

Corneal and Conjunctival Fibroblasts Are Major Sources of Eosinophil-Recruiting Chemokines

Kazumi Fukagawa^{1,2,3}, Naoko Okada^{1,2}, Hiroshi Fujishima^{1,4}, Toshiharu Nakajima², Yoji Takano^{1,4}, Mari Tanaka¹, Murat Dogru¹, Yoshiyuki Satake³, Kazuo Tsubota¹ and Hirohisa Saito²

ABSTRACT

Background: Differential expression of chemokine genes were investigated in various types of ocular surface cells.

Methods: Primary cultures of human corneal epithelial cells ($n = 3$), corneal fibroblasts ($n = 2$), conjunctival epithelial cells ($n = 2$) and conjunctival fibroblasts ($n = 2$) were established and incubated with or without interleukin (IL)-4 (30 ng/ml) and tumor necrosis factor (TNF)- α (30 ng/ml) for 24 hours. Gene transcription levels of 33 chemokines and production of 4 chemokines were analyzed.

Results: After stimulation, chemokine expression increased for 18 of 33 coded chemokine gene transcripts. In stimulated conjunctival and corneal cells, CC chemokine genes increased in fibroblasts (expression of 6 out of 8 genes), while CXC chemokine genes increased in both epithelial cells (expression of 4 out of 9 genes in conjunctival epithelial cells and 7 out of 9 genes in corneal epithelial cells) and in fibroblasts (expression of 8 out of 9 genes in conjunctival and corneal fibroblasts). Except for MCP-1, gene transcription levels for most CC chemokines were inducible and, except for IP-10 and I-TAC, most CXC chemokines were constitutively expressed. Corneal epithelial cell and fibroblast production patterns for eotaxin-1, MCP-1 and IP-10 were comparable to the mRNA expression pattern.

Conclusions: Corneal and conjunctival fibroblasts exhibited marked increases in the expression of chemokines upon stimulation with TNF- α and IL-4, suggesting that fibroblasts may be one of the primary sources of chemokines in allergic conjunctival diseases. Therefore, regulation of chemokine production from these cells may be an effective strategy for treating such diseases.

KEY WORDS

chemokine, conjunctiva, epithelial cells, fibroblast, gene expression

INTRODUCTION

Allergic inflammation may be augmented by many types of inflammatory cells recruited by the ocular surface cells, such as T cells, eosinophils and neutrophils. Chemokines undoubtedly play major roles in the recruitment of these inflammatory cells.¹⁻³ Ocular tissue constituent cells along with tissue resident mast cells and recruited inflammatory cells are

thought to be the major sources of these chemokines.^{4,5}

In 1993, interleukin (IL)-8 production from corneal epithelial cells was first reported,⁶ followed by several reports on IL-8 production from corneal fibroblasts⁷ and conjunctival epithelial cells.^{8,9}

In 1995, "regulated upon activation, normal T cell expressed and secreted" (RANTES) was reported to occur in the tears of allergic conjunctivitis patients.¹⁰

¹Department of Ophthalmology, Keio University, ²Department of Allergy & Immunology, National Research Institute for Child Health and Development, ³Ryogoku Eye Clinic and ⁴Department of Ophthalmology, International University of Health and Welfare, Tokyo, Japan.

Author contributions: Kazumi Fukagawa drafted the paper. Naoko Okada conducted the gene chip experiment and ELISA assay. Hiroshi Fujishima supervised and contributed to the primary culture of human cells. Toshiharu Nakajima supervised the gene chip analysis. Yoji Takano, Mari Tanaka, Murat Dogru, and Yoshiyuki

Satake contributed to the primary culture of human cells. Kazuo Tsubota supervised the study plan. Hirohisa Saito generally supervised the study plan.

Correspondence: Kazumi Fukagawa, MD, Ryogoku Eye Clinic, 4-33-12 Ryogoku, Sumida-ku, Tokyo 130-0026, Japan.

Email: BR5K-FKGW@asahi-net.or.jp

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We have previously reported that eotaxin-1 concentrations in tears correlate with the severity of allergic corneal damage.¹¹ These findings suggest CC chemokine involvement in the pathogenesis of the eosinophilic tissue damage in allergic conjunctival diseases.¹² Although we have previously reported that anti-CC chemokine receptor (CCR) 3 and anti-CCR1 antibody suppressed eosinophil chemotaxis induced by tear samples, this suppression was not complete.¹³ This suggests that in addition to eotaxin-1 and RANTES, other chemokines or factors are also present in tears and contribute to eosinophil recruitment.

Primary cultures and cell lines of corneal and conjunctival cells have been widely used in studies on CC chemokine production.^{7,14,15} We have reported that IL-4 and tumor necrosis factor (TNF)- α induced eotaxin-1 production in corneal fibroblasts, but not in corneal epithelial cells.⁵ Although there may be differences in chemokine production patterns between the cell types, comprehensive studies on chemokine production from ocular surface constituent cells have yet to be reported.

The chemokine production profile of the ocular surface constituent cells is of great interest to ophthalmologists, as this has the potential to lead to new treatment strategies directed towards suppressing chemokine production or towards blocking chemokine receptors in allergic tissue.² In this study, we used high-density oligonucleotide probe arrays (GeneChip®) and an enzyme-linked immunosorbent assay (ELISA) to investigate comprehensive chemokine gene expression and production in primary cultures of corneal epithelial cells, corneal fibroblasts, conjunctival epithelial cells and conjunctival fibroblasts.

METHODS

CULTURE OF HUMAN CORNEAL EPITHELIAL CELLS AND FIBROBLASTS

Human corneas were obtained from the American Eye Bank Association. Cultures of human corneal epithelial cells ($n = 3$ for gene chip and $n = 3$ for ELISA) were established as has been previously reported.¹⁶ In brief, corneas with adjacent limbus were dissected into small pieces and cultured in collagen-coated 35-mm culture dishes (Iwaki Co., Tokyo, Japan) in supplemental hormonal epithelial medium (SHEM).¹⁷ The medium consisted of an equal volume of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12, which contained bicarbonate, 0.5% dimethyl sulfoxide, 10 ng/mL human EGF, 5 μ g/mL insulin, 0.1 μ g/mL cholera toxin A subunit, 10% fetal calf serum (FCS), and penicillin G-streptomycin. Cultures were performed using standard conditions (37°C, 95% humidified air and 5% CO₂) until confluence was obtained.

Human corneal fibroblasts were established in culture ($n = 2$ for gene chip and $n = 3$ for ELISA) as described by Cubitt *et al.*⁶ In brief, dissected corneal pieces were cultured in DMEM/F12 (1 : 1 mixture of DMEM and Ham's F12; Gibco, Grand Island, NY, USA) with 10% FCS on collagen-coated 35-mm culture dishes (Iwaki Co.).

Cultured cells were studied between the third and fifth passages. Purity of each cell type was assessed by cell morphology and differential activities towards anti-cytokeratin antibodies; i.e., corneal fibroblasts were positive for anti-vimentin antibodies (Boehringer Mannheim, Indianapolis, IN, USA), but not anti-keratin AE1/AE3 (Progen Biotechnik GmbH, Heidelberg, Germany).

CULTURES OF HUMAN CONJUNCTIVAL EPITHELIAL CELLS AND FIBROBLASTS

All human subjects in this study provided written informed consent. The study was approved by the Ethical Review Board at the Tokyo Dental College Ichikawa Hospital (Chiba, Japan) and adhered to the principles embodied in the Declaration of Helsinki of 1995 (as revised in Edinburgh 2000). Approximately 1×3 mm of conjunctival tissue was obtained during chalazion or cataract operations. Primary cultures of conjunctival epithelial cells ($n = 2$) and fibroblasts ($n = 2$) were established as corneal epithelial cells and fibroblasts, as described above.

ACTIVATION OF CORNEAL AND CONJUNCTIVAL CELLS

At the third to fifth passage, cells were removed from the culture dishes by diluting the cultures (1 : 4) with 0.25% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) (Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS). Corneal and conjunctival fibroblasts were resuspended with DMEM/F12 containing 10% FCS and preincubated overnight in 150-cm² flasks for the gene chip experiments (5×10⁶ cells/flask) or on 96-well culture plates for the ELISA assay (1×10⁴ cells/well). Epithelial cells were resuspended with SHEM medium containing 10% FCS and preincubated overnight in 75-cm² flasks for the gene chip experiments (1×10⁷ cells/flask) or on 96-well culture plates for the ELISA assay (5×10⁴ cells/well). After washing with PBS, the culture medium was changed to DMEM without FCS for the next 24 hours.

Cells were then incubated with recombinant (r) human (h) IL-4 (R&D Systems, Minneapolis, MN, USA) (30 ng/mL) and rhTNF- α (R&D Systems) (30 ng/mL) for 24 hours. Samples were collected by pipetting, with the remaining cells removed by centrifugation at 15000 rpm for 5 minutes. The RNeasy Mini Kit (Qiagen, Valencia, CA, USA) was used to obtain the total RNA from the collected cells, after which the samples were stored at -20°C until performance of

further gene chip analysis.

For ELISA, cells were incubated with the combinations of IL-4 (0.3, 3, 30 ng/ml) and TNF- α (0.3, 3, 30 ng/ml) for 48 hours. Supernatants were stored at -70°C until further ELISA assays were performed.

OLIGONUCLEOTIDE ARRAY SEQUENCE ANALYSIS

The Human Genome U95A probe array (GeneChip®, Affymetrix, Santa Clara, CA, USA), which contains an oligonucleotide probe set for approximately 12,000 full-length genes, was used to examine the human genome-wide gene expression in accordance with the manufacturer's protocol (Expression Analysis Technical Manual) and a previous report.¹⁸

Total RNA (5 µg) was extracted from approximately 10⁷ cells. Double-stranded cDNA was synthesized using a SuperScript Choice System (Life Technologies, Rockville, MD, USA) and a T7-(dT) 24 primer (Amersham Pharmacia Biotech, Buckinghamshire, UK). The cDNA was subjected to *in vitro* transcription in the presence of biotinylated nucleoside triphosphates using a BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Inc., Farmingdale, NY). The biotinylated cRNA was hybridized with a probe array for 16 hours at 45°C. After washing, the hybridized biotinylated cRNA was stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA) and then scanned with a HP Gene Array Scanner. The fluorescence intensity of each probe was quantified using a computer program, GeneChip® Analysis Suite 4.0 (Affymetrix). The expression level of a single mRNA was determined as the average fluorescence intensity among the intensities obtained by 6-20-paired probes (perfect-matched and single nucleotide-mismatched), and which consisted of 25-base oligonucleotides. If the intensities of the mismatched probes were extremely high, gene expression was judged to be absent even if there was a high average fluorescence obtained with the GeneChip® Analysis Suite 4.0 program. The GeneChip® software was employed to determine the level of the gene expression, which was calculated as the average difference (AD). The percentages for the specific AD levels versus the mean AD levels of the six probe sets for the housekeeping genes (β -actin and glyceraldehyde-3-phosphate dehydrogenase) were then calculated.

ELISA FOR CHEMOKINES

Human eotaxin-1, MCP-1, IP-10 and GRO- α concentrations were measured by ELISA kits (R&D). The detection limits of these assay kits were approximately 3 pg/ml.

STATISTICAL ANALYSIS

Differences between two paired groups were analyzed by a paired Student's *t*-test with *p* < 0.05 consid-

ered to be significant. Values are expressed as the mean $\pm$ SD.

RESULTS

ELEVATED TRANSCRIPTS IN ACTIVATED CORNEAL AND CONJUNCTIVAL CELLS

We used GeneChip® to examine the expression levels of approximately 12,000 genes in the corneal and conjunctival cells before and after stimulation with IL-4 and TNF- α (*n* = 2-3 for each of the cell types). Fold increase was obtained by calculating the AD levels of the activated cells versus those of the resting cells. The "average fold increase" was obtained by averaging the fold increase for each sample. After elimination of the redundant transcripts, we selected the 30 most upregulated transcripts for each of the cell types.

In the conjunctiva, three genes for the CC chemokines (RANTES, TARC and eotaxin-1) were listed among the top 30 most increased genes for the fibroblasts, while only one (RANTES) was noted to increase in the epithelial cells (Table 1). In the cornea, three genes for the CC chemokines (eotaxin-1, MCP-2 and RANTES) were among the top 30 most increased genes for the fibroblasts, while only one (LARC) was observed for the epithelial cells (Table 2).

COMPARISON OF CHEMOKINE GENE EXPRESSIONS BETWEEN CELL TYPES

As seen in Table 3, we selected 33 chemokine-associated genes and 15 chemokine receptor-associated genes. Fold increases were calculated and judged as "increased" when there was a two-fold increase in the AD for the stimulated cells.

In both the stimulated conjunctival and corneal cells, CC chemokine-associated genes primarily increased in the fibroblasts, while CXC chemokine-associated genes increased in both the epithelial cells and the fibroblasts. In six out of eight of the "increased" CC chemokine genes, a dominant increase was noted in the fibroblasts, while in six out of nine of the "increased" CXC chemokine genes, increases in both the fibroblasts and the epithelial cells were noted. In conjunctival cells, one gene for CC chemokine (RANTES) and four genes for CXC chemokines (GRO- α , CKA-3, IL-8 and IP-10) were judged as being "increased" in the epithelial cells, while six genes for CC chemokines (MCP-1, 2, 4, RANTES, eotaxin-1 and TARC) and eight genes for CXC chemokines (GRO- α , β , γ , CKA-3, IL-8, MIG, IP-10 and I-TAC) were judged as "increased" in the fibroblasts. In corneal cells, three genes for the CC chemokines (RANTES, LARC and TECK) and seven genes for the CXC chemokines (GRO- α , β , γ , ENA-78, CKA-3, IL-8, and IP-10) were judged as "increased" in the epithelial cells, while six genes for the CC chemokines (MCP-1, 2, 4, RANTES, eotaxin-1, and

Table 1 Top 30 increased transcripts in the conjunctival epithelial cells and fibroblasts

Conjunctival epithelial cells (n=2)			Conjunctival fibroblasts (n=2)		
Name of transcript (protein)	Accession number [†]	Average fold increase [‡]	Name of transcript (protein)	Accession number	Average fold increase
Squamous cell carcinoma antigen 2	U19557	2997.3	Diubiquitin	AL031983	6533.9
3-beta-hydroxysteroid dehydrogenase	M38180	766.2	Bone marrow stromal cell antigen 2	D28137	2527.8
Occludin	U49184	511.6	Viperin (cig5)	AF026941	2303.4
Interleukin-13 receptor	U70981	382.5	<u>IP-10</u>	<u>X02530</u>	1694.7
IFN-beta 2a mRNA	X04430	362.4	Endothelial Cell Growth Factor 1	HG544-HT544	1495.3
<u>CKA-3[§]</u>	<u>U81234</u>	344.2	2-5' oligoadenylate synthetase	AJ225089	1467.5
I cell membrane-associated protein hT1a-2	AF030428	329.9	<u>I-TAC</u>	<u>AF030514</u>	944.3
DGCR6 protein	X96484	329.5	<u>RANTES</u>	<u>M21121</u>	843.8
RNA polymerase II	L37127	283.6	Thyroid receptor interactor	L40387	587.6
Cholesterol 25-hydroxylase	AF059214	260.3	Metalloproteinase	L23808	563.8
Ras associated with diabetes	L24564	254.6	Plasminogen activator-inhibitor 2	Y00630	559.2
Tec-interacting proteins-3	AB000734	253.1	IP gene for prostacyclin receptor	D38128	523.2
HSP27	X67325	253.0	Prostaglandin transporter (SLC21A2)	AF056732	464.7
CCAAT transcription binding factor subunit gamma	Z74792	236.2	Human interleukin-13 receptor	U70981	461.0
Collagen VI alpha-1 C-terminal globular domain	X15880	167.1	I cell membrane-associated protein hT1a-2	AF030428	437.5
Tumor necrosis factor-inducible protein (TSG-6)	M31165	162.9	<u>TARC</u>	<u>D43767</u>	371.5
KIAA0664 protein	AB014564	122.6	<u>MIG</u>	<u>X72755</u>	325.0
Human cbl-b mRNA	U26710	42.5	Metallothionein-IG	J03910	323.4
Carbonic anhydrase II	J03037	41.8	Oxysterol 7alpha-hydroxylase	AF029403	275.6
Calcium and diacylglycerol-regulated guanine nucleotide exchange factor II	AF081195	38.8	Vascular cell adhesion molecule 1	M30257	264.2
Human sulfate transporter of Diastrophic dysplasia	U14528	32.1	Tenascin-C	X78565	245.5
Squamous cell carcinoma antigen	S66896	27.8	DKFZp564A032	AL050267	218.4
ICAM-2	M24283	27.7	Peptide supply factor-2	M74447	213.5
Beta-galactoside alpha-2,6-sialyltransferase	X62822	21.6	Adenosine A2 receptor	S46950	185.7
Membrane-associated protein 17	U21049	21.5	Human polymorphic epithelial mucin	J05581	173.4
Inhibitor of apoptosis protein 1	U45878	13.4	HEM45	U88964	171.3
Natural killer cell transcript 4	AA631972	11.9	TIP3	AB000734	157.4
Proto-Oncogene Met, Alt. Splice Form 2	HG1747-HT1764	10.9	Collagen type IV alpha 4 chain	X81053	156.5
<u>RANTES</u>	<u>M21121</u>	9.9	Usurpin-beta	AF015451	136.0
<u>IL8</u>	<u>M28130</u>	8.6	<u>Eotaxin</u>	<u>U46573</u>	132.7

[†] The GeneBank accession number (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide>) of each transcript is shown.

[‡] The level of gene expression was obtained as the AD (average difference) using GeneChip® software. The percentages of the specific AD level versus the mean AD level of 6 probe sets for housekeeping genes (beta-actin and glyceraldehyde-4-phosphate dehydrogenase) were then calculated. The fold increases of each experiment were averaged and are shown as the *average fold increase*.

[§] Data indicating chemokine gene are underlined.

TARC) and eight genes for the CXC chemokines (GRO- α , β , γ , ENA-78, CKA-3, IL-8, IP-10 and I-TAC) were judged as “increased” in the fibroblasts. CX3C chemokine (fractalkine) was judged as “increased” in both the conjunctival and corneal fibroblasts. Except for MCP-1, gene transcription levels were inducible

for most CC chemokines and, except for IP-10 and I-TAC, most CXC chemokines were constitutively expressed. No gene transcripts for the chemokine receptors were judged to be “increased”.

Table 2 Top 30 increased transcripts in the corneal epithelial cells and fibroblasts

Corneal epithelial cells (n=3)			Corneal fibroblasts (n=2)		
Name of transcript (protein)	Accession number [†]	Average fold increase [‡]	Name of transcript (protein)	Accession number	Average fold increase
Natural killer cell transcript 4	AA631972	2883.1	Diubiquitin	AL031983	7474.7
Diubiquitin	AL031983	2258.7	<u>Eotaxin</u>	<u>U46573</u>	5479.9
Inter cellular adhesion molecule-1	M24283	735.0	<u>IP-10</u>	<u>X02530</u>	2124.9
Ras associated with diabetes	L24564	660.2	<u>I-TAC</u>	<u>AF030514</u>	1468.4
Nuclear factor kappa-B2	U20816	225.8	Claudin5	AF000959	1028.0
Nuclear factor kappa-B	S76638	217.0	<u>MCP-2</u>	<u>Y16645</u>	791.2
Prolactin-inducible protein	Y10179	111.7	Interleukin-13 receptor	U70981	785.0
<u>IP-10</u> [§]	<u>X02530</u>	101.8	Brain Natriuretic Protein	AL021155	600.9
Human carbonic anhydrase II	J03037	97.7	Mevalonate pyrophosphate decarboxylase	U49260	530.6
H.sapiens p27 mRNA	X67325	87.8	Ras associated with diabetes	L24564	476.0
U-snRNP-associated cyclophilin	AF016371	79.7	Toll-like receptor 5	AF051152	207.3
Interleukin-13 receptor	U70981	72.1	Collagen type IV alpha 4 chain	X81053	206.1
Osteoblast specific factor 2	D13666	59.7	Hyaluronan synthase 2	U54804	197.0
Diphthamide biosynthesis protein-2	AF053003	55.3	Solute carrier family 21	AF056732	195.7
<u>CKA-3</u>	<u>U81234</u>	47.8	KIAA0869 protein	AB020676	180.3
Metalloproteinase-like, disintegrin-like, cysteine-rich family II	AJ242015	39.9	Porc-PI gene similar to yeast CDC45	AJ223728	173.6
Matrix metalloproteinase 9	J05070	38.7	IL-17 receptor	U58917	149.4
Superoxide dismutase 2	X07834	30.4	Peptide supply factor-2	M74447	147.6
Human squamous cell carcinoma antigen 2	U19557	20.2	Cluster Incl A1865431: wk11h09.x1	A1865431	115.3
K12 protein precursor	U77643	18.6	Nuclear factor kappa-B2	U20816	111.1
KIAA0374 gene, syntaphilin 20p13	AB002372	16.0	Metallothionein-IG	J03910	106.8
Tenascin-C	X78565	14.8	<u>RANTES</u>	<u>M21121</u>	101.6
Homo sapiens GSTT1 mRNA	X79389	14.3	Kynureninase	A1148772	92.7
IFN-beta 2a	X04430	13.7	Epstein-Barr virus-induced protein	U19261	92.5
Sialyltransferase 1	X62822	13.6	Tec-interacting proteins 3	AB000734	73.2
Tumor necrosis factor-inducible protein (TSG-6)	M31165	11.6	HEM45	U88964	66.5
<u>IL8</u>	<u>M28130</u>	10.2	Platelet-derived endothelial cell growth factor	M63193	62.4
BCG-induced gene in monocytes	AL049963	9.5	FLT3/FLK2 ligand	U04806	49.2
Tumor necrosis factor	X02910	8.2	Human vascular cell adhesion molecule 1	M30257	45.7
<u>LARC</u>	<u>U64197</u>	8.0	Vhitinase	U58515	32.6

[†] The GeneBank accession number (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide>) of each transcript is shown.

[‡] The level of gene expression was obtained as the AD (average difference) using GeneChip[®] software. The percentages of the specific AD level versus the mean AD level of 6 probe sets for housekeeping genes (beta-actin and glyceraldehyde-3-phosphate dehydrogenase) were then calculated. The fold increases of each experiment were averaged and are shown as the *average fold increase*.

[§] Data indicating chemokine gene are underlined.

CHEMOKINE PRODUCTION FROM CORNEAL EPITHELIAL CELLS AND FIBROBLASTS

Because the gene expression patterns between the conjunctival and corneal cells were similar, we decided to investigate chemokine production from the corneal cells at the protein level, as assessed by ELISA. We selected two CC chemokines (eotaxin-1, which only increases in fibroblasts that are induced upon stimulation, and MCP-1, which is more promi-

nently increased in fibroblasts and is constitutively expressed) and two CXC chemokines (IP-10, which increases in both epithelial cells and fibroblasts that are inducible upon stimulation, and GRO- α , which increases in both the epithelial cells and fibroblasts and is constitutively expressed).

Eotaxin-1, MCP-1 and IP-10 production patterns in the corneal epithelial cells and fibroblasts were comparable to the mRNA expression pattern, while the

Table 3 Fold increase of the chemokine transcripts in the corneal and conjunctival cells

CHEMOKINES		Accession number†	Conjunctival epithelial cells	Conjunctival fibroblasts	Corneal epithelial cells	Corneal fibroblasts
Name of transcript (protein)			Fold‡	Fold	Fold	Fold
CCL1	I-309	M57506	1.0	0.9	0.7	0.5
CCL2	MCP-1	M28225	2.1	3.4	1.6	2.2
CCL3	MIP-1alpha	D90144	1.1	1.9	0.8	1.3
CCL4	MIP-1beta	J04130	1.0	0.9	1.0	0.8
CCL5	RANTES	M21121	9.9	843.8	3.7	101.6
CCL7	MCP-3	X72308	54.2	2.3	1.2	5.0
CCL8	MCP-2	Y16645	1.0	70.0	0.7	791.2
CCL11	eotaxin	U46573	1.6	132.7	26.2	5479.9
CCL13	MCP-4	AJ001634	0.8	15.6	28.4	12.7
CCL14	HCC-1	AF088219	7.9	0.1	1.0	0.8
CCL16	LEC	AB018249	6.5	0.3	1.1	1.3
CCL17	TARC	D43767	4.5	371.5	1.0	37.5
CCL18	PARC	Y13710	2.4	0.5	1.0	4.2
CCL19	ELC	AB000887	1.0	0.9	1.0	0.8
CCL20	LARC	U64197	1.2	22.7	8.0	6.3
CCL21	SLC	AB002409	1.0	0.9	1.0	0.8
CCL22	MDC	U83171	1.0	0.9	1.0	0.8
CCL23	MIP-3	AF088219	2.8	0.1	0.8	0.4
CCL25	TECK	U86358	68.6	0.8	3.6	1.5
CCL27	CTACK	AJ243542	66.9	0.9	1.0	0.8
CX3CL1	fracralkine	U84487	0.7	14.6	1.4	30.7
CXCL1	GRO-alpha	X54489	3.6	4.4	2.7	2.3
CXCL2	GRO-beta	M36820	1.3	5.8	2.3	3.5
CXCL3	GRO-gamma	M36821	0.6	14.2	4.1	6.5
CXCL4	PF4	M25897	0.7	2.1	1.5	1.4
CXCL5	ENA-78	X78686	1.3	2.3	5.2	4.2
CXCL6	CKA-3	U81234	344.2	12.4	47.8	3.8
CXCL8	IL-8	M28130	8.6	48.7	10.2	7.7
CXCL9	MIG	X72755	1.0	325.0	0.9	3.2
CXCL10	IP-10	X02530	8.5	1694.7	101.8	2124.9
CXCL11	I-TAC	AF030514	3.0	944.3	5.3	1468.4
CXCL12	SDF-1b	L36033	0.8	0.3	0.9	0.4
CXCL13	BCL	AF044197	1.0	0.9	0.8	0.2
CCR1	CCR1	L09230	0.9	2.4	1.7	1.2
CCR2	MCP-1RB	U03905	1.0	1.5	0.7	1.4
CCR3	CCR3	U28694	0.7	0.5	0.7	4.8
CCR4	CCR4	X85740	1.0	22.6	1.0	0.8
CCR5		U95626	8.5	0.9	0.5	5.3
CCR6	CCR6	U45984	0.9	2.4	9.8	1.9
CCR8	CCR2	D49919	1.3	5.6	0.9	0.2
CCR9	CCR9	AJ132337	1.0	22.7	0.8	15.8
CCR10	CCR10	U94888	1.2	0.8	0.9	1.3
	CRTH2	AB008535	1.0	18.0	10.0	22.5
CX3CR1		U20350	0.0	40.6	3.5	0.8
CXCR1	IL-8RA	U11870	0.7	0.7	1.1	32.3
CXCR2	IL-8RB	L19593	0.6	1.7	2.9	0.8
CXCR3		X95876	1.5	1.0	1.0	32.3
CXCR4		L06797	0.6	112.8	7.9	0.1

† The GeneBank accession number (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide>) of each transcript is shown.

‡ The level of gene expression was obtained as the AD (average difference) using GeneChip® software. The percentages of the specific AD level versus the mean AD level of 6 probe sets for housekeeping genes (beta-actin and glyceraldehyde-3-phosphate dehydrogenase) were then calculated. The primary cultured cells were incubated with IL-4 (30 ng/ml) and TNF-alpha (30 ng/ml) for 24 hr. Bold numerals stand for "presence" transcripts, which were judged by the GeneChip® software, while normal numerals stand for "marginal" or "absent" transcripts. Fold increase was obtained by calculating the AD levels of activated cells versus those of resting cells. Gray-colored columns mean "present" and over 3-fold increased transcripts.

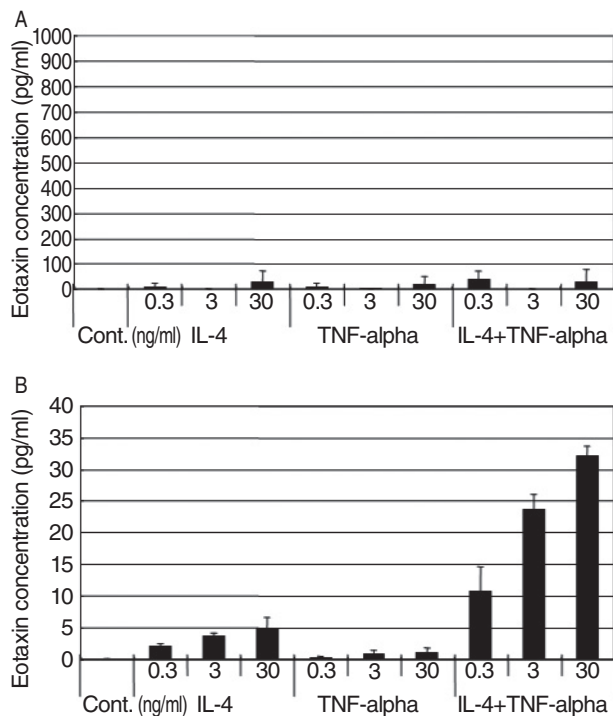


Fig. 1 Eotaxin-1 production from corneal epithelial cells (A) and fibroblasts (B). Cells were incubated with IL-4 and TNF- α for 48 hours. Eotaxin-1 concentrations in the supernatants were measured by ELISA. Data represent the mean $\pm$ SD from three experiments.

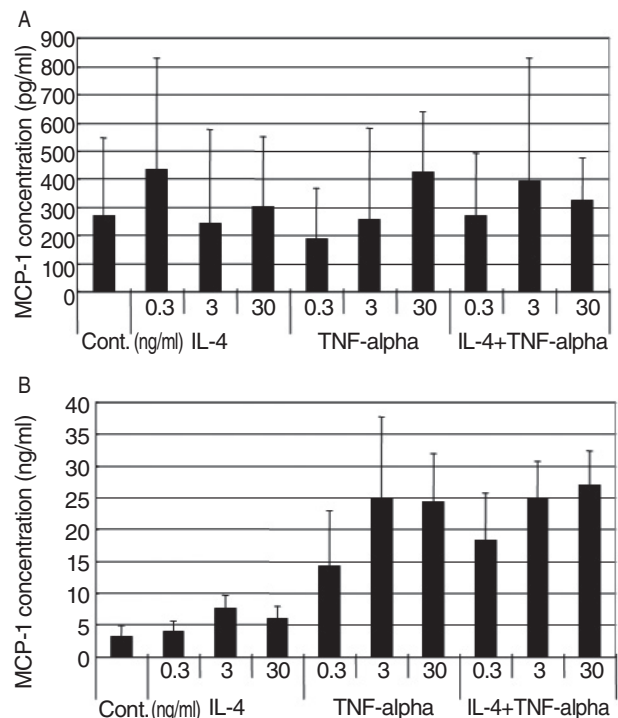


Fig. 2 MCP-1 production from corneal epithelial cells (A) and fibroblasts (B). Cells were incubated with IL-4 and TNF- α for 48 hours. Eotaxin-1 concentrations in the supernatants were measured by ELISA. Data represent the mean $\pm$ SD from three experiments.

production pattern for GRO- α was slightly different from its mRNA expression pattern. IL-4 induced eotaxin-1 production and TNF- α enhanced eotaxin-1 production in fibroblasts but not in epithelial cells (Fig. 1). The epithelial cells constitutively produced small amounts of MCP-1, with marked increases noted in the fibroblasts after TNF- α stimulation (Fig. 2). GRO- α was induced by TNF- α in the epithelial cells, while it was constitutively produced by fibroblasts (Fig. 3). IP-10 was induced by TNF- α in both cells (Fig. 4).

DISCUSSION

This is the first time that a comprehensive investigation has been undertaken on the chemokine gene expression in primary cultures of the four types of cells that constitute the ocular surface; i.e., the corneal epithelial cells, corneal fibroblasts, conjunctival epithelial cells and conjunctival fibroblasts.

GeneChip[®] is a high-density oligonucleotide expression probe array that is designed to measure the absolute levels of >10,000 transcripts by using the same set of inner standards. Since competition with another cell types is not required for the GeneChip[®],¹⁹ this makes it possible to be able to compare expression levels of >10,000 transcripts in

different types of cells. In the current study, there were three and six chemokine-associated genes listed among the top 30 most increased genes for the conjunctival epithelial cells and fibroblasts, respectively. In the cornea, four and five chemokine-associated genes were among the top 30 most increased genes for the corneal epithelial cells and fibroblasts, respectively. These findings indicate the importance of chemokines in environments that are influenced by IL-4 and TNF- α . CC chemokines were mostly inducible and dominant in the fibroblasts, while CXC chemokines were mostly constitutive and expressed in both the epithelial cells and the fibroblasts.

Corneal tissue damage is among the most painful and vision-threatening complication that is associated with severe ocular allergic diseases such as atopic keratoconjunctivitis (AKC)^{20,21} and vernal keratoconjunctivitis (VKC).^{22,23} Eosinophils as well as eosinophil cationic protein (ECP) have been found in the conjunctival tissue and in the tears of patients with AKC and VKC.^{24,25} Eosinophil major basic protein (MBP), which has been detected in corneal plaques in VKC,²⁶ was shown to inhibit epithelial migration.²⁷ Therefore, eosinophils appear to participate in the pathogenesis of corneal damage in ocular allergic diseases. In addition, both CC chemokine-attracting in-

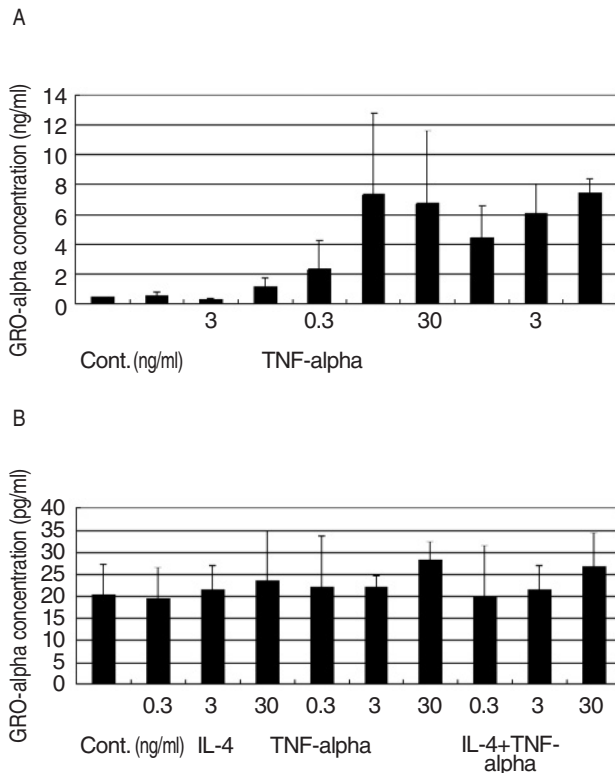


Fig. 3 GRO- α production from corneal epithelial cells (A) and fibroblasts (B). Cells were incubated with IL-4 and TNF- α for 48 hours. Eotaxin-1 concentrations in the supernatants were measured by ELISA. Data represent the mean $\pm$ SD from three experiments.

flammatory cells such as T-cells and basophils, and CXC chemokine-attracting neutrophils have roles in ocular allergic diseases.

CC chemokines such as eotaxin-1^{28,29} and the protein RANTES³⁰ are important in recruiting eosinophils into tissue affected by allergy. Eotaxin-1 has been found in tears from allergic patients with severe corneal damage, and has been shown to be correlated with the number of eosinophils that are observed in tears.¹¹ We have also reported that interleukin (IL)-4 induces eotaxin-1 production in human corneal keratocytes but not in corneal epithelial cells.⁵ In the current study, we found that corneal and conjunctival fibroblasts induced CC chemokine gene expression, while epithelial cells expressed only a few CC chemokine transcripts. Corneal and conjunctival fibroblasts may be a major source of the chemokines that are responsible for recruiting eosinophils. In order to prevent eosinophil infiltration of the ocular surface, treatment strategies need to focus on regulating the fibroblast chemokine production.

Our previous clinical experience has demonstrated that while AKC and VKC without corneal epithelial lesions are relatively controllable, the disease becomes

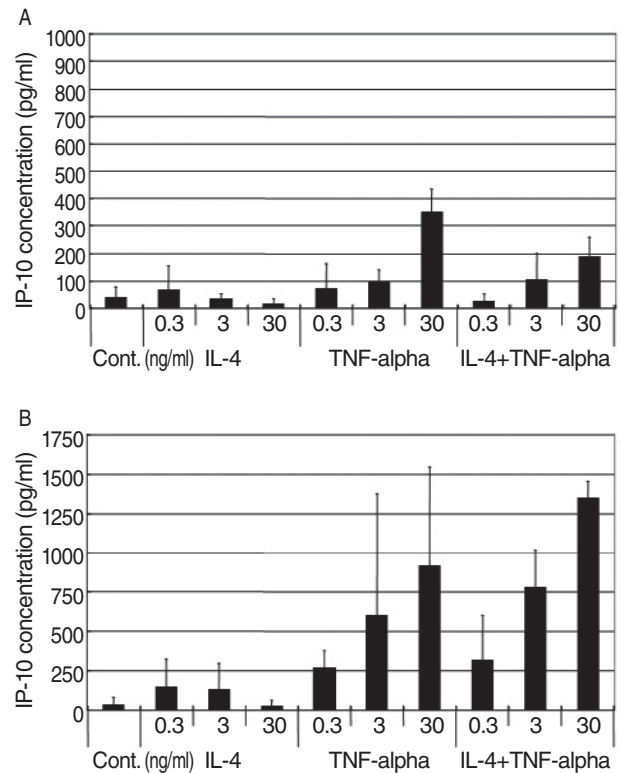


Fig. 4 IP-10 production from corneal epithelial cells (A) and fibroblasts (B). Cells were incubated with IL-4 and TNF- α for 48 hours. Eotaxin-1 concentrations in the supernatants were measured by ELISA. Data represent the mean $\pm$ SD from three experiments.

more severe and is often refractory to treatment when the corneal epithelial integrity is compromised. Based on this clinical impression, the CC chemokine expression pattern found in this study (which occurred mainly in the fibroblasts and not in the epithelial cells) led us to hypothesize that corneal epithelial cells may act as a barrier against eosinophil recruitment and severe tissue damage. Maintaining corneal epithelial integrity in patients with severe ocular allergy should therefore be considered to be an important part of the treatment strategy in such diseases.

Eosinophils are attracted when chemokines interact with the CC chemokine receptor (CCR)-1 and CCR-3, which are located on their surfaces.³¹ While RANTES activates eosinophils through both CCR-1 and CCR-3, eotaxin-1 is a specific ligand for CCR-3.³² We have previously reported that the anti-CCR-1 antibody (ab) and anti-CCR-3 ab are able to effectively, although not completely, suppress the eosinophil chemotaxis that is induced by the culture supernatant of stimulated corneal fibroblasts.¹³ This incomplete suppression suggests the existence of eosinophil chemoattractants other than eotaxin-1 and RANTES. In the current study, we noted that stimulation of the

fibroblasts resulted in an increase of MCP-1, MCP-2 and IP-10, which are all known to be able to recruit eosinophils. Since CCR-2³³ and CXCR3^{34,35} have been found to be located on the eosinophil surface, further experiments are needed to investigate the interactions of MCP-1/CCR2, MCP-2/CCR2 and IP-10/CXCR3. Liu *et al.* have found that BAL eosinophils, but not blood eosinophils, are able to slightly express CXCR3.³⁵ This suggests that CXCR3 signaling is involved in local inflammations, such as AKC and VKC. Furthermore, while we found that the production of GRO- α was inducible by TNF- α in corneal epithelial cells, it was constitutive in the corneal fibroblasts. These findings indicate that there is induction of limited GRO- α production in corneal epithelial cells when inflammation occurs. Moreover, when the epithelial damage is severe, production of GRO- α can cause a much more intensive inflammation.

In this study, CC chemokines were mostly inducible and selectively expressed in fibroblasts. CXC chemokines were mostly constitutive and expressed both in the epithelial cells and in the fibroblasts. CC chemokines seem to have a more selective function with regard to ocular allergic inflammation, while CXC chemokines may have more of a general defensive function against insults such as infections. LARC and RANTES were the only two CC chemokines that were constitutively expressed in the epithelial cells in this study. LARC recruits T cells and dendritic cells via CCR6,^{36,37} while RANTES attracts various inflammatory cells such as eosinophils, basophils, T cells, monocytes, and dendritic cells, in addition to neutrophils via CCR1, 3, 4 and 5.^{30,38} Thus, these two CC chemokines may have a more general function as compared to the other CC chemokines.

MIG, IP-10 and I-TAC are the CXC chemokines that are induced by interferon γ and attract T cells bearing the specific receptor CXCR3. These chemokines have been reported to augment the inflammation that occurs in atopic dermatitis.³⁹ MIG has been reported to play a role in T cell recruitment in VKC.⁴⁰ In the present study, MIG and I-TAC expressions were inducible and selective in the fibroblasts, while all cell types induced IP-10 expression. The T cell that recruits the CXC chemokines that are induced in the fibroblasts may play a role in late-phase ocular allergic inflammation.

We found only a few mRNA expressions for the chemokine receptors in the conjunctival and corneal cultured cells. In addition, only low mRNA expressions of CCR3, CCR10 and CXCR3 were found, and these were not subject to upregulation by any type of stimulation. Thus, while chemokines may act on inflammatory cells, they do not act on any of the tissues that constitute the ocular surface.

In conclusion, we were able to obtain a comprehensive overview of chemokine and receptor expression in cells that constitute the ocular surface. Corneal and

conjunctival fibroblasts exhibited marked increases in the expression of chemokines upon stimulation. Since fibroblasts exist on the ocular surface, they may be one of the primary sources for the eosinophil-recruiting chemokines that are present in allergic conjunctival disease. Therefore, regulation of chemokine production from the surface of such cells might be an effective strategy for treating these diseases.

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