The Implications of the Upregulation of ICAM-1/VCAM-1 Expression of Corneal Fibroblasts on the Pathogenesis of Allergic Keratopathy

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OBJECTIVE. The present study investigated the expression of ICAM-1 and VCAM-1 on fibroblasts with interleukin (IL)-4 and/or tumor necrosis factor (TNF)-α stimulation and assessed the effect of eosinophil adhesion on fibroblast viability.

METHODS. Primary cultured human corneal fibroblasts were incubated with IL-4, TNF-α, or their combination for 24 hours. Expression of ICAM-1 and VCAM-1 was examined by real-time quantitative PCR and flow cytometric analysis. Purified eosinophils were cocultured with activated fibroblasts, and the number of eosinophils adhered to fibroblasts and the number of damaged fibroblasts were counted using microscopy. In a separate trial, conjunctival and corneal impression cytology was performed on patients with atopic keratoconjunctivitis and corneal ulcers (eight eyes) to assess the status of the ocular surface epithelium and the presence of inflammatory cell infiltrates.

RESULTS. Real-time quantitative PCR and flow cytometric analysis revealed that both mRNA and protein of VCAM-1 and ICAM-1 were upregulated by IL-4 and TNF-α. IL-5-primed eosinophils adhered to the corneal fibroblasts treated with IL-4 and TNF-α, and the fibroblasts were damaged by eosinophil adherence. Anti–ICAM-1 antibody and anti–VCAM-1 antibody inhibited the eosinophil adherence to fibroblasts and the fibroblast damage. Impression cytology revealed extensive infiltration of neutrophil and eosinophils among isolated ocular surface epithelial cells with advanced squamous metaplasia.

CONCLUSIONS. Corneal fibroblasts expressed ICAM-1 and VCAM-1 when activated with IL-4 and TNF-α. Eosinophils can adhere to the activated fibroblasts and can induce subsequent fibroblast damage through these adhesion molecules. Eosinophil adhesion to fibroblasts may possibly contribute to the pathogenesis of severe persistent allergic corneal ulcers. (*Invest Ophthalmol Vis Sci.* 2005;46:4512–4518) DOI:10.1167/iovs.04-1494

Eosinophils are thought to exacerbate the late-phase inflammatory response in immediate-type allergic reactions by releasing leukotrienes and highly cytotoxic proteins, such as major basic protein (MBP) and eosinophilic cationic protein (ECP).[1] These proteins may cause a variety of corneal disorders, including superficial punctate keratopathy and corneal ulcer in vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC).[2-5] The presence of eosinophils and the deposition of ECP have already been observed in conjunctival tissues and in tears of patients with AKC and VKC.[6-10] MBP deposits were also observed in allergic corneal ulcers.[11] Purified MBP and ECP reduced corneal epithelial cell viability and caused morphologic changes in vitro.[12] These findings strongly suggest that eosinophils play an important role in the pathogenesis of allergic corneal ulcer. However, the precise mechanisms by which eosinophils damage the corneal tissue remain unclear.

Allergic reactions in the conjunctiva induce the release from inflammatory cells of various types of cytokines, including proinflammatory cytokines and helper type 2 T-cell (Th2) cytokines. Levels of interleukin (IL)-4 and tumor necrosis factor (TNF)-α in the tears of patients with allergies were found to be significantly higher than in healthy subjects.[13,14] These cytokines are known to modulate various functions of fibroblasts, such as eotaxin production and adhesion molecule expression.[15,16] Eotaxin is known to induce further recruitment of eosinophils.[17] On the other hand, adhesion molecules are thought to play an important role in the binding of eosinophils, through which eosinophils are believed to be stimulated further to release inflammatory mediators.[18,19]

Recently, it has been found that CD11/18-dependent adhesion is a critical step in human eosinophil degranulation.[20,21] Eosinophils express all four members of the CD18 (β2) leukocyte integrin family, CD11a to -d, which allow them to bind to their ligands, ICAM-1 to -3.[22-27] Eosinophils also express CD49d/CD29, which bind to the ligand VCAM-1.[28] In human lung fibroblasts, IL-4– and TNF-α–dependent expression of ICAM-1 and VCAM-1 and the influence of eosinophil-fibroblast adhesion on eosinophil degranulation have been reported.[29] However, it is still unclear how the eosinophil-fibroblast interactions influence allergic corneal inflammation, especially the course of corneal ulcer formation. We believed that actual adhesion of eosinophils to corneal fibroblasts through ICAM-1 and/or VCAM-1 might induce subsequent activation and might contribute to the evolution of persistent allergic corneal ulcers. Therefore, we initially looked into the changes of expression of ICAM-1 and VCAM-1 on stimulating corneal fibroblast cultures by IL-4 and TNF-α by employing flow cytometry and real-time PCR. We also cocultured corneal fibroblasts with eosinophils to assess the adhesion between the two cell types. We then investigated the timewise cell damage on corneal fibroblasts after eosinophil binding, as well as the effects of anti–ICAM-1 and anti–VCAM-1 applications on eosinophil adhesion and eosinophil-induced damage to the corneal fibroblasts. In addition, we performed conjunctival and corneal impression cytology on patients with AKC and corneal ulcers to evaluate the status of the ocular surface epithelium and the presence of eosinophils in the inflammatory response, if any.

FIGURE 1. Immunohistochemistry staining of corneal fibroblasts by antivimentin antibodies. (**A**) Image shows diffuse positive staining with antivimentin antibodies (rabbit polyclonal, 1:2000 dilution; Lab Vision Corporation, Fremont, CA) and a lack of impurities from the presence of any other cell types in the slide chamber (magnification 200×). (**B**) Image shows the absence of staining in the negative control specimen (magnification 200×).
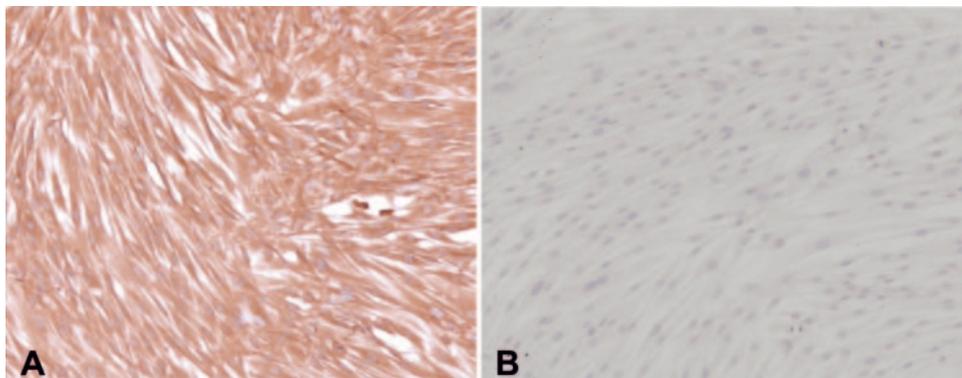
MATERIALS AND METHODS

Primary Cell Cultures

Three human corneas were obtained from the American Eye Bank Association. Briefly, after the center of each cornea was punched out for transplantation, the remaining rim of tissue was used to prepare corneal fibroblasts. The human tissue was used in strict accordance with the tenets of the Declaration of Helsinki. Corneal tissue was cut into pieces and then placed on collagen-coated 35-mm culture dishes (Iwaki, Tokyo, Japan). Corneal fibroblasts were isolated from corneal tissue explants and cultured with fibroblast culture medium composed of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM-F12; Gibco-BRL, Grand Island, NY), supplemented with 10% fetal calf serum (FCS; Gibco), 100 IU/mL penicillin, and 100 μg/mL streptomycin (all supplied by Invitrogen-Gibco Life Technologies, Paisley, UK) at 37°C with 5% (vol/vol) CO_2 in air. Cultures of passages 3 and 6 were used in the present study. The purity of the cell cultures was assessed on the basis of both the distinctive morphology of corneal fibroblasts and their reactivity with antibodies to vimentin and isotype control in immunohistochemistry analysis. No contamination by corneal epithelial cells was detected (Fig. 1).

Isolation of Human Eosinophils

Human granulocytes were isolated from heparin-anticoagulated venous blood of atopic volunteers. The granulocytes underwent Percoll density gradient centrifugation (490g) at room temperature, and CD16-positive cells were removed using immunomagnetic beads (Miltenyi Biotec GmbH, BergischGladbach, Germany) as previously described.[30] Eosinophil purity of cytocentrifuge preparations was determined by staining (Diff-Quick; American Scientific Products, McGraw Park, IL) and was confirmed to be greater than 98%.

Quantitative Real-Time PCR

We confirmed whether ICAM-1 and VCAM-1 mRNA expressions were correlated with the concentrations of IL-4 and TNF-α by performing quantitative measurements of ICAM-1 and VCAM-1 mRNA with real-time PCR. The primary human corneal fibroblasts from three different donors were cultured for 24 hours with various cytokine concentrations, after which total RNA was extracted. RNA was extracted from corneal fibroblasts cultured in the presence or absence of 0.3 to 30 ng/mL IL-4 and TNF-α for 24 hours. A commercially available sequence detection system (ABI PRISM 7700; Applied Biosystems, Warrington, UK) and gene expression assay mixes (TaqMan Universal PCR Master Mix and Assay-on-Demand Gene Expression Assay Mix; Applied Biosystems) were used for real-time quantitative PCR to measure for ICAM-1, VCAM-1, and GAPDH. The thermal profile consisted of 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 94°C for 15 seconds and 60°C for 1 minute. Results were analyzed by the comparative cycle threshold method.[31,32]

Flow Cytometry Analysis

To examine the ICAM-1 and VCAM-1 protein expression on the cell surface of cultured fibroblasts, flow cytometric analysis was performed 24 hours after cytokine stimulation. IL-4– and/or TNF-α–stimulated corneal fibroblasts were gently removed from the six well-culture dishes with cell dissociation buffer (Gibco-BRL), washed, and diluted in PBS containing 1% BSA and 0.1% NaN_3. Cells were stained by monoclonal antibodies against mouse IgG_1 (Sigma, St. Louis, MO), ICAM-1 (84H10; Immunotech, Marseille, France) or VCAM-1 (1G11; Immunotech), respectively, and analyzed by flow cytometry (FACScan, Becton Dickinson, Franklin Lane, NJ) and analysis software (CellQuest; Becton Dickinson, Mountain View, CA).

Eosinophil-Fibroblast Adhesion Assay

To determine the functional significance of ICAM-1 and VCAM-1 expression on cultured fibroblasts, in vitro eosinophil adhesion experiments were performed. Corneal fibroblasts from three different donors were cultured in 96-well culture plates (Becton-Dickinson Labware, Lincoln Park, NJ) for 48 hours After starvation for 24 hours, the cells were stimulated with IL-4 (1–100 ng/mL) and/or TNF-α (1–100 ng/mL)
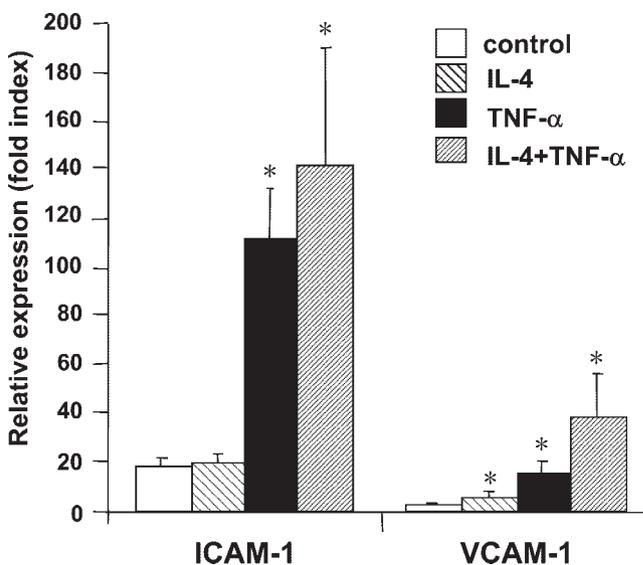
FIGURE 2. Relative expression of mRNA encoding ICAM-1 and VCAM-1 on corneal fibroblasts compared to controls. Corneal fibroblasts were incubated with IL-4 (0.3–30 ng/mL), TNF-α (0.3–30 ng/mL), or their combination (0.3–30 ng/mL) for 24 hours Total RNA was isolated and real-time quantitative PCR for ICAM-1, VCAM-1, and GAPDH was performed. Data were analyzed by the comparative cycle threshold method and expressed as the fold index relative to the nonstimulated control (three trials). Values are means ± SD; *$P < 0.05$, Student's *t*-test.

for 24 hours. Purified eosinophils from three different donors were preincubated with 1 ng/mL IL-5 for 15 minutes, then cocultured with the cultured fibroblasts (1×10^5 cells per well) for 3 hours The wells were washed gently with PBS to remove nonadhered eosinophils, and the eosinophils adhered to the fibroblasts were counted using the light microscope field. To determine the effect of two adhesion molecules, ICAM-1 and VCAM-1, on eosinophil-fibroblast interactions, an inhibition assay was performed using anti-ICAM-1 monoclonal antibody (mAb) and anti–VCAM-1 mAb. After stimulation of fibroblasts with IL-4 and TNF-α for 24 hours, 50 μL anti-mouse IgG_1 (Sigma), anti–ICAM-1 mAb, and anti-VCAM-1 mAb (Immunotech) were added and reacted for 30 minutes at 37°C before coculturing.

Morphologic Study of Fibroblasts Cocultured with Eosinophils

By a similar method as the adhesion assays, fibroblasts were cultured in 96-well plates and stimulated with IL-4 and/or TNF-α for 24 hours. Preactivated eosinophils were added to the cultured fibroblasts, and they were cocultured for 72 hours. After coculturing, fibroblasts were gently washed twice with PBS, then 20 μL of 0.5% trypan blue solution was added, and the cells were stained for 1 minute. After removing the solution with a pipet, nonstained cells, regarded as intact, were immediately counted under the microscope.

TUNEL Assay for Detection of Apoptosis in Fibroblasts

Apoptosis in the cocultures of fibroblasts with eosinophils was detected employing the TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated deoxyuridine triphosphate [dUTP] nick-end labeling) assay, using a commerically available kit (In Situ Cell Death Detection Kit, Peroxidase; Boehringer Mannheim, Mannheim, Germany) per manufacturer's instructions.

Repeatability of the Individual Experiments

Gene chip analysis, real-time PCR and flow cytometry experiments, adhesion assays, and morphologic studies were repeated three times in this study.

Conjunctival and Corneal Impression Cytology

Conjunctival and corneal impression cytology was performed on eight patients with AKC and corneal ulcers (8 eyes; 7 males, 1 female; mean age 24 years, range 9–34 years). The impression cytology specimens were obtained after administration of topical anesthesia with 0.4% oxybuprocaine. Strips of cellulose acetate filter paper (HAWP 304; Millipore, Bedford, MA) that were soaked in distilled water for a few hours and dried at room temperature were applied on upper palpebral conjunctiva and corneal ulcers, pressed gently by a glass rod, and then removed. The specimens were then fixed with formaldehyde. The specimens were stained with periodic acid schiff, dehydrated in ascending grades of ethanol and then with xylol, and finally coverslipped. The status of epithelial cells was determined by taking photographs using a light microscope at a magnification of 400×. The same researcher, who was masked to the identity of the specimen donors, evaluated the specimens for goblet cell counts, epithelial squamous metaplasia grades, and presence of inflammatory cell infiltrates.

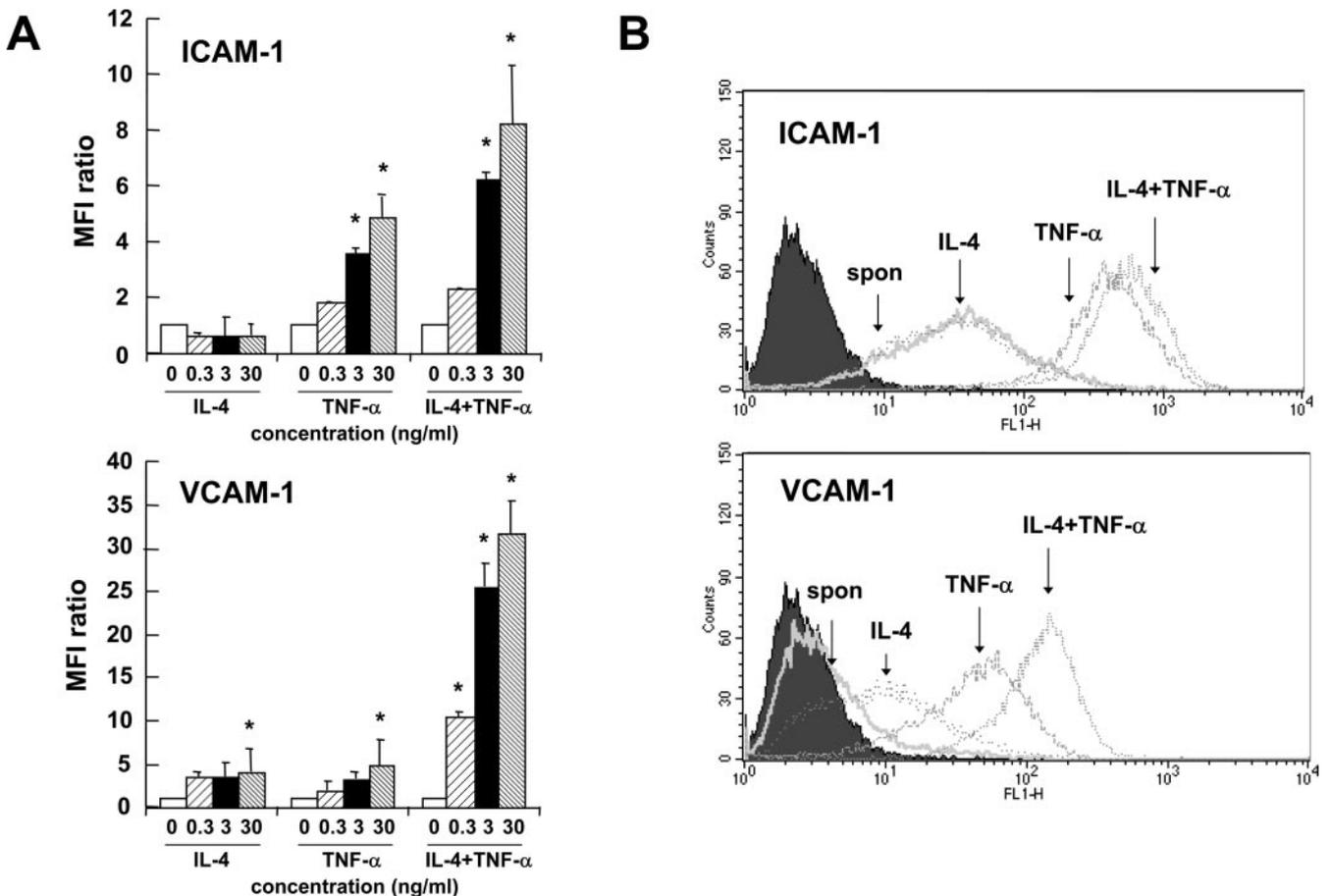FIGURE 3. Flow cytometry analysis of the expression of ICAM-1 and VCAM-1 on corneal fibroblasts. Corneal fibroblasts were incubated with IL-4 (0.3–30 ng/mL), TNF-α (0.3–30 ng/mL), or their combination (0.3–30 ng/mL) at 37°C for 24 hours. Cells were washed and stained with anti–ICAM-1 mAb and anti–VCAM-1 mAb and analyzed by flow cytometry. IL-4 and TNF-α induced the expression of ICAM-1 and VCAM-1 on corneal fibroblasts. (A) Mean fluorescence intensity (MFI) of ICAM-1 and VCAM-1 in flow cytometry ($n = 3$); *$P < 0.05$. (B) Representative individual fluorescence intensity profile of ICAM-1 and VCAM-1 in flow cytometry.

Statistical Analysis

All data are presented as means $\pm$ SEM. Depending on the distribution of the individual data, the significance of differences between groups was determined by either Student's t-test or a Mann–Whitney rank sum test. Repeated-measures ANOVA was used where more than one comparison was made. Values of $P < 0.05$ were considered significant.

RESULTS

Real-Time PCR Analysis of ICAM-1 and VCAM-1 Expression after IL-4 and TNF-α Stimulation of Fibroblasts

A significant increase in both ICAM-1 and VCAM-1 mRNA expression over basal levels was observed with TNF-α (0.3–30 ng/mL) stimulation (4.8- and 4.6-fold, compared to control, at 30 ng/mL) as shown in Figure 2. Although IL-4 (0.3–30 ng/mL) alone did not enhance ICAM-1 mRNA expression, a combination of TNF-α and IL-4 showed a slight additive effect on ICAM-1 mRNA expression. In contrast, VCAM-1 mRNA expression was increased by IL-4 alone to the same extent as with TNF-α stimulation (4.0-fold, at 30 ng/mL). A combination of 30 ng/mL TNF-α and IL-4 resulted in the greatest augmentation of VCAM-1 mRNA expression (32-fold).

Flow Cytometry Analysis of ICAM-1 and VCAM-1 Expression on Corneal Fibroblasts

As shown in Figure 3, ICAM-1 was constitutively expressed on corneal fibroblasts, and significantly enhanced by TNF-α stimulation alone ($P < 0.05$) but not by IL-4 stimulation. Stimulation with the combination of IL-4 and TNF-α showed an additive effect on ICAM-1 expression. On the other hand, VCAM-1 was scarcely expressed on resting corneal fibroblasts, but was induced by IL-4 or TNF-α stimulation. A combination of IL-4 and TNF-α showed an additive effect on VCAM-1 expression.

Morphologic Analysis of Eosinophil Adhesion to Corneal Fibroblasts

When unstimulated fibroblasts were incubated with activated eosinophils (1×10^5 cells per well), slight spontaneous eosin-ophil adhesion to fibroblasts was noted at 20.3 ± 4.9 cells/high power field (hpf) (Fig. 4A). After preincubation of fibroblasts with IL-4 (1–100 ng/mL) or TNF-α (1–100 ng/mL) for 24 hours, the number of adhered eosinophils increased in a dose-dependent manner ($P < 0.001$). A combination of IL-4 and TNF-α showed an additive effect on eosinophil adhesion. After stimulation with both 100 ng/mL IL-4 and 100 ng/mL TNF-α, eosinophil adhesion was most enhanced at 99.0 ± 15.6 cells/hpf (a fivefold increase, compared with nonstimulated controls).

Morphologic Analysis of the Viability of Corneal Fibroblasts Cocultured with Eosinophils

In eosinophil adhesion assays, we observed slight morphologic changes in fibroblasts cocultured with eosinophils after 3 hours of incubation. Therefore, we continued the coculture study and observed the eosinophil-dependent morphologic changes in the fibroblasts. At 72 hours, corneal fibroblasts were partially damaged, and a number of cells were detached from the culture dish. After cocultured fibroblasts were gently washed with PBS, a trypan blue exclusion test was performed. The number of intact fibroblasts incubated without eosinophils was 44.0 ± 3.4 cells/hpf, and the viability was consistently 100%. It was also confirmed that IL-4 and TNF-α scarcely affected the viability of fibroblasts in our system (data not shown). Preincubation of fibroblasts with IL-4 or TNF-α increased the number of damaged cells in a concentration-dependent manner when cocultured with eosinophils (Fig. 4B). Preincubation with both 100 ng/mL IL-4 and 100 ng/mL TNF-α showed an additive effect on cell damage (53.7%).

All fibroblasts in IL-4– and TNF-α–stimulated cocultures with eosinophils were TUNEL negative and did not reveal specific features of apoptosis, such as shrinkage of cells or nuclear changes.

Morphologic Changes of Eosinophil Adhesion to Corneal Fibroblasts and Eosinophil-Induced Fibroblast Damage with Anti–ICAM-1 or Anti–VCAM-1 Treatment

The addition of anti–ICAM-1 mAb or anti–VCAM-1 mAb (10 μg/mL) significantly inhibited the adhesion of eosinophils to
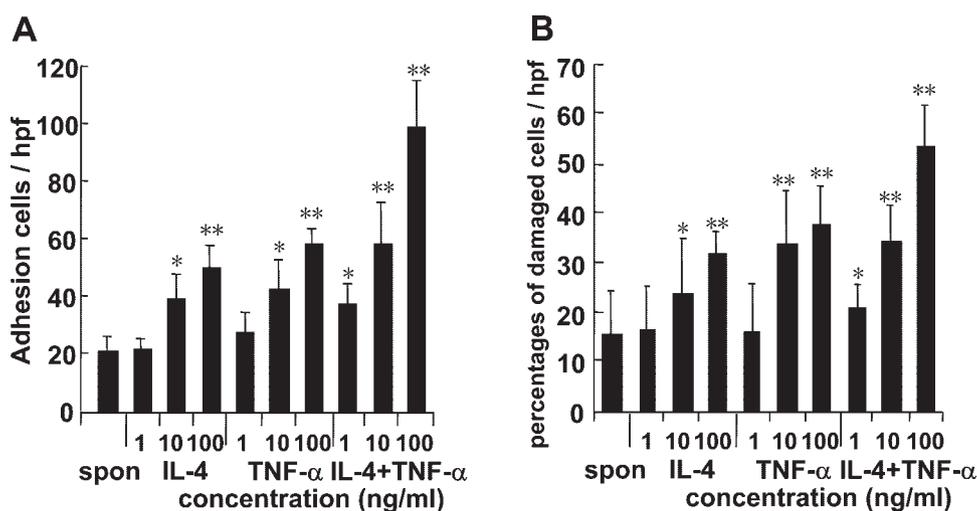

FIGURE 4. Morphologic analysis of eosinophil adhesion and fibroblast damage. Corneal fibroblasts were preincubated for 24 hours with IL-4 and/or TNF-α. Eosinophils primed with IL-5 were then added. (**A**) Graph shows the number of adhered eosinophils counted by light microscopic observation; eosinophil adhesion to corneal fibroblasts is significantly increased in stimulated cultures compared to control cultures ($n = 3$); *$P < 0.05$, **$P < 0.005$. (**B**) Eosinophils were incubated for 72 hours. Graph shows a significant increase of corneal fibroblast damage compared to control cultures. Percentages of damaged cells were calculated by subtraction of trypan blue–negative cells (100% = viability of control fibroblasts without addition of eosinophils and cytokines; $n = 3$); *$P < 0.05$, **$P < 0.005$. spon, spontaneous.

activated fibroblasts (Fig. 5A). Preincubation with anti–ICAM-1 or anti–VCAM-1 mAb significantly inhibited the eosinophil adhesion by approximately 80%, compared to the level observed with mouse IgG$_1$ application.

Eosinophil-induced cell damage was also assayed after 72 hours of coculturing. Treatment with anti–ICAM-1 mAb and anti–VCAM-1 mAb significantly reduced the fibroblast damage (57% and 43%, respectively) to a similar extent as with eosinophil adhesion (Fig. 5B). An additive reduction of fibroblast damage was observed when fibroblasts were treated with both anti–ICAM-1 and anti–VCAM-1 mAbs.

Conjunctival and Corneal Impression Cytology

To provide further evidence for our in vitro findings, conjunctival and corneal impression cytology was carried out on eight patients with AKC and sterile shield corneal ulceration (eight eyes). Conjunctival imprints from all eyes contained sheets of conjunctival epithelial cells with advanced squamous metaplasia, inflammatory cell infiltrates, predominant neutrophils and eosinophils, variable amounts of goblet cells, and mucin pickup. Figure 6A shows an abundance of inflammatory cell infiltrates, consisting mainly of neutrophils and eosinophils, adjacent to conjunctival epithelial cells with advanced squamous metaplasia (Dogru M, et al. *IOVS* 2002;46:ARVO E-Abstract 939). The red arrows indicate eosinophils, the black arrow points to a conjunctival epithelial sheet with Nelson's grade 2 squamous metaplasia and decreased cellular cohesion, and the black stars indicate a total loss of cellular cohesion between the focus of inflammation and the surrounding epithelial cells. The orange arrow indicates a loss of cellular cohesion between the conjunctival epithelial cells lying under the inflammatory infiltrates.

Imprints from eyes of patients with corneal ulcers revealed extensive infiltration by both neutrophils and eosinophils among isolated corneal epithelial cells with advanced squamous metaplasia. Figure 6B, a representative corneal impression cytology imprint obtained from the ulcer edge in the same patient as Figure 6A, shows extensive infiltration by eosinophils (red arrows) and neutrophils and isolated corneal epithelial cells with Nelson's grade 3 squamous metaplasia.

DISCUSSION

Eosinophils have long been blamed for the morbidity of corneal complications in patients with allergies. Indeed, eosinophils have been shown to accumulate on the ocular surface in patients with allergic conjunctivitis and to exert cytotoxic effects through degranulation. We have previously reported higher concentrations of eotaxins in the tears of patients with severe ocular allergies and observed that tears of such patients had chemotactic activity for eosinophils in vitro.[6] Although the current literature suggests some evidence in relation to eosinophil-induced ocular damage in ocular surface allergies, the precise mechanisms of the formation of severe corneal damage, especially corneal ulceration, are still not clear.

Therefore, we performed quantitative real-time PCR and flow cytometric analysis to confirm the expression of ICAM-1 and VCAM-1 related to eosinophils at the mRNA and protein levels. We also looked into the in vitro morphologic alterations of corneal fibroblasts cocultured with eosinophils by assessing eosinophil adhesion to corneal fibroblasts, fibroblast viability, and the effects of anti–ICAM-1 and anti–VCAM-1 treatment on fibroblast morphology.

It is well known that keratocyte apoptosis is one the first events to follow corneal epithelial injury. Virtually any source of corneal epithelial injury, such as a mechanical scrape, LASIK surgery, or an infectious process, causes the release of cytokines from the epithelium, resulting in the activation and trans-
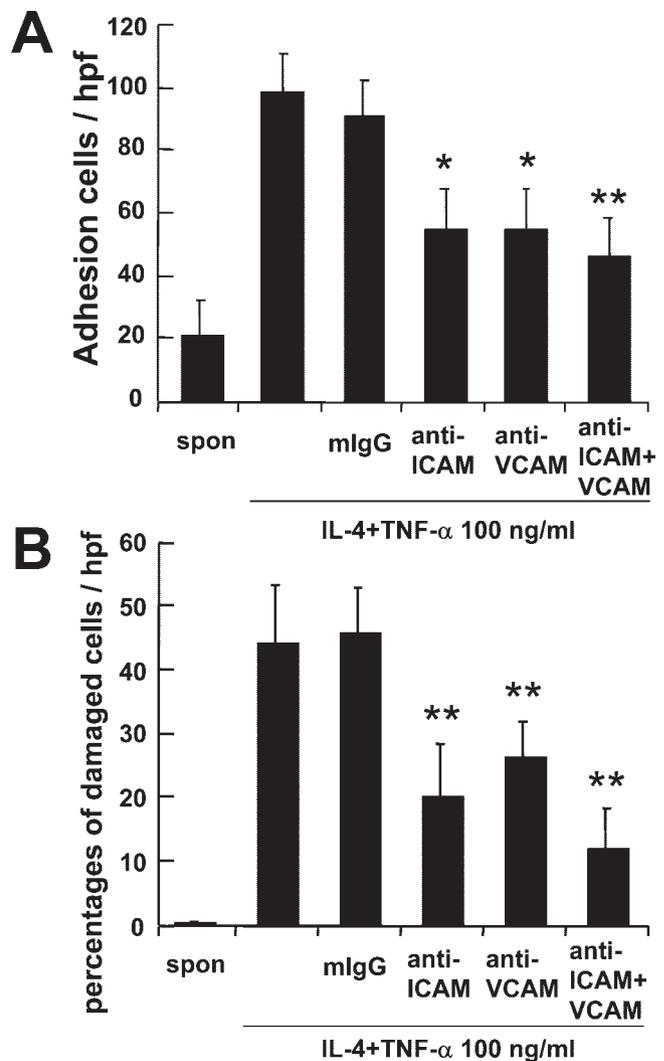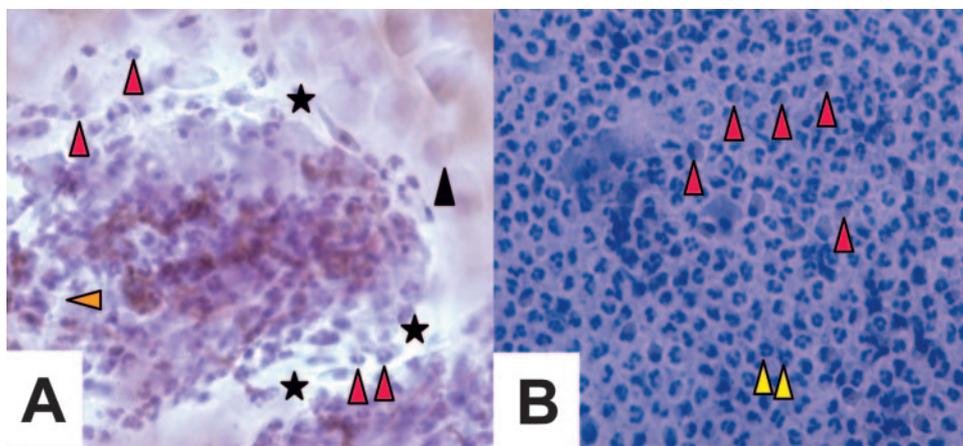

FIGURE 5. The effects of anti–ICAM-1 and anti–VCAM-1 mAb on cell adhesion and fibroblast damage. Cultured fibroblasts were treated with IL-4 and TNF-α (100 ng/mL each) and mouse IgG1 (mIgG; control), anti–ICAM-1, and anti–VCAM-1 antibody (10 mg/mL each) for 30 minutes. **(A)** Primed eosinophils were cocultured for 3 hours, and adhered eosinophils were counted ($n = 3$); *$P < 0.05$, **$P < 0.005$. **(B)** Primed eosinophils were cocultured for 72 hrs, and the viability of keratocytes was evaluated by counting trypan blue–negative cells. Percentage of damaged cells was calculated by subtraction of trypan blue–negative cells ($n = 3$); **$P < 0.005$. spon, spontaneous.

formation of keratocytes into a repair phenotype of fibroblasts that release enzymes such as collagenases, matrix metalloproteinases involved in stromal modeling.[33] We thus chose to use fibroblast instead of keratocyte cultures in this study to investigate active fibroblast-related events that may be important in allergic wound-healing response. To support the findings from the in vitro experiments of this study, we performed conjunctival and corneal impression cytology on AKC patients with corneal ulcers as well.

We initially confirmed the expression of two adhesion molecules, ICAM-1 and VCAM-1, by real-time PCR and flow cytometry. Real-time PCR provided evidence that ICAM-1 and VCAM-1 mRNA expression were upregulated with IL-4 and TNF-α stimulation. Flow cytometry analysis revealed further evidence of increased corneal fibroblast expression of ICAM-1 and VCAM-1 on stimulation by IL-4 and TNF-α. We thought that increased expression of ICAM-1 and VCAM-1 on corneal fibroblasts on stimulation with IL-4 and TNF-α, two cytokines

FIGURE 6. **(A)** Conjunctival impression cytology specimen from a patient with AKC and corneal ulceration, showing the abundance of inflammatory cell infiltrates, consisting mainly of neutrophils and eosinophils (*red arrows*), adjacent to conjunctival epithelial cells with advanced Nelson's grade 2 squamous metaplasia and decreased cellular cohesion (*black arrow*), as well as the total loss of cellular cohesion between the focus of inflammation and the surrounding epithelial cells (*black stars*) and the loss of cellular cohesion between the conjunctival epithelial cells lying under the inflammatory infiltrates (*orange arrow*) (magnification, ×100). **(B)** Representative corneal impression cytology imprint obtained from the ulcer edge in the same patient, showing extensive eosinophilic infiltration (*red arrows*) and neutrophils (*yellow arrows*) and isolated corneal epithelial cells with Nelson's grade 3 squamous metaplasia (Wiegert's iron hematoxylin staining; magnification, ×100) (Dogru M, et al. *IOVS* 2002;46:ARVO E-Abstract 939).

known to be present in high amounts in allergic ocular surfaces, prepared a background which would ease the adhesion of eosinophils to corneal fibroblasts.

Indeed, eosinophils are known to bind ICAM-1 via all four members of the CD18 integrin family, CD11a to -d, which are expressed on the eosinophil cell sulface.[22–27] Eosinophils also express a number of β1 integrins, of which α4β1, the ligand of VCAM-1, is best characterized.[28] Observations from previous studies suggest that ICAM-1 and VCAM-1 are the potent molecules involved in eosinophil-fibroblast adhesion. The present study is the first to describe the morphologic alterations of corneal fibroblasts exposed to IL-4 and TNF-α, and bound by eosinophils.

Our morphologic observations of the cocultures showed that both IL-4 and TNF-α increased eosinophil adhesion to corneal fibroblasts solitarily and in combination. Likewise, applications of both cytokines were associated with significant increases in corneal fibroblast cell damage in the cultures. Our TUNEL assay findings suggested that fibroblast cellular damage was due to eosinophil-induced necrosis rather than apoptosis. Interestingly, blocking experiments performed with anti–ICAM-1 and anti–VCAM-1 applications revealed partial reversal of cellular damage. The reversal effect was enhanced when anti–ICAM-1 and anti–VCAM-1 were applied concomitantly.

Our results suggest that eosinophil adhesion to corneal fibroblasts via ICAM-1 and VCAM-1 may lead to degranulation of eosinophils. Indeed, eosinophils contain cytotoxic proteins such as ECP and MBP, which are released on activation and have been reported to cause damage to corneal cellular structures.[12] However, it should be noted that eosinophil adhesion was not completely inhibited with the anti–ICAM-1 and anti–VCAM-1 monoclonal antibodies in this study, indicating that other molecules may be associated with corneal fibroblasts. Recent evidence indicates that cell adhesion through CD11b/CD18 (Mac-1) is a crucial step for the activation, signaling, and effector function of eosinophils.[20,21] On the other hand, it has been reported that VLA-4 (CD49d)-mediated adhesion augments stimulated eosinophil degranulation. Therefore, ICAM-1/β2 and VCAM-1/VLA-4 interactions seem to be involved in the mechanisms of eosinophil cytotoxicity.

Conjunctival impression cytology findings in all subjects revealed inflammatory cell infiltrates, consisting mainly of neutrophils and eosinophils, adjacent to conjunctival epithelial cells with advanced squamous metaplasia, providing clinical evidence on the adverse effects of the inflammatory process on the epithelial cells and backing up our findings from the in vitro experiments. Interestingly, imprints obtained from corneal ulcers showed extensive eosinophilic and neutrophilic infiltration among isolated corneal epithelial cells with advanced squamous metaplasia, suggesting adverse effects of the infiltrates on cellular cohesion and integrity.

In conclusion, we found that corneal fibroblasts express ICAM-1 and VCAM-1 when activated with IL-4 and TNF-α, and the expression is highly selective among all adhesion-related molecules. Eosinophils can adhere to the activated fibroblasts and can induce subsequent fibroblast damage through the adhesion molecules. Eosinophil adhesion to fibroblasts may contribute to the pathogenesis of severe persistent allergic corneal ulcers.

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