Growth Factors, Cytokines, Cell Cycle Molecules

Conjunctival Interleukin-13 Expression in Mucous Membrane Pemphigoid and Functional Effects of Interleukin-13 on Conjunctival Fibroblasts *in Vitro*

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Interleukin-13 (IL-13) is the dominant effector cytokine of fibrosis in pulmonary and liver disease. Excessive conjunctival fibrosis in the immunobullous disease ocular mucous membrane pemphigoid (MMP) causes blindness; the pathogenesis of scarring in this disease is incompletely understood. To determine whether IL-13 is involved in conjunctival fibrosis in MMP, we studied the expression of IL-13 in ocular MMP patients before and after systemic immunosuppression and examined the effects of IL-13 on normal human conjunctival fibroblasts. We found high stromal cell expression of IL-13 in active ocular MMP by immunohistochemistry; 80% of these cells were CD3-positive T cells. Following immunosuppression, in clinically uninflamed, treated, ocular MMP patients, the number of IL-13 positive cells was significantly reduced, but this was still fourfold greater than in normal conjunctiva. IL-13 stimulated collagen lattice contraction and migration, and decreased production of mmp-3 and mmp-10 by human conjunctival fibroblasts. The addition of T cell culture supernatant to IL-13 synergistically augmented fibroblast migration. IL-13 also up-regulated surface expression of HLA-DR, CD80, CD40, and CD154 by conjunctival fibroblasts, suggesting a potential mechanism for fibroblast-T cell cross talk, via which fibroblasts may actively engage in perpetuating chronic inflammation and continued fibrosis. Together, these findings suggest that IL-13 is involved in conjunctival fibrosis in MMP, and that IL-13 has both profibrotic and pro-inflammatory effects on human conjunctival fibroblasts. (Am J Pathol 2009, 175:2406–2415; DOI: 10.2353/ajpath.2009.090579)

Autoimmune conjunctivitis caused by ocular mucous membrane pemphigoid (MMP), also known as ocular cicatricial pemphigoid, is a blinding disease characterized by recurrent episodes of inflammation and aggressive conjunctival fibrosis. It is part of a spectrum of immunobullous disease associated with linear deposition of IgG and IgA autoantibodies directed against epithelial basement membrane zone proteins, which most commonly involves oral and ocular mucosa. In ocular mucosa, unlike oral mucosa, scarring is a clinical hallmark of the disease.¹ Although the mechanisms have not been clearly demonstrated, autoantibodies are thought to cause blistering by disrupting basement membrane adherence via activation of complement, neutrophils and pro-inflammatory cytokines.² In the eye, blisters are infrequently seen, and the disease manifests as chronic recurrent cicatricial conjunctivitis.

The functional consequences of the inflammatory lesions that heal with scarring are most evident in the eye, where patients with severe conjunctival fibrosis become blind in 30% of cases.³ Developing effective anti-fibrotic agents would therefore have therapeutic potential, but is challenging because the cellular and molecular mechanisms of conjunctival scarring in MMP are incompletely understood. Given that conjunctival fibrosis can still progress, despite apparent clinical control of inflammation by conventional immunosuppressive therapy,^{4,5} better understanding of the changes in treated tissue compared with actively inflamed tissue might assist and guide in the development of adjunctive local therapies to target conjunctival fibrosis.

Interleukin-13 is a key profibrotic mediator that has been identified as the dominant effector cytokine of fibrosis in experimental models of skin, hepatic, and pulmonary fibrosis and airway remodeling in asthma.^{6–9} It is

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produced by type 2 helper T cell (Th2) cells, mast cells, and basophils, and is a potent stimulator of eosinophil-, lymphocyte- and macrophage-rich inflammation, mucosal metaplasia, tissue fibrosis, and parenchymal remodeling.^{10,11} Fibrosis associated with repetitive injury in chronic inflammatory disease is strongly linked with CD4⁺ Th2 responses involving interleukin (IL)-4, IL-5, IL-13, and IL-21.¹² As CD4⁺ and CD8⁺ T cells are the predominant cells in the conjunctival subepithelial infiltrate in ocular MMP,^{13–15} it has been hypothesized that T cells play an important role in the pathogenesis of conjunctival fibrosis in MMP. As in most chronic immune-mediated fibroproliferative disorders,¹⁶ both Th1 (interferon [IFN] γ) and Th2 cytokines (IL-4, IL-5) are present in MMP lesions,^{17–20} but it is not known whether the Th2 cytokine IL-13 plays a role in the autoimmune conjunctivitis and fibrosis observed in ocular MMP.

IL-13 is unique in that it is not thought to exert any control over T cell function,¹⁰ so unlike IL-4, it does not appear to be important in the initial differentiation of CD4⁺T cells into Th2-type cells, but rather appears to be important in the effector phase of inflammation and repair, via its effects on monocytes/macrophages, dendritic cells, fibroblasts, and other inflammatory and stromal cells. IL-13 has been shown to exert direct pro-fibrotic effects on fibroblasts isolated from human lung and skin.^{21–24} Fibroblasts isolated from different bodily sites exhibit different functional properties and site-specific gene expression,²⁵ so these effects cannot necessarily be extrapolated to fibroblasts from other tissues such as the conjunctiva. Although the effects of IL-13 on normal human conjunctival fibroblast proliferation, apoptosis, and matrix metalloproteinase expression have been reported,^{26,27} its effects on other functional activities carried out by normal conjunctival fibroblasts have not been fully investigated.

Fibrosis is an excessive repair response, where the replacement of normal parenchymal tissue by connective tissues is uncontrolled, and there is an imbalance between accumulation of extracellular matrix, and remodeling and collagen turnover. Fibrosis typically results from chronic inflammation due to a persistent irritant that damages tissues.^{12,28} Inflammatory cells, including macrophages and Th2 lymphocytes, play important roles in the chronic inflammation resulting in fibrosis, by releasing cytokines, chemokines, and growth factors, and also through direct interaction with fibroblasts.^{12,16,28} Rather than simply being structural cells, it is also recognized that fibroblasts play an active role in the persistence of inflammation by secreting cytokines and chemokines, recruiting and maintaining the survival of T cells and other inflammatory cells via both soluble factors, and by direct cross talk with lymphocytes via the CD40/CD154 pathway.^{25,29} It has previously been shown that IFN γ up-regulates CD40 expression on human lung fibroblasts, and that IL-13 increases CD154 levels in human lung fibroblasts, but the effects of IL-13 on co-stimulatory molecule expression by human conjunctival fibroblasts has yet to be elucidated.

In the present study, we sought to explore the potential role of IL-13 in ocular MMP, and its effects on

conjunctival fibroblast-related profibrotic and pro-inflammatory activity.

Materials and Methods

Conjunctival Tissues

Bulbar conjunctival biopsies obtained from patients with ocular MMP were used to study the expression of IL-13. The patients were classified according to ocular disease activity as having active disease with acute inflammation ($n = 11$), or chronic disease ($n = 10$) without clinically apparent inflammation following treatment with immunosuppressive therapy. The diagnosis of MMP was based on clinical presentation and direct immunofluorescence of the conjunctiva showing linear IgG, IgA, and/or complement deposition along the basement membrane zone. Conjunctivae from nine normal individuals undergoing routine cataract surgery were used as controls. Details regarding the patients and normal controls are shown in Table 1.³⁰ Institutional research and ethics committee approval was granted and informed consent was obtained from all patients and normal controls participating in the study.

Immunohistochemistry

Immunohistochemistry was performed on glycol methacrylate resin-embedded sections of conjunctiva, prepared as described previously.¹³ In brief, fresh biopsy tissue was fixed overnight in acetone and protease inhibitors at -20°C , post-fixed in acetone then dehydrated with methylbenzoate. The specimens were embedded in glycol methacrylate resin using the JB4 kit (TAAB Laboratories, Aldermaston, UK) and stored at -70°C . Serial sections $2\ \mu\text{m}$ thick were cut, mounted on poly-L-lysine-coated slides and air dried. Endogenous peroxidase was inhibited using 0.3% hydrogen peroxide in 0.1% sodium azide, followed by incubation with 10% fetal calf serum to block nonspecific binding. The sections were incubated overnight at room temperature with a rabbit antibody against human IL-13 (Biogenesis Ltd, Poole UK [5378–8530]), 1:100 dilution. After washing with PBS, biotinylated goat anti-rabbit immunoglobulin (Dako, Cambridgeshire UK) was applied to the sections, followed by incubation with streptavidin-peroxidase (Dako), and finally amino-ethyl carbazole (AEC, Dako), forming a red AEC reaction product. For IL13-CD3 double staining, the slides were washed with PBS and the immunohistochemistry process repeated using a mouse antibody against human CD3 (Dako) 1:10 dilution, and 3,3'-diaminobenzidine (DAB), forming a brown DAB reaction product. The specimens were counterstained with Meyer's hematoxylin (Dako) and mounted with glycerol mounting medium (Dako). Double staining was identified by a brown or combined red-black color, due to the combination of a positive reaction to AEC and DAB. Human tonsil sections were used as positive controls, and the two negative controls used were fetal calf serum and an isotype matched, irrelevant monoclonal antibody (Dako). The

Table 1. Details of Patients and Controls,³⁰ and IL-13 Staining Results

Diagnosis	Age	Sex	Disease duration (yrs)	Bulbar inflammation grade (0 to 4)	Tauber stage ³⁰ (Upper stage/Lower stage)	Topical therapy
Active MMP	54	F	0.5	3	IIcIIIId/IIcIIIId	Hypromellose, acetylcysteine, yellow soft paraffin
	59	M	3	2.5	I/IIa	Hypromellose, lacrilube
	76	M	5	3	IIaIIa(1)/IIbIIb(2)	Hypromellose, chloramphenicol
	80	M	10	3	IIbIIc(2)/IIIdIIId(2)	Carmellose
	55	M	13	3	IIcIIc(2)/IIIdIIId(1)	Dexamethasone, carmellose
	60	F	2	3	I/IIcIIc(3)	Carmellose
	76	F	2	2.5	IIc/IIbIIa(2)	Prednisolone, hypromellose, carmellose
	51	F	0.1	3	I/IIa(1)	Nil
	57	M	0.5	3	IIb/IIcIIb(2)	Dexamethasone, lacrilube
	64	F	1	2	IIa/IIbIIc(2)	Prednisolone
	83	F	7	3	IIa/IIbIIb(2)	Brimonidine, latanoprost, timolol, dorzolamide, carmellose
Treated uninfamed MMP	59	F	10	1	IIbIIb(2)/IIIdIIId(2)	Carbomer 980
	86	F	15	1	IIb/IIcIIb(2)	Nil
	78	F	10	1	IIbIIb(2)/IIIdIIId(2)	Nil
	84	F	2	0	IIb/IIcIIc(2)	Nil
	66	F	2	0	IIb/IIcIIb(2)	Nil
	59	M	4	1	I/IIa	Hypromellose, carmellose, liquid paraffin, chloramphenicol
	76	M	6	1.5	IIIdIIId(2)/IIbIIb(2)	Chloramphenicol, hypromellose, retinoic acid, acetylcysteine
	60	F	3	1	I/IIaIIa(1)	Carmellose
	62	F	0.75	0.5	IIa/IIcIIa(1)	Hyaluronate
	51	F	1	1	I/IIa(1)	Nil
Normal control	50	F	—	0	—	Nil
	76	M	—	0	—	Nil
	65	M	—	0	—	Nil
	70	F	—	0	—	Nil
	82	F	—	0	—	Nil
	65	M	—	0	—	Nil
	75	F	—	0	—	Nil
	73	F	—	0	—	Nil
	62	M	—	0	—	Nil
						(table continues)

number of cells stained in the stroma were counted in a masked fashion in five representative high power fields per patient, using a microscope with a graticule (Leica, Milton Keynes UK).

Isolation of Conjunctival Fibroblasts

Fibroblasts were grown from the conjunctival biopsies of normal human subjects patients as previously described.³¹ The explants were placed into tissue culture wells under a coverslip, and cultured with fibroblast culture medium (FCM) composed of Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat inactivated fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (all from Gibco Invitrogen Ltd, Paisley, UK) at 37°C with 5% (v/v) CO₂ in air. The cells were passaged 1:3

with trypsin/EDTA. Cultures were used between passages 3 and 7 for experiments, and cultures were assessed for typical fibroblast morphology by phase contrast microscopy before every experiment. By passage 3 onwards, contaminating epithelial cells had been eliminated in cultures that had been fed only FCM, due to the fastidious nutritional requirements of epithelial cells.

Serum-free medium (SFM) composed of Dulbecco's modified Eagle's medium supplemented with 0.1% bovine serum albumin (Sigma-Aldrich, Dorset, UK) was used where possible in all experiments where the effect of the addition of IL-13 was being evaluated. Testing under serum-free conditions eliminates the possibility that the observed responses could be due to the activity of unspecified cytokines and growth factors present in serum-containing medium. The optimal con-

Table 1. *Continued*

Systemic therapy	Other eye pathology	Stromal IL-13 staining (grade N to ++++)	Epithelial IL-13 staining (grade N to ++++)
Nil		+++	++++
Nil		++++	++
Mycophenolate	Amblyopia	++	+/N
Mycophenolate + dapsone		++++	++
Mycophenolate + dapsone + deflazocort		+++	+
Mycophenolate + dapsone		+++	N
Mycophenolate + dapsone	Sicca, blepharitis	+	N
Nil		++	N
Nil		+++	N
Nil		++++	+++
Mycophenolate + doxycycline	Glaucoma	++	+/N
Dapsone		++	+++
Nil		+	+
Nil		+	N
Mycophenolate		+	+
Dapsone		+	+++
Cyclophosphamide		+	+++
Cyclophosphamide	Blepharitis	+	++
Cyclophosphamide + dapsone		+	++
Prednisolone		++	+++
Cyclophosphamide + prednisolone		++	+++
Nil	Cataract	++	++
Nil	Cataract	N	+
Nil	Cataract	N	++
Nil	Cataract	+/N	N
Nil	Cataract	+/N	N
Nil	Cataract	+/N	N
Nil	Cataract	N	+
Nil	Cataract	N	N
Nil	Cataract	+/N	+

MMP mucous membrane pemphigoid, F = female, M = male, N = Nil.

+, minimal extent and intensity of staining; ++, mild extent and intensity of staining; +++, moderate extent and intensity of staining; +++++, marked extent and intensity of staining.

centration of IL-13 used in experiments was initially determined from previous results.^{32,33} If this level did not result in a significant response, a range of IL-13 concentrations (1, 10, 100 ng/ml) was then evaluated in a preliminary dose-response experiment. The concentration that showed the greatest response in this preliminary experiment was then selected as the optimal concentration for that assay. 3T3 cells (a fibroblast cell line) were used for initial calibration experiments that compared the negative control SFM versus the positive control FCM.

Collagen Contraction

To assess matrix contraction in the presence of IL-13, free-floating collagen lattice models were used. Three-dimensional fibroblast-populated type I rat tail collagen

(5 mg/ml; Sigma-Aldrich, Dorset, UK) lattices were prepared with 16.7×10^5 cells/ml of lattice mixture as previously described.³⁴ The collagen lattices were incubated for 2 hours at 37°C to set in the culture wells, then the lattices were detached immediately after feeding with either 1) 10 ng/ml IL-13 (recombinant human IL-13, R&D systems, Abingdon, UK) in SFM, or 2) the positive control of 10% fetal calf serum-containing FCM, or 3) the negative control of SFM alone. Gelatin sepharose beads (Gelatin Sepharose 4B, Amersham Biosciences, Buckinghamshire, UK) were incubated in serum at a concentration of 10% with gentle rocking for 2 hours to produce gelatinase-free serum. Reduction in lattice area at days 1, 3, and 7 due to contraction was digitally photographed, and the gel areas calculated using image analysis software (Image J public domain Java image processing program <http://rsb.info.nih.gov/ij/>).

Matrix Metalloproteinase Protein Levels

Conditioned medium collected from contracting lattices was analyzed for matrix metalloproteinases (mmp)-1, mmp-2, mmp-3, mmp-8, mmp-10, and mmp-13; and tissue inhibitor of matrix metalloproteinases (timp)-1, timp-2, and timp-4 protein levels by using an antibody-coated membrane array (Raybiotech Inc, Norcross, GA) in accordance with the manufacturer's instructions. The mmp and timp levels in baseline control medium were subtracted from the conditioned medium results.

Collagen Type I Secretion

Conditioned medium collected from contracting lattices under both serum-free and serum-containing conditions, in the presence and absence of IL-13 [10 ng/ml], was analyzed for secretion of the C-terminal propeptide of type I collagen using an enzyme-linked immunosorbent assay (Quidel Corp, San Diego CA) performed according to the manufacturer's instructions.

Proliferation

Fibroblast cell division in the presence or absence of IL-13 was analyzed by flow cytometry using the fluorescent probe carboxyfluorescein diacetate, succinimidyl ester (CFSE; Vybrant CFDA SE Cell Tracer Kit, Invitrogen, Paisley UK). Serum-deprived fibroblasts were incubated with 1 μ M CFSE in serum-free medium at 37°C for 10 minutes, and ice-cold FCM was added to stop the reaction. The cell pellet was then resuspended in ice-cold FCM for 30 minutes to allow free CFSE to come out of the cells. The cell pellet was then resuspended at 2×10^5 cells/ml in FCM, and the cells were seeded into 6-well tissue culture plates at a density of 4×10^5 cells per well and stimulated with IL-13 10 ng/ml in FCM. After 72 hours, the cells were detached with trypsin and 10,000 events acquired for flow cytometry (FacsCalibur, BD Pharmingen). Winlist software (Verity Software House, Topsham, ME) was used to analyze the number of divided cells per 5000 undivided cells.

Migration

Cell culture inserts incorporating polyethylene terephthalate membranes with a pore size of 8 μ m (BD Falcon, WVR International, Leicestershire UK), which fit into wells in a tissue culture plate, were used to assess fibroblast migration in the presence and absence of IL-13, with or without the addition of T cell culture supernatant (see below). Cells were seeded into the inserts at a density of 8000 per insert in 200 μ l SFM and allowed to attach to the upper surface of the membrane for 4 hours. A volume of 700 μ l of IL-13 [10 ng/ml] in SFM was added to the well in the tissue culture plate, so that the IL-13-containing medium was in contact with the undersurface of the membrane in the culture insert. SFM was used as a negative control, and 10% bovine serum FCM as a positive control. The cells were incubated for 16 hours to permit migration through the pores, to the undersurface of the membrane. The culture

inserts were then washed with PBS to remove excess protein, fixed in 70% (v/v) methanol for 5 minutes, then stained with Mayer's hematoxylin (Dako) for 30 minutes and rinsed in tap water. Settled cells on the upper surface of the membrane in the culture inserts were removed using a cotton swab. The number of migrated cells on the undersurface of the membrane was counted per $\times 10$ objective field (average of five fields) was counted using an inverted microscope and cell counting software (Image J).

T Cell Culture Supernatant

Supernatant from culture of a conjunctival T cell line derived from an atopic keratoconjunctivitis patient stimulated with phytohaemagglutinin was obtained from previous experiments.³⁵ Previous testing by enzyme-linked immunosorbent assay indicated that this supernatant contained IL-4, IL-5, IL-13, IL-10, and IFN γ . This supernatant was tested as a chemoattractant in the lower chamber of the migration assay tissue culture plate at a concentration of 1:10 in SFM, either alone or in combination with IL-13 10 ng/ml.

Immunofluorescence Staining and Flow Cytometry

Confluent fibroblasts were cultured in 6-well plates in the presence of various concentrations of IL-13 [1, 10, 100 ng/ml] or IFN γ [1 ng/ml] (Peprotech, London UK) in SFM for 72 hours. A range of IL-13 concentrations was used in this assay to detect any dose-dependent effect. For three-color immunofluorescence staining, fibroblasts were washed with Ca²⁺ and Mg²⁺-free Hanks' balanced salt solution and detached with collagenase [1 mg/ml] in Hanks' balanced salt solution. The cell suspension containing at least 1×10^5 cells was centrifuged at 4000 rpm for 4 minutes, the cell pellet resuspended in 10% mouse serum for 20 minutes to block nonspecific binding, then the cells were stained in suspension with 5 μ l each of either anti-ICAM-1^{PE}, anti-CD80^{FITC} and anti-HLA-DR^{PerCP}, or anti-CD154^{FITC}, anti-CD86^{PE}, and anti-CD40^{PECy5} (all from BD Pharmingen) for 30 minutes at 4°C before washing twice and acquiring 10,000 events for flow cytometry (FacsCalibur, BD Pharmingen). Isotype matched, irrelevant monoclonal antibodies were used as negative controls (BD Pharmingen). Winlist software (Verity Software House, Topsham, ME) was used for analysis. Analyses were performed on a population of live cells gated by forward and side scatter to include the fibroblast population. At least 10,000 events were acquired in the live region, and the percentages of positive cells were calculated after subtracting background staining.

Statistical Analysis

Differences between two groups were examined for statistical significance using the Mann-Whitney *U*-test and one-way analysis of variance (analysis of variance) was used to compare the means of three or more unmatched groups. *P* < 0.05 was considered to be statistically significant.

Figure 1. Human conjunctiva of ocular MMP patients show IL-13 expression. Immunohistochemistry of bulbar conjunctival sections showing a negative isotype mAb control of a normal subject (A), very little IL-13 or CD3 staining in a normal subject (B), a negative isotype mAb control of a patient with actively inflamed MMP (C), and positive IL-13 (red, AEC), and CD3 (brown, DAB) double staining in a patient with actively inflamed MMP (D). **Thick arrows** indicate double-stained IL-13⁺CD3⁺ cells; **thin arrow** indicates single-stained IL-13⁺CD3⁻ cell. Scale bar = 100 μ m. AEC amino-ethyl carbazole, DAB 3,3'-diaminobenzidine. **E:** Cell counts of positive stromal IL-13/CD3 staining in the conjunctiva of human ocular MMP and normal controls. Many stromal IL-13-expressing cells are present in patients with active MMP (39 cells/mm², 80% (31/39) of which also express the T cell marker CD3). Following immunosuppressive therapy, in clinically uninfamed treated ocular MMP, the number of stromal IL-13-expressing cells is significantly reduced to 44% (17/39) of the number of cells present in active disease (17 cells/mm²), but this is still fourfold greater than the number of stromal IL-13-expressing cells present in normal controls (4 cells/mm²). * $P < 0.001$. Results are the mean and SE from at least nine individuals per group, counting at least nine fields per individual.

Results

IL-13 Is Expressed in MMP Conjunctiva in Both Active Disease and Clinically Uninfamed Treated Disease

There was minimal expression of IL-13 in the substantia propria of normal conjunctiva (Figure 1, A and B). In contrast, in active autoimmune conjunctivitis due to MMP there was a significant increase in the number of stromal cells expressing IL-13 (39 ± 1 cells/mm²) (Figure 1, C and D), 80% (31/39) of which were also positive for the T cell marker CD3 (Figure 1E). Following immunosuppressive therapy, in clinically uninfamed, treated, MMP patients, the number of stromal IL-13-expressing cells was significantly reduced to 44% (17/39) of the number of cells present in active disease (17 ± 0.5 cells/mm², $P < 0.001$), but this was still fourfold greater than the number of stromal IL-13-expressing cells present in normal con-

Figure 2. **A:** Contraction of fibroblast-populated collagen lattice gels by normal human conjunctival fibroblasts in response to the addition of IL-13 under serum-free conditions. SFM containing 0.1% BSA was the negative control. FCM containing 10% fetal calf serum was the positive control. Results are the mean and SE from six or more individual donors with at least two separate experiments per donor. IL-13 stimulates collagen contraction by normal human conjunctival fibroblasts. **B:** Matrix metalloproteinase (mmp) and tissue inhibitor of matrix metalloproteinase (timp) levels in conditioned medium of conjunctival fibroblast-populated collagen gels incubated over 7 days in the presence of IL-13, as detected by antibody-coated membrane array. Data are relative values compared with the membrane array negative control. There was a significant decrease in mmp-3 ($P = 0.05$) and mmp-10 ($P = 0.01$) levels in the conditioned medium from IL-13-treated collagen gels, in comparison with the untreated control 0.1% BSA, SFM collagen gels. Results are the mean and SE from six individual donors. * $P \leq 0.05$. **C:** Effect of IL-13 on normal human conjunctival fibroblast migration. Conjunctival fibroblasts were seeded into culture inserts incorporating porous membranes, and allowed to migrate overnight toward the lower well chamber containing the test substance. SFM containing 0.1% BSA was the negative control. FCM containing 10% fetal calf serum was the positive control. IL-13 stimulated fibroblast migration under serum-free conditions. Results are the mean and SE of five individual experiments. * $P = 0.01$.

junctiva (4 ± 0.3 cells/mm², $P < 0.001$). The proportion of CD3-positive cells was similar across the three groups: 80% (31/39), 76% (13/17), 75% (3/4) (Figure 1E). Stromal cell IL-13 staining was present in 11/11 patients with active ocular MMP and 10/10 patients with treated ocular MMP (Table 1).³⁰ Occasional stromal cells stained positive in 5/9 normal controls. Epithelial IL-13 staining was present in 7/11 patients with active MMP, 9/10 patients with treated MMP, and 5/9 normal controls.

Figure 3. Effect of IL-13 combined with T cell supernatant on normal human conjunctival fibroblast migration. Conjunctival fibroblasts were seeded in the upper chambers of porous membranes and allowed to migrate overnight toward the lower chamber containing the test substance. The negative control was SFM containing 0.1% BSA. T cell supernatant (SN) was diluted 1:10 in SFM and 10 ng/ml IL-13 was used. The combination of IL-13 and T cell supernatant acts synergistically to significantly enhance fibroblast migration. Results are the mean and SE of three individual experiments. * $P = 0.03$, one way analysis of variance.

IL-13 Stimulates Collagen Contraction, Chemotaxis and Reduced Secretion of mmp-3 and mmp-10 by Normal Human Conjunctival Fibroblasts

To investigate the effect of IL-13 on direct profibrotic activity by normal human conjunctival fibroblasts, the addition of IL-13 under serum-free conditions to fibroblast-populated collagen gel lattices was investigated, and the conditioned medium from the lattices was analyzed for secretion of mmps, timp, and the C-terminal propeptide of type I collagen. We also investigated the effect of IL-13 on fibroblast proliferation and migration. We found that IL-13 stimulated collagen contraction by normal human conjunctival fibroblasts (Figure 2A), and that mmp-3 and mmp-10 secretion by normal human conjunctival fibroblasts is decreased in the presence of IL-13 (Figure 2B). It is not clear whether the observed small differences in mmp-3 and -10 represent physiologically relevant changes in these enzymes. We also found that IL-13 stimulates chemotaxis of these cells (Figure 2C).

No significant change in type I collagen production was detected, in response to the addition of IL-13 [10 ng/ml] in both serum-free and serum-containing collagen lattice experiments (data not shown). We also found no significant differences in fibroblast proliferation in the presence of 10 ng/ml IL-13 compared with the FCM negative control (data not shown).

IL-13 Augments the Stimulatory Effect of T Cell Culture Supernatant on Fibroblast Chemotaxis

To evaluate whether IL-13 had a synergistic effect, in combination with T cell-derived mediators, on normal human conjunctival fibroblasts, we investigated the effect of the addition of T cell culture supernatant to the chemotaxis assay. We found that the combination of IL-13 and T cell culture supernatant significantly augmented

Figure 4. A: Normal human conjunctival fibroblasts were treated with various concentrations of IL-13 [1, 10, 100 ng/ml] or IFN γ [1 ng/ml] for 72 hours under serum-free conditions, and the percentage of conjunctival fibroblasts expressing on their cell surface HLA-DR, ICAM, CD80, CD86, CD40, and CD154 was determined by flow cytometry. SFM containing 0.1% BSA was the negative control. 100 ng/ml IL-13 significantly up-regulated the surface expression of HLA-DR, CD80, CD40, and CD154. As a comparison, 1 ng/ml IFN γ up-regulated HLA-DR, CD40, and CD154 expression. Results are the mean $\pm$ SE from six individual donors. * $P < 0.05$. B: Representative density plots presenting flow cytometric analyses of the up-regulated surface expression of CD40, CD154, CD80, and HLA-DR in the presence of 100 ng/ml IL-13 compared with the untreated control 0.1% BSA SFM by a normal human conjunctival fibroblast primary cell line.

the effect of either treatments alone (Figure 3). Given that IL-13 was also present in the T cell supernatant, the increased migration observed could have been due to an increase in IL-13, or it could have been due to synergism between IL-13 and the other mediators present in the supernatant.

IL-13 Up-Regulates Surface Expression of HLA-DR, CD80, CD40, and CD154 on Normal Human Conjunctival Fibroblasts

Interactions between tissue fibroblasts and infiltrating T lymphocytes via the CD40/CD40L pathway have been shown to powerfully co-stimulate T lymphocyte proliferation and to activate fibroblasts, thus potentially promoting fibrogenesis.³⁶ We investigated the surface expression of human leukocyte antigen (HLA)-DR, intercellular adhesion molecule (ICAM)-1, and the T cell-costimulatory molecules CD80, CD86, CD40, and CD154 by normal human conjunctival fibroblasts in response to 72 hours' stimulation with a range of IL-13 concentrations [0, 1, 10, 100 ng/ml] or IFN γ [1 ng/ml] under serum-free conditions. ICAM was constitutively expressed by a high percentage

of fibroblasts ($84.3\% \pm 13.5\%$), and there was no significant change in the percentage of cells expressing ICAM in the presence of IL-13 (Figure 4A). In contrast, HLA-DR, CD80, CD40, and CD154 were minimally expressed under basal serum-free conditions (HLA-DR $0.7\% \pm 0.4\%$, CD80 $7.2\% \pm 2.5\%$, CD40 $0.3\% \pm 1.8\%$, CD154 $1.28\% \pm 2.0\%$) and all were significantly up-regulated in response to stimulation by IL-13, but only at the maximum concentration of 100 ng/ml (HLA-DR $7.5\% \pm 3.9\%$ $P = 0.01$; CD80 $12.7\% \pm 9.4\%$ $P = 0.04$; CD40 $52.8\% \pm 9.9\%$ $P = 0.03$; CD154 $22.4\% \pm 5.6\%$ $P = 0.03$, Figure 4B). No significant change in either the percentage of expression of CD86 was detected, nor in the intensity of expression of ICAM (data not shown). In comparison, IFN γ [1 ng/ml] was found to significantly up-regulate HLA-DR, CD40, and CD154 expression, but not CD80.

Discussion

In this study we have shown that IL-13 is expressed in the conjunctiva in the scarring condition MMP and that it has direct effects in stimulating normal human conjunctival fibroblast collagen contraction and migration and modifying matrix metalloproteinase secretion *in vitro*, which could lead to connective tissue remodeling. IL-13 also promotes the active involvement of these cells in pro-inflammatory activities by upregulating HLA-DR, CD80, CD40, and CD154 expression, thus potentially enabling the fibroblasts to cross talk with lymphocytes and other inflammatory cells. IL-13 also appears to act synergistically with lymphocyte-secreted products to augment conjunctival fibroblast chemotaxis.

Our findings of increased collagen contraction and migration by conjunctival fibroblasts in response to IL-13 are similar to reports from other studies where human lung fibroblasts were investigated.^{22,23} Reduced matrix metalloproteinase-3 secretion by normal human conjunctival fibroblasts in response to IL-13 has similarly been described by Fukuda et al,²⁷ but they did not investigate matrix metalloproteinase-10 secretion, which we also found was reduced by IL-13. Moreover, they also found increased release of timp-2, which was not detected by our assay method. These differences are probably due to the different culture conditions. Matrix metalloproteinase-3 is a stromelysin that cleaves pro-mmps 1, 7, 8, 9, and 13, laminin and fibronectin.³⁷ It is thus a key regulator of mmp activity, and reduced secretion of mmp-3 is consistent with reduced degradation of extracellular matrix and a net increase in collagen. Matrix metalloproteinase-10 is also a stromelysin, and has similar activities to mmp-3.

An exciting observation from this study is that IL-13 up-regulates expression of the T cell co-stimulatory molecules CD80, CD40, and CD154 (CD40 ligand) on human conjunctival fibroblasts, as well as HLA-DR expression. It would be useful to confirm this up-regulated expression by additional methods such as Western blotting, immunocytochemistry, confocal microscopy, and reverse transcription PCR. Up-regulated surface molecule expression only occurred at a concentration of 100

ng/ml IL-13. A similar threshold response to IL-13 has previously been demonstrated.³⁸ It may be that a threshold exists, above which the signaling pathway to induce the transcriptional changes necessary for surface molecule expression is active. While the CD40/CD154 pathway has been implicated in the development of fibrosis in the lung, it has not previously been investigated with respect to conjunctival fibroblasts. The CD40/CD154 interaction is a potent activator of fibroblasts, and we hypothesize that the promotion of autocrine and paracrine activation of conjunctival fibroblasts via this pathway by IL-13, which up-regulates both expression of the ligand (CD40) and the receptor (CD154) on these cells, plays a role in the perpetuation of inflammation and wound healing responses in MMP. Up-regulated expression of CD154 in human lung fibroblasts by IL-13 has previously been reported by Kaufman et al,³⁹ who detected CD154 more readily intracellularly than on the surface of lung fibroblasts. It is possible that they were less able to detect surface CD154 because they used trypsin to detach fibroblasts for flow cytometry rather than collagenase. Trypsin cleaves surface molecules more readily than collagenase.⁴⁰ Given that the expression of IL-13 was still significantly elevated in conjunctiva that appeared 'white' and clinically uninfamed following systemic immunosuppressive treatment, progressive fibrosis may be driven by this underlying chronic inflammation, and fibroblasts may be playing an active role in perpetuating this chronic inflammation.

We also detected up-regulated expression of CD80 (B7-1) on conjunctival fibroblasts. While binding of CD80 to its receptor CD28 is an essential co-stimulatory step in T cell activation, there is an inhibitory receptor for the B7 ligands, CTLA-4 (cytotoxic T lymphocyte-associated antigen 4/CD152),⁴¹ which is up-regulated after T cell activation has peaked, and limits the degree of activation. It is possible that engagement of the inhibitory receptor CTLA-4 by the CD80 expressed on conjunctival fibroblasts regulates the activation process. Tesavibul et al⁴² have previously reported significantly high CD86 (B7-2) expression in the conjunctival substantia propria of MMP, compared with normal conjunctiva, and minimal detection of CD80. The CD86 positive cells detected in their study were thought to be mostly tissue macrophages and Langerhans' cells. Differences in CD80/CD86 expression between their study and our findings may also reflect differences in regulation of expression, given that B7-1 (CD80) is expressed in a tightly regulated manner, but B7-2 (CD86) is constitutively expressed on the cell surface of antigen presenting cells.

The principal mediators responsible for the observed synergistic enhancement of the chemotactic effect of IL-13 with T cell supernatant are most likely IL-2, IL-4, IL-5, and/or IFN γ .⁴³ Antibody neutralization experiments would aid in identifying the responsible mediators. It is possible that other actions of IL-13 on fibroblasts require synergism with other growth factors.

In contrast with previous studies which used the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to assess cell proliferation,^{26,32} we were not able to detect any increase in

proliferation of normal conjunctival fibroblasts in response to IL-13 using CFSE flow cytometry. This may have been due to differences in the method used.

Leonardi et al³² showed increased procollagen I production in response to IL-13 by vernal keratoconjunctivitis fibroblasts grown on culture plates in fresh ascorbic acid with 0.4% serum. Unlike Leonardi et al, we were not able to detect any change in collagen secretion in the conditioned medium from contracting collagen lattices, either under serum-free conditions or in the presence of 10% serum. There could be several reasons for the difference in our findings: in our experiments the fibroblasts were actively contracting the collagen lattices and thus perhaps unlikely to divert their metabolic activities to synthesizing collagen at the same time, or alternatively the culture medium conditions were not optimal for collagen synthesis, or because of phenotypic differences between vernal keratoconjunctivitis and normal conjunctival fibroblasts, or because IL-13 alone has only a modest effect on collagen synthesis, and synergism with another factor such as transforming growth factor- β is necessary.⁴⁴

In summary, the present study indicates that IL-13 contributes to conjunctival fibrosis in MMP, and that by promoting the ability for cross talk between conjunctival fibroblasts and other inflammatory cells, it may facilitate fibroblast-generated chronic inflammation and perpetuate fibrosis. Development of a suitable animal model of conjunctival fibrosis will enable testing of hypotheses raised by these findings, and guide development of effective inhibitory therapies, used in conjunction with systemic immunosuppression, to target conjunctival fibrosis in this disease.

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