Protection of Human Conjunctival Fibroblasts from NO-Induced Apoptosis by Interleukin-4 or Interleukin-13

Youichiro Fujitsu,¹ Ken Fukuda,² Kazubiro Kimura,¹ Keisuke Seki,² Naoki Kumagai,¹ and Teruo Nishida¹

PURPOSE. To examine the possible roles of T helper type 2 (Th2) cell–derived cytokines in formation of the giant papillae characteristic in individuals with vernal keratoconjunctivitis, the effects of these cytokines on the proliferation and apoptosis of cultured human conjunctival fibroblasts were investigated.

METHODS. Apoptosis was induced by the NO donor sodium nitroprusside. Cell viability was determined by measurement of mitochondrial metabolic activity, and apoptotic cells were identified on the basis either of nuclear morphology after staining with 4′,6-diamidino-2-phenylindole or of TUNEL staining. The activation of antiapoptotic signaling mediated by the protein kinase Akt was assessed by immunoblot analysis and by an in vitro kinase assay. Expression of interleukin (IL)-4 and IL-13 receptor subunits was examined by reverse transcription and polymerase chain reaction analysis and by flow cytometry.

RESULTS. IL-4 and IL-13, but not IL-5, IL-9, or IL-10, induced the proliferation of conjunctival fibroblasts as well as protecting these cells from NO-induced apoptosis. Both IL-4 and IL-13 induced the phosphorylation of Akt and increased the kinase activity of this enzyme in a manner that was sensitive to the phosphatidylinositol 3-kinase inhibitors LY294002 or wortmannin. These inhibitors also blocked the antiapoptotic effects of IL-4 and IL-13. Transcripts encoding IL-4 and IL-13 receptor components were detected in conjunctival fibroblasts, and the proteins were expressed at the cell surface.

CONCLUSIONS. Among the various Th2 cytokines tested, only IL-4 and IL-13 induced the proliferation of human conjunctival fibroblasts and protected these cells from apoptosis. These effects may contribute to the formation of giant papillae in individuals with vernal keratoconjunctivitis. (Invest Ophthalmol Vis Sci. 2005;46:797–802) DOI:10.1167/iovs.04-01016

Vernal keratoconjunctivitis (VKC) is a severe and chronic ocular allergic disease that threatens vision as a result of the associated corneal disorders such as corneal epithelial erosion and shield ulcer.¹ A specific clinical characteristic of VKC is the formation of conjunctival giant papillae at the upper tarsus. These giant papillae develop as the result of overgrowth of resident conjunctival fibroblasts,² infiltration of inflammatory cells such as eosinophils, mast cells, and T helper type 2 (Th2) cells,³ as well as excessive deposition of extracellular matrix proteins such as fibronecetin and collagen types I and III.²

Cell proliferation and programmed cell death (apoptosis) play important roles in the maintenance of tissue integrity. An imbalance between these two processes contributes to the pathogenesis of various diseases.⁴ Certain cytokines and growth factors promote cell proliferation, whereas others protect cells against apoptosis by triggering specific survival signals. The overgrowth of conjunctival fibroblasts that contributes to the development of giant papillae might thus result from an imbalance between cell proliferation and cell death induced by cytokines or growth factors released during allergic inflammation.

Interleukin (IL)-4 and IL-13 are Th2 cell–derived cytokines that induce immunoglobulin (Ig) isotype switching in B cells and maintain the production of IgE.⁵ These cytokines thus play important roles in the development of both ocular and systemic allergic reactions. The concentration of IL-4 is increased in the tear fluid of patients with VKC,⁶ and the number of T cells and mast cells that express IL-15 is also increased in such individuals.⁷,⁸ The functional receptors for these cytokines are expressed on various cell types including epithelial cells, endothelial cells, fibroblasts, and smooth muscle cells.⁹ Two types of IL-4 receptor (IL-4R) have been identified: The type I receptor is composed of the IL-4Rα chain and the IL-2 receptor common γ chain (IL-2Rγc). The type II receptor comprises IL-4Rβ and the IL-13 receptor α1 chain (IL-13Rα1) and also functions as a receptor for IL-13. IL-4 and IL-13 act not only on inflammatory cells but also on tissue-resident fibroblasts. These cytokines thus appear to play important roles in the initiation and amplification of ocular allergic inflammation by inducing both the production of chemokines such as eotaxin (CCL11)¹⁰–¹² and thymus- and activation-regulated chemokine (CCL17),¹³,¹⁴ as well as the expression of vascular cell adhesion molecule-1 in human corneal fibroblasts. Interleukin-4 may also contribute to the formation of conjunctival giant papillae by stimulating the proliferation and the production of extracellular matrix by human conjunctival fibroblasts.¹⁶

To provide further insight into the pathogenesis of giant papillae, we have now investigated the possible survival-promoting (antiapoptotic) effects of Th2 cytokines in human conjunctival fibroblasts.

METHODS

Materials

Minimum essential medium (MEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY), sodium nitroprusside (SNP) and propidium iodide were from Sigma (St. Louis, MO), and 4′,6-diamidino-2-phenylindole (DAPI) and Alexa Fluor 488–conjugated donkey antibodies to mouse or goat immunoglobulin G (IgG) were from Molecular Probes (Eugene, OR). Tissue culture dishes and cell culture plates (Falcon) were from Becton-Dickinson (Franklin Lakes, NJ). Recombinant human IL-4, IL-5, and IL-10 were from Genzyme Teche (Minneapolis, MN); recombinant human IL-9 and IL-13, a mouse monoclonal antibody to human IL-13Rα1, goat polyclonal anti-

From the Departments of ¹Biomolecular Recognition and Ophthalmology and ²Ocular Pathophysiology, Yamaguchi University School of Medicine, Ube City, Yamaguchi, Japan.

Submitted for publication August 24, 2004; revised November 9, 2004; accepted November 17, 2004.

Disclosure: Y. Fujitsu, None; K. Fukuda, None; K. Kimura, None; K. Seki, None; N. Kumagai, None; T. Nishida, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertised" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Ken Fukuda, Department of Ocular Pathophysiology, Yamaguchi University School of Medicine, 1-1-1 Minami-Kogushi, Ube City, Yamaguchi 755-8505, Japan; k.fukuda@yamaguchi-u.ac.jp.
bodies to human IL-13Rα2, normal mouse IgG1, and normal goat IgG were from R&D Systems (Minneapolis, MN); a rat monoclonal antibody to human IL-2Rγc was from Sumitomo Electric (Osaka, Japan); and normal rat IgG and fluorescein isothiocyanate–conjugated goat antibodies to rat IgG were from ICN Pharmaceuticals (Aurora, OH). An Akt kinase assay kit, rabbit polyclonal antibodies to Akt and to phospho-Akt (phosphoserine-473), as well as LY294002 were obtained from Cell Signaling (Beverly, MA); wortmannin was from Calbiochem (LA Jolla, CA); and horseradish peroxidase–conjugated donkey antibodies to rabbit IgG and a terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling (TUNEL) assay kit were from Promega (Madison, WI). All reagents used for cell culture were endotoxin minimized.

Isolation and Culture of Human Conjunctival Fibroblasts

Three normal human conjunctivas were obtained after informed consent from individuals undergoing eyelid or strabismus surgery, and were processed separately. The human tissue was used in strict accordance with the basic principles of the Declaration of Helsinki. Conjunctival fibroblasts were isolated and cultured as described previously.16

Apoptosis Induction

The nitric oxide (NO) donor SNP was used as a proapoptotic agent because of its ability to induce apoptosis in fibroblasts.17 Cells were cultured for 24 hours in MEM supplemented with 0.5% FBS, for 48 hours in fresh medium further supplemented with cytokines, and for 6–24 hours in fresh medium supplemented with cytokines and 3 mM SNP. In some experiments, LY294002 or wortmannin was included in all incubations with cytokines.

Measurement of Cell Survival

Conjunctival fibroblasts were cultured and stimulated in 96-well plates (5 × 10^5 cells per well) as described above. After incubation for 24 hours with 3 mM SNP, the relative number of viable cells was estimated by measurement of mitochondrial 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)–metabolizing activity with the use of a Celltiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega), as described previously.16

Morphologic Evaluation and TUNEL Analysis of Apoptosis

The number of apoptotic cells was determined by morphologic evaluation18 as well as by the TUNEL assay17 as described previously. In brief, cells were cultured and stimulated in 8-well plates (5 × 10^4 cells per well) as described above, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and subjected to the TUNEL assay. They were then counterstained with propidium iodide (1 μg/mL) and DAPI (0.3 μM) before observation with a fluorescence microscope (Axioskop 50; Zeiss, Oberkochen, Germany). The mean percentages of TUNEL-positive (apoptotic) cells as well as of apoptotic cells identified by characteristic chromatin condensation were determined by examination of three microscopic fields in each of three replicate cultures.

Immunoblot Analysis of Akt and Phospho-Akt

The phosphorylation of Akt was examined by immunoblot analysis as described previously.17 In brief, cells were cultured in 60-mm dishes (3 × 10^4 cells per dish) first for 48 hours in MEM supplemented with 0.5% FBS and then for an additional 2 hours in serum-free medium. They were then incubated for 5 minutes at 37°C with fresh serum-free medium containing various cytokines before lysis by scraping and sonication. The lysate was centrifuged, and the resulting supernatant was fractionated by SDS-polyacrylamide gel electrophoresis on a 10% gel. The separated proteins were transferred to a polyvinylidene difluoride membrane, which, after the blocking of nonspecific sites, was incubated with antibodies to phospho-Akt. Immune complexes were detected with horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence. The membrane was then reprobed with antibodies to Akt.

Assay of the Kinase Activity of Akt

The kinase activity of Akt was assayed with a kit. In brief, cells were cultured in 100-mm dishes and were exposed to cytokines for 15 minutes at 37°C in serum-free medium. They were then washed once with ice-cold phosphate-buffered saline and lysed by lysis buffer. Total cellular proteins were subjected to immunoprecipitation overnight at 4°C with agarose-conjugated monoclonal antibodies to Akt. The resulting precipitates were washed three times with lysis buffer and twice with kinase buffer and were then assayed for kinase activity for 30 minutes at 30°C with continuous agitation in kinase buffer containing 200 μM ATP and 1 μg of a glycoprotein synthase kinase-3 (GSK-3) fusion protein as substrate. The reaction was terminated by the addition of SDS sample buffer, and the reaction mixtures were then subjected to immunoblot analysis with polyclonal antibodies to phosphorylated GSK-3α or β. Equal loading of samples was confirmed by reprobing of the membrane with monoclonal antibodies to Akt.

RT-PCR Analysis of IL-4 and IL-13 Receptor Components

The abundance of mRNAs for IL-4R and IL-13R components was determined by reverse transcription (RT) and polymerase chain reaction (PCR) analysis as described previously.12,16 Transcripts of the constitutively expressed gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. The sequences of the PCR primers for IL-4Rα, IL-13Rα1, IL-13Rα2, IL-2Rγc, and GAPDH were as described previously.12,19

Flow Cytometric Analysis of Cell Surface Expression of IL-4 and IL-13 Receptor Components

Cell surface expression of cytokine receptors was analyzed by flow cytometry as described previously.12,19 In brief, cells were incubated for 30 minutes at 4°C with antibodies to human IL-2Rγc, IL-13Rα1, or IL-13Rα2. The cells were then washed, incubated for 30 minutes at 4°C with FITC-conjugated goat antibodies to rat IgG or Alexa Fluor 488–conjugated donkey antibodies to mouse or goat IgG, respectively,
washed again, and fixed with 1% paraformaldehyde in phosphate-buffered saline. As a negative control, cells were incubated under similar conditions with rat, mouse, or goat IgG in place of the primary antibodies. Cells were then analyzed by flow cytometry with an EPICS-XL instrument; at least 10,000 cells were analyzed for each sample.

Statistical Analysis
Data were analyzed by Dunnett’s test or Scheffé’s multiple comparison test. A P value of <0.05 was considered statistically significant.

RESULTS
Antiapoptotic Effects of IL-4 and IL-13 in Human Conjunctival Fibroblasts
We first investigated the effects of Th2 cytokines on the proliferation of human conjunctival fibroblasts and on the induction of apoptosis in these cells by NO. Measurement of mitochondrial MTS-metabolizing activity revealed that IL-4 and IL-13, each at a concentration of 10 ng/mL, stimulated the proliferation of conjunctival fibroblasts, consistent with our

FIGURE 2. Effects of Th2 cytokines on SNP-induced apoptosis in conjunctival fibroblasts. (A–L) Cells were pretreated or not with the indicated cytokines (10 ng/mL) for 48 hours and then incubated in the additional absence (A–C, G–I) or presence (D–F, J–L) of 3 mM SNP for 24 hours. They were then subjected to the TUNEL assay and stained with DAPI (insets) before examination with a fluorescence microscope; for the TUNEL assay, cells were counterstained with propidium iodide. Scale bar, 100 μm. (M) The percentage of TUNEL-positive cells under the various conditions was determined. Data are means ± SEM of values from three independent experiments. *P < 0.01 versus the corresponding value (plus SNP) for no cytokine addition (Scheffé’s test).
previous observations with IL-4, whereas IL-5, IL-9, and IL-10 had no such effect (Fig. 1). Exposure to the NO donor SNP (3 mM) for 24 hours resulted in the death of most cells, as revealed by the MTS survival assay (Fig. 1). However, this assay also revealed that pretreatment of the cells with IL-4 or IL-13, but not with IL-5, IL-9, or IL-10, protected them from NO-induced cell death (Fig. 1). Evaluation of nuclear morphology by DAPI staining showed that NO-induced chromatin condensation characteristic of apoptosis in most cells and that this effect was blocked by IL-4 or IL-13 but was not inhibited by IL-5, IL-9, or IL-10 (Fig. 2); none of the cytokines tested alone affected nuclear morphology. To confirm that the cell death induced by SNP was indeed apoptosis, we performed the TUNEL assay. Exposure to SNP rendered most cells TUNEL positive, or apoptotic, and pretreatment of the cells with IL-4 or IL-13, but not IL-5, IL-9, or IL-10, protected them from SNP-induced apoptosis (Fig. 2). These results thus demonstrated that IL-4 and IL-13 each protected human conjunctival fibroblasts from SNP-induced apoptosis.

Phosphorylation and Activation of Akt Induced by IL-4 or IL-13 in Conjunctival Fibroblasts

We next examined whether IL-4 or IL-13 induced the activation (phosphorylation) of Akt, which inhibits apoptosis and promotes cell survival. Conjunctival fibroblasts were incubated for 5 minutes with cytokines (100 ng/mL), lysed, and subjected to immunoblot analysis with antibodies to phospho-Akt and to Akt. The abundance of Akt was not affected by exposure of conjunctival fibroblasts to any of the cytokines examined. However, IL-4 and IL-13, but not IL-5, IL-9, or IL-10, induced a marked increase in the amount of phosphorylated Akt (Fig. 3A). To determine whether the IL-4- or IL-13-induced activation of Akt in conjunctival fibroblasts was mediated by phosphatidylinositol (PI) 3-kinase, we examined the effects of the PI 3-kinase inhibitors LY294002 and wortmannin. Preincubation of cells with LY294002 (50 μM) or wortmannin (200 nM) almost completely inhibited the phosphorylation of Akt induced by IL-4 or IL-13 (Fig. 3B). We confirmed the effects of IL-4 and IL-13 on Akt activation by measuring the kinase activity of the enzyme directly with an in vitro immune-complex kinase assay. Both IL-4 and IL-13 induced a marked increase in the kinase activity of Akt as determined with a GSK-3 fusion protein as substrate (Fig. 4A). Preincubation of conjunctival fibroblasts with the PI 3-kinase inhibitors LY294002 (50 μM) or wortmannin (200 nM) blocked the increase in Akt activity induced by IL-4 or IL-13 (Fig. 4B), suggesting that the activation of Akt by these cytokines is indeed mediated by PI 3-kinase.

Role of PI 3-Kinase in the Antiapoptotic Effects of IL-4 and IL-13 in Conjunctival Fibroblasts

We then examined the effect of the PI 3-kinase inhibitors on the antiapoptotic action of IL-4 or IL-13 in conjunctival fibroblasts. Pretreatment of the cells with IL-4 or IL-13 in the presence of LY294002 or wortmannin resulted in marked inhibition of the protective effect of these cytokines against SNP-induced apoptosis (Fig. 5).

Expression of IL-4 and IL-13 Receptors in Conjunctival Fibroblasts

Finally, whether human conjunctival fibroblasts express receptors for IL-4 and IL-13 was investigated. With the use of RT-PCR analysis and flow cytometry, we previously showed that these cells contain transcripts for the IL-4R α chain and express IL-4R and IL-13R chains (Fig. 6A). Flow cytometric analysis also revealed the presence of transcripts encoding IL-2Rγc, IL-13Rα1, and IL-13Rα2 (Fig. 6B).

Discussion

We have shown that the Th2 cytokines IL-4 and IL-13 each significantly inhibit SNP-induced apoptosis in human conjunctival fibroblasts. Pretreatment of the cells with IL-4 or IL-13 in the presence of LY294002 or wortmannin resulted in marked inhibition of the protective effect of these cytokines against SNP-induced apoptosis (Fig. 5).

Expression of IL-4 and IL-13 Receptors in Conjunctival Fibroblasts

Finally, whether human conjunctival fibroblasts express receptors for IL-4 and IL-13 was investigated. With the use of RT-PCR analysis and flow cytometry, we previously showed that these cells contain transcripts for the IL-4R α chain and express IL-4R and IL-13R chains (Fig. 6A). Flow cytometric analysis also revealed the presence of transcripts encoding IL-2Rγc, IL-13Rα1, and IL-13Rα2 (Fig. 6B).

Discussion

We have shown that the Th2 cytokines IL-4 and IL-13 each significantly inhibit SNP-induced apoptosis in human conjunctival fibroblasts. Pretreatment of the cells with IL-4 or IL-13 in the presence of LY294002 or wortmannin resulted in marked inhibition of the protective effect of these cytokines against SNP-induced apoptosis (Fig. 5).
tival fibroblasts and that this effect appears to be mediated by the activation of PI 3-kinase and Akt. As we previously demonstrated for IL-4,16 these two cytokines also stimulated the proliferation of conjunctival fibroblasts. These observations thus suggest that the mitogenic and antiapoptotic effects of IL-4 and IL-13 in conjunctival fibroblasts might contribute to the formation of giant papillae in individuals with VKC.

The interaction of IL-4 and IL-13 with their receptors in immune cells results in activation of signaling pathways mediated by STAT6 and PI 3-kinase. These observations thus suggest that the mitogenic and antiapoptotic effects of IL-4 and IL-13 in conjunctival fibroblasts might contribute to the formation of giant papillae in individuals with VKC.

The interaction of IL-4 and IL-13 with their receptors in immune cells results in activation of signaling pathways mediated by STAT6 and PI 3-kinase.21 We have now shown that two inhibitors of PI 3-kinase activity, wortmannin and the more specific compound LY294002, blocked the ability of IL-4 or IL-13 to protect conjunctival fibroblasts from SNP-induced apoptosis. Neither LY294002 nor wortmannin affected the phosphorylation or nuclear translocation of STAT6 induced by IL-4 or IL-13 in these cells (unpublished data). These results are consistent with previous observations with colonic epithelial cells and FDCP2 myeloid cells.22 The activation of STAT6 and that of PI 3-kinase in conjunctival fibroblasts thus appear to be distinct events, and the activation of PI 3-kinase seems to mediate the antiapoptotic effects of IL-4 and IL-13 in these cells.

The serine-threonine protein kinase Akt is a key downstream target of PI 3-kinase in the promotion of hematopoietic cell survival.23 Hematopoietic cells are dependent on the presence of specific cytokines for their continued proliferation and survival.24 In the absence of cytokines, the cells thus cease to proliferate and undergo apoptosis. We have now shown that tissue-resident fibroblasts were protected from NO-induced apoptosis by IL-4 or IL-13 as a result of the activation of the PI 3-kinase–Akt signaling pathway. Other Th2 cytokines, including IL-5, IL-9, and IL-10, did not activate Akt in human conjunctival fibroblasts, nor did they protect these cells from NO-induced apoptosis. The abundance of several growth factors has been shown to be increased in giant papillae.25 Given that Akt is activated by growth factors such as platelet-derived growth factor and insulin-like growth factor–1,17 these growth factors also might exert both mitogenic and antiapoptotic effects in giant papillae.

Oxidative stress caused by the production of superoxide, the hydroxyl radical, or NO is characteristic of many allergic disorders.26 NO is synthesized from L-arginine by a family of NO synthase enzymes and its production is increased in individuals with allergic asthma, allergic rhinitis, or atopic dermatitis.27 In addition, the amounts of NO and NO synthase are increased in an animal model of allergic conjunctivitis.28 NO induces apoptosis in many inflammatory cell types, including eosinophils and Th2 cells.27,29 Both of which are major effector cells in allergic inflammation. We have now shown that the NO donor SNP induces apoptosis in human conjunctival fibroblasts. Although the contribution of NO to the pathogenesis of giant papillae remains to be determined, NO, together with IL-4 and IL-13, might be a determinant of the balance between

![Figure 6](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932929/)

**Figure 6.** Expression of IL-4 and IL-13 receptor components in conjunctival fibroblasts. (A) RT-PCR analysis. Total RNA isolated from conjunctival fibroblasts (CF) and from human monocytes as a positive control (P) was subjected to RT-PCR analysis with primers specific for IL-4Rα, IL-13Rα1, IL-13Rα2, IL-2Rγc, and GAPDH (internal control) cDNAs. No PCR products were detected in the absence (−) of reverse transcription (RT). The leftmost lanes contain DNA size markers. Data are representative of three experiments. (B) Flow cytometric analysis. Conjunctival fibroblasts were stained either with antibodies to human IL-2Rγc, IL-13Rα1, or IL-13Rα2 (shaded histograms) or with isotype-matched control Ig (open histograms). Data are representative of two experiments.
the proliferation and apoptosis of human conjunctival fibroblasts in individuals with VKC.

The IL-4 and IL-13 receptors share a common chain, IL-4Rα. Conjunctival fibroblasts express IL-2Rγc, IL-13Ra1, and IL-13Ra2 chains at the mRNA and protein levels in addition to IL-4Rα. These cells thus appear to express both type I (IL-4Rα and IL-2Rγc) and type II (IL-4Rα and IL-13Ra1) receptors for IL-4, the latter of which also functions as a receptor for IL-13.

In conclusion, IL-4 and IL-13 each promote the proliferation of human conjunctival fibroblasts as well as protect these cells from apoptosis. This latter effect appears to be mediated by activation of the PI 3-kinase-Akt signaling pathway. These actions of IL-4 and IL-13 might thus contribute to the overgrowth of conjunctival fibroblasts that is characteristic of the development of giant papillae in individuals with VKC.

References


