunoreactivity was also analyzed in surgically excised conjunctival tissue from three patients with VKC and three normal volunteers. Immediately after excision, the conjunctival specimens were frozen, embedded in OCT compound, and stored at −80°C until the assay. The specimens were cut with a cryostat, air dried, and fixed in acetone for 2 minutes at room temperature. After they were washed in TBS (0.1 M, pH 7.4), the sections were incubated with a mouse anti-PAR-2 monoclonal antibody (10 μg/mL), anti-human mast cell tryptase antibody (2 μg/mL), or preimmune mouse IgG1 and IgG2a for 1 hour at room temperature. Then, the samples were processed according to the manufacturer's protocol (Vectastain ABC Universal kit; Vector Laboratories) and counterstained with hematoxylin.

Statistical Analysis

In the cell proliferation study, the mean ± SD was calculated. The variation between the data sets was tested with ANOVA, and the significance was analyzed with an unpaired *t*-test and the Dunnett test. $P < 0.05$ were considered statistically significant.

RESULTS

Cell Proliferation by Tryptase Stimulation

We investigated the dose-dependent effect of tryptase on the proliferation of cultured conjunctival fibroblasts. Conjunctival fibroblasts were stimulated with 0.1, 1.0, 10, or 100 ng/mL or 1.0 μg/mL of tryptase for 48 hours. Each experiment was

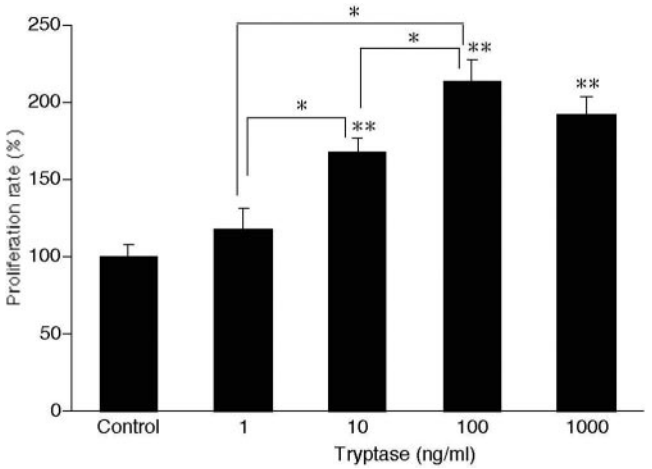

FIGURE 1. Proliferation of cultured conjunctival fibroblasts incubated with various concentration of tryptase. Tryptase increased the proliferation of conjunctival fibroblasts in a statistically significant and dose-dependent manner (*$P < 0.05$; **$P < 0.05$ compare to control). No further increase above 100 ng/mL was observed. The data are representative of experiments using fibroblasts from three different donors.

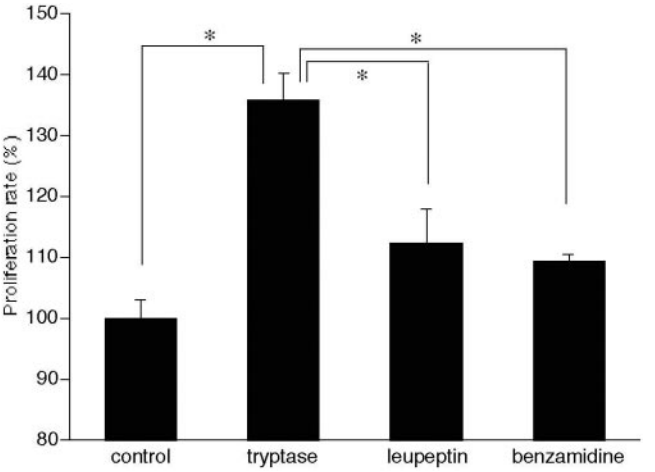

FIGURE 3. Effects of the protease inhibitors leupeptin and benzamidine on tryptase-induced conjunctival fibroblast proliferation. Both leupeptin (10^{-4} M) and benzamidine (10^{-4} M) significantly inhibited 100 ng/mL of tryptase-induced conjunctival fibroblast proliferation (*$P < 0.05$).

performed in triplicate, using fibroblasts from three different donors. Tryptase produced a statistically significant increase in conjunctival fibroblast proliferation in a dose-dependent manner, with maximum proliferation observed at a dose of 100 ng/mL ($P < 0.05$; Fig. 1).

Existence of PAR-2 on Conjunctival Fibroblasts

We investigated the existence of mRNA for the known tryptase receptor, PAR-2, in human conjunctival fibroblasts using RT-PCR. Primary cultured fibroblasts from three different donors were used for the assay. PAR-2 mRNA was detected in all specimens from three different donors (Fig. 2A).

Next, we examined the existence of PAR-2 protein on human conjunctival fibroblasts using Western blot analysis. Primary cultured fibroblasts from three different donors were used for the assay. PAR-2 protein was detected in the specimens from all three different donors (Fig. 2B).

Inhibition of Cell Proliferation by Tryptase Inhibitors

We next examined the inhibition of tryptase inhibitors on the proliferative activity of tryptase-stimulated conjunctival fibroblasts. Leupeptin and benzamidine are known to inhibit the catalytic activity of tryptase. After conjunctival fibroblasts were cultured in 96-well culture plates (5000 cells per well) for 24 hours in DMEM/F12 supplemented with FCS, the medium was replaced with serum-free DMEM/F12 containing 100 ng/mL of tryptase with or without inhibitors. The culture medium was preincubated with 100 ng/mL of tryptase, or 100 ng/mL of tryptase and 10^{-4} M of each inhibitor at 37.0°C for 1 hour before use. Both leupeptin and benzamidine significantly in-

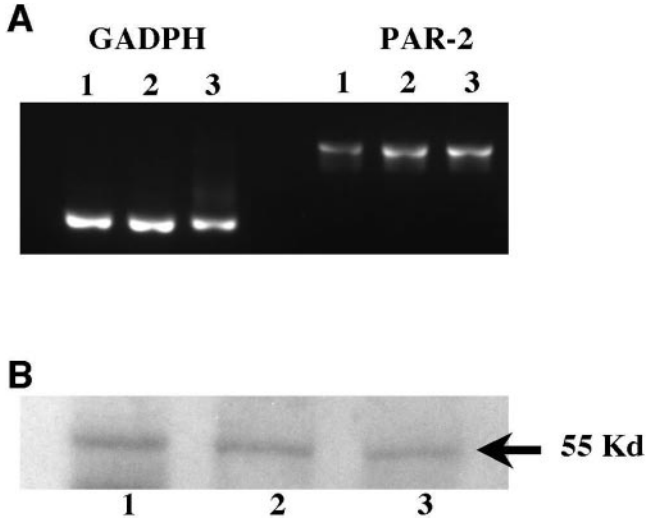

FIGURE 2. Existence of PAR-2 mRNA and protein on human conjunctival fibroblasts. (**A**) RT-PCR analysis shows PAR-2 mRNA expression in cultured conjunctival fibroblasts. (**B**) Western blot analysis shows the presence of a 55-kDa protein (PAR-2) in cultured conjunctival fibroblasts.

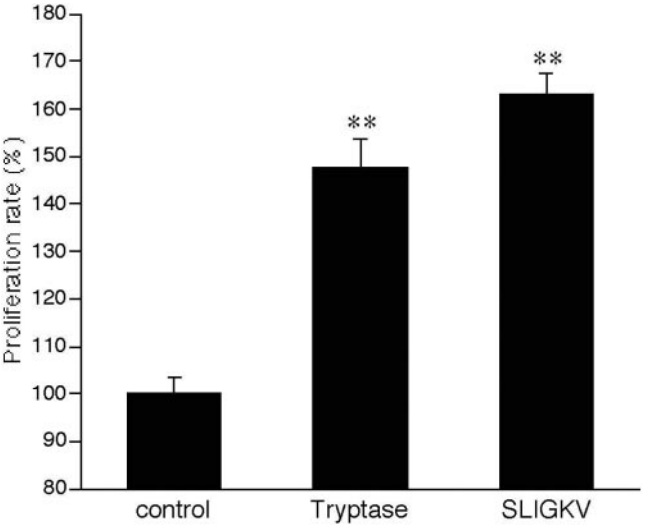

FIGURE 4. Effect of the PAR-2 agonist SLIGKV on conjunctival fibroblast proliferation. The PAR-2 agonist SLIGKV (10^{-4} M) mimicked 100 ng/mL of tryptase's effect and significantly increased the proliferation of conjunctival fibroblasts (*$P < 0.05$).

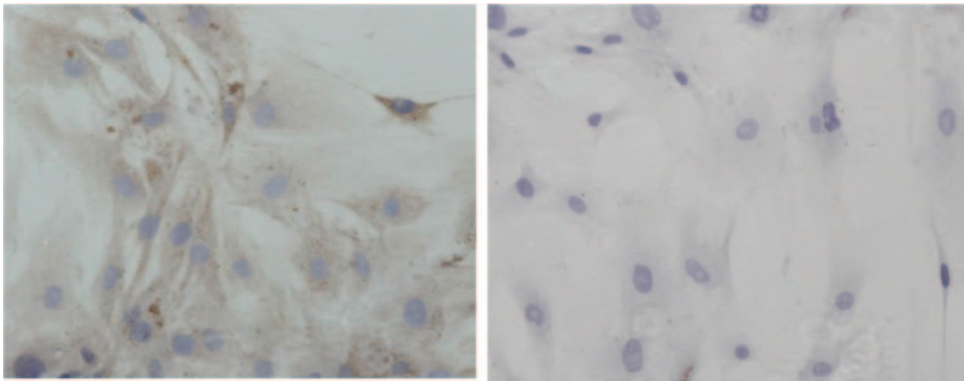

FIGURE 5. Immunoreactivity of PAR-2 receptor protein in cultured conjunctival fibroblasts. *Left*: immunohistochemistry shows that PAR-2 immunoreactivity was observed in cultured conjunctival fibroblasts. *Right*: isotype control.

hibited effect of tryptase on the proliferative activity of the cells (Fig. 3; $P < 0.05$).

The Effect of PAR-2 Agonist on Conjunctival Fibroblast Proliferation

Human PAR-2 is known to be activated by a synthetic peptide, SLIGKV. SLIGKV is designed based on the amino acid sequence of the tethered ligand, which directly binds to the body of PAR-2 without cleaving the N-terminal peptide, acting as an agonist for PAR-2.[14,15] SLIGKV mimicked the effect of tryptase (100 ng/mL of dose) and significantly increased the proliferative activity of cultured conjunctival fibroblasts (Fig. 4; $P < 0.05$).

Immunoreactivity of PAR-2 in Conjunctival Fibroblasts

We assessed the existence of PAR-2 receptor protein in conjunctival fibroblasts. In cultured conjunctival fibroblasts, PAR-2 immunoreactivity was observed except cell nucleus (Fig. 5). Because the anti-PAR-2 antibody, SAM-11 binds to the amino acid sequence 37-50, which is contained in the amino terminal of exodomain of human PAR-2, we strongly suspected that this staining pattern reflected PAR-2 localization on the cell membrane.

In the surgically excised papillae from patients with VKC, there were many tryptase-positive cells within the inflammatory cell infiltration site, whereas conjunctival tissues from normal volunteers contained no tryptase-positive cells. In both surgically excised papillae from patients with vernal keratoconjunctivitis and conjunctival tissue from normal control subjects, fibroblast-like cells with a flattened appearance were positively stained by the PAR-2 antibody (Fig. 6).

DISCUSSION

In this study, we showed that tryptase upregulated the proliferative activity of cultured conjunctival fibroblasts and that this stimulation was mediated by the known tryptase receptor, PAR-2. To the best of our knowledge, this is the first report indicating that conjunctival fibroblasts, in addition to fibroblasts derived from lung or dermal tissue, express PAR-2 and proliferate after stimulation with tryptase.

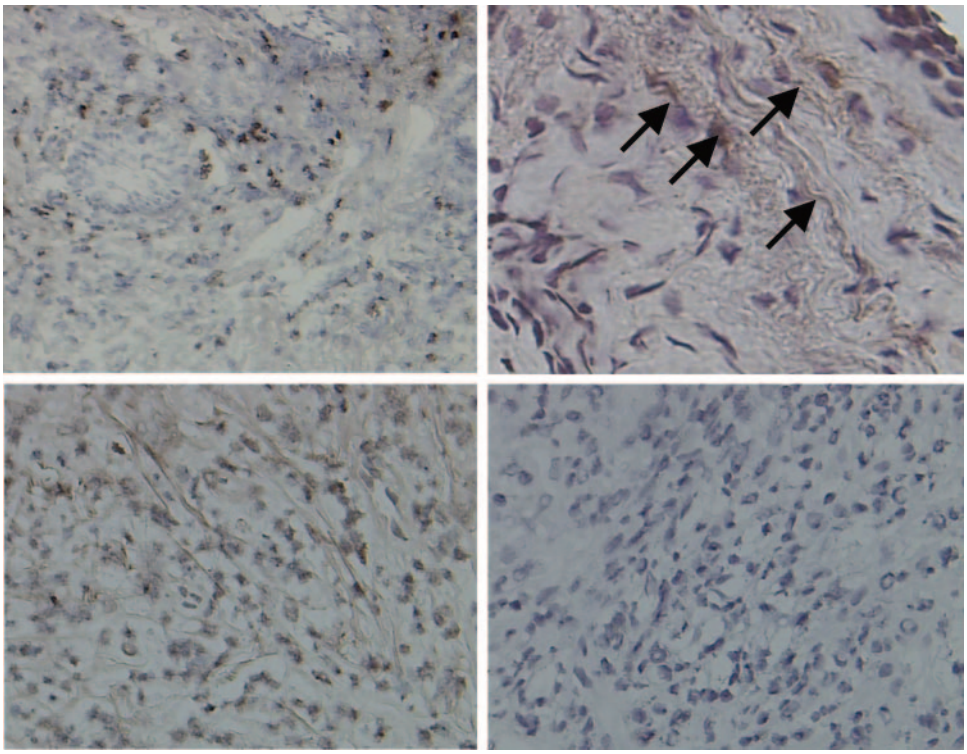

FIGURE 6. Immunoreactivity of human mast cell tryptase and PAR-2 receptor protein in conjunctival fibroblasts from surgically excised papillae. *Top left*: immunohistochemistry shows tryptase immunoreactive cells are observed within the area with inflammatory cell infiltration in the papillae from VKC specimens. *Top right*: PAR-2 immunoreactive stromal fibroblasts (*arrows*) are observed in conjunctival tissues from normal volunteers. *Bottom left*: PAR-2 immunoreactivity is seen in spindle-shaped cells, thought to be fibroblasts, in surgically excised papillary specimens from patients with VKC. *Bottom right*: isotype control shows no immunoreaction.

Tryptase is mainly released by mast cells. Mast cells activated by IgE binding to FcεRI play a critical role in the early phase of allergic inflammation, releasing preformed mediators like histamine, chymase, cathepsin-G, and mast-cell–specific carboxypeptidase A. Human mast cells produce α and β forms of tryptase. α-Tryptase is constitutively produced, while β-tryptase is stored in the secretory granules and released by exocytosis.[11] Our current results revealed that β-tryptase upregulated fibroblast proliferation, indicating that mast cells can affect the mitogenic acceleration of fibroblasts, when activated.

Like fibroblasts derived from lung or skin, conjunctival fibroblasts are known to produce various factors and affect other type of cells, including leukocytes. Previous investigation reported that conjunctival fibroblasts secreted Th-2 cytokines,[19] eotaxin,[20] procollagen type I and III,[21–23] MMPs,[23] and VEGF.[24] Solomon et al.[25] reported that conjunctival fibroblasts enhanced the survival and functional activity of eosinophils through IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor. Leonardi et al.[26] reported that histamine, which is accumulated in preformed granules in mast cells as well as tryptase, increases the proliferative and productive activity of conjunctival fibroblasts in patients with VKC. These facts may indicate a hypothesis that mast cells activate residential conjunctival stromal cells through the degranulation of preformed mediators, resulting in massive leukocyte infiltration and excess fibrovascular proliferation, causing giant papillary formation. Thus, mast cells may affect tissue remodeling[27] in allergic conjunctiva.

PAR-2 is known to be the sole receptor of tryptase. The current results show that the proliferative activity upregulated by tryptase was almost totally inhibited by leupeptin and benzamidine and that the PAR-2 antagonist peptide SLIGKV completely mimicked the effect of tryptase. These findings suggest that the local administration of a PAR-2-blocking peptide may effectively inhibit fibroblast proliferation in conjunctival tissue in vivo. We also demonstrated the existence of PAR-2 mRNA and protein on cultured conjunctival fibroblasts derived from normal volunteers, as well as on stromal fibroblasts in giant papillae from patients with VKC. We speculate that under allergic conditions, when Th-2 cytokines or proinflammatory factors are abundant, PAR-2 expression on conjunctival stromal cells may be upregulated. A quantitative assessment of PAR-2 expression on conjunctival fibroblasts would provide interesting data.

In conclusion, tryptase upregulates conjunctival fibroblast proliferation, and this effect appeared to be mediated via the PAR-2 receptor. Mast cells may play an important role in giant papillary formation during late-phase allergic conjunctivitis by means of releasing tryptase. Inhibitors of tryptase or peptides that block PAR-2 on the surfaces of fibroblasts may offer therapeutic benefits by selectively preventing the activation of fibroblasts and consequently the formation of giant papillae, in patients with allergic conjunctivitis. Further investigations are required in this field.

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