Alarmins From Corneal Epithelial Cells Upregulate CCL11 and VCAM-1 in Corneal Fibroblasts

Ken Fukuda,1 Waka Ishida,1 Hiroshi Tanaka,2 Yosuke Harada,1 Akira Matsuda,3 Nobuyuki Ebihara,3 and Atsuki Fukushima1

1Department of Ophthalmology, Kochi Medical School, Nankoku City, Kochi, Japan
2Machida Hospital, Kochi City, Kochi, Japan
3Department of Ophthalmology, Juntendo University School of Medicine, Tokyo, Japan

Purpose. Severe ocular allergic diseases are characterized by pronounced conjunctival inflammation triggered by T helper 2 (Th2) cells and corneal epithelial damage induced by eosinophils. To examine the role of alarmins released by damaged corneal epithelial cells in tissue eosinophilia, we investigated the effects of a supernatant derived from necrotic human corneal epithelial (HCE) cells on expression of the chemokine CCL11 (eotaxin) and the adhesion molecule VCAM-1 in human corneal fibroblasts.

Methods. An alarmin preparation was obtained as the material released from HCE cells after three cycles of freezing and thawing. CCL11 released into culture medium and cell surface expression of VCAM-1 were measured by reverse transcription and real-time polymerase chain reaction analysis. Signaling by the transcription factor NF-kB was evaluated by immunoblot and immunofluorescence analyses.

Results. The combination of the necrotic HCE cell supernatant and either interleukin (IL)-4 or IL-13 induced synergistic increases in CCL11 release, VCAM-1 expression, and the amounts of CCL11 and VCAM-1 mRNAs were quantitated by reverse transcription and real-time polymerase chain reaction analysis. Signaling by the transcription factor NF-kB was evaluated by immunoblot and immunofluorescence analyses.

Conclusions. Alarmins including IL-1 released from necrotic corneal epithelial cells cooperate with Th2 cytokines to induce CCL11 production and VCAM-1 expression in corneal fibroblasts, and may thereby play an important role in tissue eosinophilia associated with ocular allergic diseases.

Keywords: alarmin, corneal fibroblasts, allergy, chemokine, adhesion molecule
inflammation has remained unclear, however. Given that the corneal epithelium is often damaged in individuals with severe ocular allergic diseases, we have now examined whether alarmins released from necrotic corneal epithelial cells might contribute to this inflammatory response.

**METHODS**

**Cells**

Human primary keratocytes obtained from ScienCell Research Laboratories (Carlsbad, CA) were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA) and manifested transdifferentiation into corneal fibroblasts. Simian virus 40–immortalized human corneal epithelial (HCE) cells were obtained from RIKEN BioResource Center (Ibaraki, Japan) and were cultured in supplemented hormonal epithelial medium (SHEM), which consists of DMEM-F12 (Life Technologies) supplemented with 15% heat-inactivated FBS; bovine insulin (5 μg/mL; Sigma-Aldrich, St. Louis, MO); human epidermal growth factor (10 ng/mL; Sigma-Aldrich); and gentamicin (40 μg/mL; Life Technologies).

**Induction of HCE Necrosis**

Necrosis was induced in HCE cells by freezing and thawing, as previously described. In brief, confluent cells cultured in 1 mL of serum-free MEM in a 35-mm culture dish were subjected to three cycles of freezing at −80°C for 20 minutes and thawing at 37°C for 20 minutes. The cell supernatant was then collected for experiments. It was found not to contain CCL11 as determined with ELISA (data not shown).

**Stimulation of Stromal Fibroblasts and Measurement of CCL11 Release**

Stromal fibroblasts were seeded in 24- or 48-well culture plates (Becton Dickinson, Franklin Lakes, NJ) and cultured for 5 days in MEM supplemented with 10% FBS. They were then washed with PBS, cultured for 24 hours in serum-free MEM, and then incubated for the indicated times in serum-free MEM supplemented with the supernatant from necrotic HCE cells or cytokines subjected to real-time PCR analysis with the use of a real-time PCR kit; Qiagen, Hilden, Germany). The resulting cDNA was subjected to reverse transcription with the use of a reverse transcription kit (QuantiTect Reverse Transcription Kit; Qiagen, Hilden, Germany). The resulting cDNA was subjected to real-time PCR analysis with the use of a real-time PCR system and power SYBR Green PCR Master Mix; Life Technologies) as well as with primers (Qiagen) for CCL11, VCAM-1, and hypoxanthine phosphoribosyltransferase (HPRT). Transcripts of the constitutively expressed gene for HPRT served to normalize the amount of target mRNA in each sample. Real-time PCR data were analyzed with commercial software (StepOne 2.2; Life Technologies). After completion of the amplification protocol, melting curve analysis was performed to confirm the specificity of amplification. The fluorescence signal (F) was plotted against temperature (T) to generate a melting curve for each sample, and the melting curve was then converted to a melting peak by plotting the negative derivative of fluorescence with respect to temperature against temperature (−dF/dT versus T). Each PCR product gave rise to a specific melting temperature.

**In Situ Whole-Cell ELISA for VCAM-1**

An in situ whole-cell ELISA for VCAM-1 was performed as described. Concanavalin A–immortalized human corneal fibroblasts were seeded in 96-well culture plates and then cultured and incubated as described above for measurement of CCL11 release. After incubation with the supernatant from necrotic HCE cells or cytokines for 24 hours, the cells were washed twice with PBS, fixed for 15 minutes at room temperature with PBS containing 1% paraformaldehyde, washed with PBS containing 0.1% bovine serum albumin (BSA), and incubated for 1 hour at 37°C with a monoclonal antibody to VCAM-1 (1:10,000 dilution; Pharmingen, San Diego, CA) in PBS–BSA (1%). The cells were washed three times with PBS–BSA (0.1%), incubated for 1 hour at 37°C with horseradish peroxidase (HRP)–conjugated goat antibodies to mouse immunoglobulin G in PBS–BSA (1%), washed three times with PBS–BSA (0.1%), and then incubated for 20 minutes in the dark with 100 μL of 3,3′,5,5′-tetramethylbenzidine solution. The reaction was terminated by the addition of 50 μL of 1 M H2SO4, and the absorbance of each well was measured at 450 nm with the use of a microplate reader.

**Immunoblot Analysis of IκB-α and STAT6**

The phosphorylation and degradation of IκB-α and the phosphorylation of signal transducer and activator of transcription 6 (STAT6) in corneal fibroblasts were examined by immunoblot analysis as described previously. In brief, the cells were washed twice with PBS and lysed in radioimmunoprecipitation (RIPA) buffer, and the lysates (10 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis on a 10% gel under reducing conditions. The phosphorylation and degradation of IκB-α and STAT6 and the degradation of IκB-α and STAT6 by immunoblot analysis with respect to temperature against temperature (−dF/dT versus T). Each PCR product gave rise to a specific melting temperature.

**Immunofluorescence Analysis of NF-κB**

Immunostaining of nuclear factor–κB (NF-κB) in corneal fibroblasts was performed as described previously. In brief, cell monolayers grown on eight-well chamber slides were washed twice with PBS, fixed with 4% paraformaldehyde in PBS, washed an additional three times, and permeabilized with 100% methanol at −20°C for 6 minutes. The cells were
then incubated at room temperature consecutively for 30 minutes with PBS containing 3% BSA, for 1 hour with antibodies to the p65 subunit of NF-κB (1:100 dilution in PBS containing 1% BSA; Santa Cruz Biotechnology), and for 30 minutes with AlexaFluor 488-conjugated secondary antibodies (1:500 dilution in PBS containing 1% BSA; Life Technologies). They were finally washed, mounted in mounting medium (Vectashield; Vector Laboratories, Burlingame, CA), and examined with a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan).

**Statistical Analysis**

Data are presented as means ± SEM and were analyzed with the unpaired Student’s t-test or the Tukey-Kramer test. Statistical analysis was performed with statistical software (StatView for Windows 5.0; SAS Institute, Cary, NC). A P value of <0.05 was considered statistically significant.

**RESULTS**

**Synergistic Effects of Necrotic HCE Cell Supernatant and Either IL-4 or IL-13 on CCL11 Release by Human Corneal Fibroblasts**

We first examined the effects of a necrotic HCE cell supernatant as well as of the Th2 cytokines IL-4 and IL-13 on the production of CCL11 by human corneal fibroblasts. Incubation of the cells for 48 hours with the necrotic HCE cell supernatant (20%) or either cytokine (each at 10 ng/mL) resulted in only a relatively small increase in the amount of CCL11 released into the culture medium (Fig. 1A). However, incubation of the cells in the presence of both the necrotic HCE cell supernatant and either IL-4 or IL-13 induced a synergistic increase in the release of CCL11 (Fig. 1A). The stimulatory effect of the necrotic HCE cell supernatant on CCL11 release was concentration dependent, and was maximal at a concentration of 20% in the presence of IL-4 or IL-13 (Fig. 1B). The time course of CCL11 release by corneal fibroblasts incubated with the combination of the necrotic HCE cell supernatant and either IL-4 or IL-13 was approximately linear for up to 24 hours and then plateaued (Fig. 1C). RT and real-time PCR analysis also revealed that exposure of corneal fibroblasts to the necrotic HCE cell supernatant or either cytokine alone induced only a relatively small increase in the amount of CCL11 mRNA (Fig. 2). Treatment of the fibroblasts with both the necrotic HCE cell supernatant and either cytokine, however, resulted in a synergistic increase in the amount of CCL11 mRNA (Fig. 2).

**Effects of Necrotic HCE Cell Supernatant and Th2 Cytokines on VCAM-1 Expression in Corneal Fibroblasts**

We next measured the effects of the necrotic HCE cell supernatant and the Th2 cytokines on expression of the adhesion molecule VCAM-1 in corneal fibroblasts. An in situ whole-cell ELISA revealed that incubation of the cells for 24 hours with the necrotic HCE cell supernatant alone induced only a small increase in VCAM-1 expression. In the presence of IL-4 or IL-13, however, the necrotic HCE cell supernatant markedly potentiated the stimulatory effect of each cytokine on VCAM-1 expression (Fig. 3A). Furthermore, whereas the necrotic HCE cell supernatant or either cytokine alone induced only a relatively small increase in the amount of VCAM-1 mRNA in corneal fibroblasts, the combination of the necrotic HCE cell supernatant and either IL-4 or IL-13 (each at 10 ng/mL), after which the concentration of CCL11 released into the culture medium was determined with an ELISA. (B) Corneal fibroblasts were incubated for 48 hours with the indicated concentrations of necrotic HCE cell supernatant and in the absence (open bars) or presence (closed bars) of necrotic HCE cell supernatant (20%) and either with or without IL-4 or IL-13 (each at 10 ng/mL), after which the concentration of CCL11 released into the culture medium was measured. (C) Corneal fibroblasts were incubated for the indicated times in the presence of necrotic HCE cell supernatant (20%) or with either IL-4 (closed triangles) or IL-13 (open triangles), each at a concentration of 10 ng/mL, after which the concentration of CCL11 released into the culture medium was determined. All data are means ± SEM of quadruplicates. **P < 0.01 (Tukey-Kramer test) versus the corresponding value for cells incubated without addition. ††P < 0.01 (Tukey-Kramer test) for the indicated comparisons.
supernatant and either IL-4 or IL-13 elicited a synergistic upregulation of this transcript (Fig. 3B).

**Effect of Necrotic HCE Cell Supernatant on NF-κB Activity in Corneal Fibroblasts**

The IκB-α-NF-κB and STAT6 pathways are thought to play key roles in the synergistic induction of CCL11 and VCAM-1 gene expression in various cell types. Therefore, we next examined the possible effects of the necrotic HCE cell supernatant on these signaling pathways in corneal fibroblasts by monitoring the phosphorylation and degradation of the endogenous IκB-α inhibitor IκB-α as well as the phosphorylation of STAT6 and the subcellular localization of the p65 subunit of NF-κB in these cells. Exposure of corneal fibroblasts to the necrotic HCE cell supernatant induced the phosphorylation and degradation of IκB-α in the absence or presence of IL-4 or IL-13 (Fig. 4A). In contrast, whereas IL-4 or IL-13 induced the phosphorylation of STAT6, the necrotic HCE cell supernatant had no such effect (Fig. 4A). The phosphorylation and degradation of IκB-α induced by the necrotic HCE cell supernatant were time dependent (Fig. 4B). The phosphorylation was thus first apparent at 5 minutes and maximal at 15 minutes, whereas a decrease in the amount of IκB-α was first apparent at 5 minutes and maximal at 30 minutes, after which the abundance of the protein gradually increased up to 2 hours after the onset of stimulation. Immunofluorescence analysis revealed that the p65 subunit of NF-κB was located predominantly in the cytoplasm of corneal fibroblasts under basal conditions, whereas treatment of the cells with the necrotic HCE cell supernatant resulted in translocation of this protein to the nucleus within 30 minutes (Figs. 4C, 4D). These various observations thus showed that the necrotic HCE cell supernatant activated the NF-κB signaling pathway in human corneal fibroblasts.

To examine the role of NF-κB activation in the synergistic stimulation of CCL11 release by the necrotic HCE cell supernatant and Th2 cytokines, we determined the effect of PDTC, an inhibitor of NF-κB signaling, on this response. PDTC (100 μM) inhibited the synergistic induction of CCL11 release (Fig. 5) and VCAM-1 expression at the cell surface (data not shown) by the necrotic HCE cell supernatant and either IL-4 or IL-13, suggesting that the activation of NF-κB by the supernatant plays a major role in this response.

**Effect of IL-1RA on CCL11 Release From Corneal Fibroblasts Induced by the Necrotic HCE Cell Supernatant and Th2 Cytokines**

Finally, we investigated what factors in the necrotic HCE cell supernatant might be responsible for the synergistic induction together with Th2 cytokines of CCL11 release from corneal fibroblasts. Various endogenous danger signals, including IL-1α, IL-33, and HMGB1, are released from dying or necrotic cells and are thought to contribute to inflammation in a sterile setting. We therefore examined the effects of IL-1α, IL-33, or HMGB1 in combination with IL-4 on CCL11 release by corneal fibroblasts. Only the combination of IL-1α and IL-4 was...
found to induce a synergistic increase in CCL11 release from these cells (data not shown). We then examined the effects of IL-1RA on CCL11 release and VCAM-1 expression induced by the necrotic HCE cell supernatant and Th2 cytokines in corneal fibroblasts. Treatment with IL-1RA (100 ng/mL) inhibited the release of CCL11 (Fig. 6) and the expression of VCAM-1 at the cell surface (data not shown) induced by the combination of the necrotic HCE cell supernatant and either IL-4 or IL-13, indicating that IL-1 derived from necrotic HCE cells mediates these responses.

DISCUSSION

We have here shown that a supernatant derived from necrotic HCE cells together with a Th2 cytokine activated corneal fibroblasts to synthesize CCL11 and VCAM-1, both of which molecules may enhance eosinophil infiltration into the cornea and thereby result in further corneal epithelial damage during ocular allergic inflammation. This action of the necrotic HCE cell supernatant appeared to be mediated by NF-κB and was attenuated by IL-1RA, suggesting that it was attributable in part to IL-1 released from the epithelial cells.

In the cornea, stromal fibroblasts, rather than epithelial cells, act as immune modulators by secreting chemokines and expressing adhesion molecules during allergic inflammation. We have previously shown that corneal fibroblasts express a high-affinity receptor complex for IL-4 and IL-13 as well as release the chemokine CCL11 and express the adhesion molecule VCAM-1 in response to combined stimulation with Th2 cytokines (IL-4 or IL-13) and either proinflammatory cytokines (tumor necrosis factor-α [TNF-α] or IL-1) or bacterial components such as peptidoglycan. Corneal epithelial cells did not secrete CCL11 on exposure to Th2 cytokines, nor did they elicit an innate immune response to bacterial components including lipopolysaccharide. Corneal epithelial cells thus appear to contribute to corneal defense by providing a barrier against invading pathogens or inflammatory mediators. We have also previously shown that ablation of the corneal epithelium increased the infiltration of eosinophils into the conjunctiva in a rat model of allergic conjunctivitis, suggesting that loss of the barrier function of the corneal epithelium and consequent exposure of corneal fibroblasts to allergic cytokines in tear fluid are likely to exacerbate ocular allergic inflammation. Our present results now suggest a new role for corneal epithelial cells during ocular allergic inflammation. The release of alarmins from epithelial cells damaged by eosinophils or mechanical scratching may thus contribute to exacerbation of eosinophilic inflammation at the ocular surface. Although the roles of resident corneal immune cells such as DCs and macrophages stimulated by alarmins released from necrotic corneal epithelial cells during ocular allergic inflammation remain unclear, alarmins are thought to contribute to the development of innate and adaptive immune responses through activation of DCs. The effects of alarmins on resident corneal immune cells therefore warrant investigation.
Synergistic induction of CCL11 and VCAM-1 by Th2 cytokines and proinflammatory cytokines such as TNF-\(\alpha\) has been described in various cell types including epithelial cells and fibroblasts.\(^{19,20}\) Putative overlapping binding sites for NF-\(\kappa\)B and STAT6 have been identified in the CCL11 gene promoter, and these two transcription factors are thought to mediate the synergistic effect of IL-4 or IL-13 together with IL-1 on CCL11 gene expression in these cells.\(^{19,20}\) We have now shown that alarmins released from HCE cells activated NF-\(\kappa\)B in corneal fibroblasts and induced CCL11 and VCAM-1 synthesis in a synergistic manner together with Th2 cytokines. In addition, an inhibitor of NF-\(\kappa\)B signaling attenuated these synergistic effects on CCL11 production and VCAM-1 expression. Alarmins as well as proinflammatory cytokines that activate NF-\(\kappa\)B such as TNF-\(\alpha\) and IL-1 are therefore potential targets for therapeutic attenuation of eosinophilic inflammation.

In addition to IL-1\(\alpha\), various other mediators are released from necrotic cells. We also examined the possible role of HMGB1 and IL-33 in the activation of corneal fibroblasts, given that these two molecules were previously found to stimulate the release of chemokines such as CXCL8 and CCL11 from fibroblasts.\(^{30,31}\) In contrast to its effect on lung fibroblasts,\(^{31}\) however, IL-33 did not stimulate CCL11 release from corneal fibroblasts either alone or in combination with Th2 cytokines. HMGB1 also did not stimulate CCL11 synthesis in these cells. We previously showed that cytokine regulation of CCL17 (TARC) expression differs among fibroblasts derived from different tissues, with corneal fibroblasts showing a greater response than lung fibroblasts.\(^{16}\) The unresponsiveness of corneal fibroblasts to IL-33 and HMGB1 might thus reflect the distinct phenotype of these cells.

In conclusion, our results suggest that IL-1 derived from necrotic corneal epithelial cells, in cooperation with Th2 cytokines, induces a synergistic increase in the production of CCL11 and VCAM-1 by corneal fibroblasts. Alarmins released from damaged corneal epithelial cells may thus play a key role in the induction of corneal damage and the vicious cycle of corneal involvement and conjunctival inflammation by activating corneal fibroblasts.

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**References**


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