CD40 Expression in Normal Human Cornea and Regulation of CD40 in Cultured Human Corneal Epithelial and Stromal Cells

Mitsuhito Iwata, Koichi Soya, Mitsuru Sawa, Takashi Sakimoto, and David G. Hwang

Purpose. To determine whether CD40–CD40 ligand (CD40L) interaction plays a role in corneal inflammatory responses, the expression of CD40 and CD40L on normal human cornea was investigated. In addition, using cultured human corneal epithelial (HCE) and human corneal stromal (HCS) cells, the regulation of CD40 expression in human corneal cells investigated, including that induced by proinflammatory cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α.

Methods. Frozen optimal cutting temperature (OCT) compound–embedded sections of corneal tissues obtained from 18 normal human corneas were examined by an immunoperoxidase staining technique with anti-CD40 and anti-CD40L monoclonal antibodies (mAbs). Also, cultured HCE and HCS cells, with IFN-γ (250–1000 U/mL) or TNF-α (500–4000 U/mL) treatment for 1 to 4 days and with no treatment, were stained by the immunofluorescence technique with mAbs and analyzed by flow cytometry.

Results. The area of positive staining for CD40 showed a topographical difference. The limbal epithelial cells were predominantly positive for CD40. Positive staining was also found to a lesser extent on the cells in the basal layer of peripheral corneal epithelium. Epithelial cells of the central cornea showed no immunoreactivity for CD40. Corneal stromal cells were negative for CD40 in most of the donor tissues (positive: 5 of the 18 corneas). Endothelial cells were distinctly negative for CD40. Cultured HCE cells were also positive but decreased in positive cell number with lengthening culture period. None or less than 5% of the cultured HCS cells were CD40 positive. IFN-γ enhanced CD40 expression on both cell types. In contrast, TNF-α enhanced CD40 on HCE but not on HCS cells. No component cells of normal human cornea or cultured HCE and HCS cells showed immunoreactivity for CD40L.

Conclusions. In the human cornea, CD40 is expressed predominantly on limbal epithelial cells and also on cultured HCE and HCS cells with high proliferative potential. In addition, the expression of CD40 is induced on cultured HCE and HCS cells differentially by proinflammatory cytokines, such as IFN-γ and TNF-α. (Invest Ophthalmol Vis Sci. 2002;43:348–357)
Methods

Immunocytochemistry

The human corneal tissues used in this study were obtained from two sources. Donor corneas for corneal transplantation were obtained from Japanese domestic local eye banks. Corneal tissues remaining after corneal transplantation were used in this study. In addition, whole corneas were obtained from the Lions Eye Bank of Oregon. This study was approved by the Ethics Committee for Human Research at Nihon University.

Corneal specimens were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Scientific, Naperville, IL) at −20°C. Frozen OCT-embedded sections were cut at 7-μm thickness and placed on poly-L-lysine coated microscope slides (Muto Pure Chemicals, Tokyo, Japan). These plates were examined by immunoperoxidase staining with anti-CD40 monoclonal antibody (mAb; G25–8; ATCC, Manassas, VA), anti-CD40L mAb (24–31; Ancell, Bayport, MN), and anti-cytokertin (CK)19 mAb (K4.62; Sigma, St. Louis, MO), using a previously described procedure. Briefly, after they were fixed in chilled acetone, the plates were incubated with horse serum for 30 minutes, washed twice with PBS containing 0.1% fetal bovine serum (FBS), and with affinity-purified goat anti-horse antibody (1:100 dilution; Vector Laboratories) for 30 minutes. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 30 minutes, followed by incubation with avidin-biotin peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) for 60 minutes. After incubation with avidin-biotin peroxidase complex, the plates were treated with either recombinant human IFN-γ (100 U/mL; Celltech, Royal Tunbridge Wells, England) or recombinant human TNF-α (Genzyme, Cambridge, MA) at 37°C under 5% CO₂. The explants were removed after 4 weeks. Cultured cells after three to five passages were used in this study. These cells were confirmed as fibroblast-like corneal stromal cells, because they were spindle shaped, vimentin positive, and negative for cytokeratin expression. Contamination by Langerhans cells was excluded by the same method used in the HCE cell culture study, as described.

Flow Cytometry

Cultured HCE and HCS cells were converted to cell suspension with trypsin-EDTA treatment. After the cells settled in DMEM with 10% FBS (10% FBS-DMEM) at room temperature for 2 hours, they were stained by the following immunofluorescence technique: The cells were washed twice with PBS containing 2% bovine serum albumin and 0.1% NaN₃ (washing buffer; Sigma), then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD40 mAb (B-B20; Diaclon, Besançon, France), or FITC-conjugated mouse IgG negative control (Dako, Glostrup, Denmark) for 45 minutes. After the cells were washed twice with washing buffer, the viable 10,000 cells were analyzed by flow cytometry (Ortho Cytoron; Ortho Diagnostic Systems, Tokyo, Japan). These staining procedures were performed at 4°C.

Results

CD40 and CD40L Expression on Normal Human Cornea

The frozen OCT sections of corneal tissues obtained from 18 normal human corneas were examined by immunoperoxidase staining with anti-CD40 and anti-CD40L mAbs. The results of the staining pattern are summarized in Table 1. The boundary between the limbus and peripheral cornea was determined by

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<tr>
<td><strong>Epithelium</strong></td>
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<tr>
<td>Basal cells</td>
<td>12</td>
<td>6</td>
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<tr>
<td>Suprabasal cells</td>
<td>1</td>
<td>10</td>
<td>5</td>
<td>1 (1)†</td>
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<tr>
<td>Superficial cells</td>
<td>/</td>
<td>5</td>
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<td>5 (5)</td>
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<td><strong>Periphery</strong></td>
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<tr>
<td>Basal cells</td>
<td>2*</td>
<td>3</td>
<td>3</td>
<td>2 (1)</td>
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<td>Suprabasal cells</td>
<td>/</td>
<td>3</td>
<td>5</td>
<td>7 (5)</td>
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<td>Superficial cells</td>
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<td>2</td>
<td>2</td>
<td>10 (4)</td>
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<td><strong>Center</strong></td>
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<td>Basal cells</td>
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<td>Suprabasal cells</td>
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<td>Superficial cells</td>
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<tr>
<td><strong>Stroma</strong></td>
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<tr>
<td>(n = 18)</td>
<td>/</td>
<td>3</td>
<td>2</td>
<td>12 (1)</td>
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<td><strong>Endothelium</strong></td>
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<td>(n = 6)</td>
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Data are the number of stained corneas. Staining intensity: +++, intense; +, moderate; ±, occasional positive cells; –, negative; /, not detected.

† Numbers in parentheses indicate number of corneas undetermined because of cell degradation.
the termination of the Bowman membrane and the appearance of underlying stromal blood vessels.

Positive staining with anti-CD40 mAb was found on corneal epithelial cells (Fig. 1). The positively stained area showed a topographical difference. The limbal epithelial cells were dominantly positive for CD40 (Fig. 1A). In most of the samples, positive staining was observed, not only on the cell surface but also in cytoplasm throughout the limbus. However, the stain-
ing on the basal epithelial cells was prominent (Fig. 1E). Peripheral corneal epithelial cells were also positive for CD40, predominantly in the basal cell layer. However, with increasing distance from the limbus, CD40-positive epithelial cells were increasingly irregular in distribution and markedly decreased in number (Fig. 1B). Epithelial cells of the central cornea showed no immunoreactivity for CD40 (Fig. 1C). No large interindividual variation of CD40 expression was observed on the epithelial cells among donor tissues examined in this study.

Corneal stromal cells were negative for CD40 in the majority of donor tissues examined here (Fig. 2A). However, in 3 of the 18 donor corneas, CD40-positive stromal cells were found throughout the cornea (Fig. 2B). In two corneas, the positive cells were present but scarce in the upper 50% of stromal depth. However, a topographical difference, such as that found in the epithelial cells, could not be detected. Corneal endothelial cells were distinctly negative for CD40, although the corneal endothelium of six donor corneas retained intact morphology (Fig. 2C).

The other cells, such as the dendritic cells located in the limbus, were positive for CD40 (Fig. 3A). Positive staining for CD40 was also found on vascular endothelial cells of limbal stromal blood vessels in 5 of the 18 donor corneas (Fig. 3B). In three of these corneas, corneal stromal cells were also positive for CD40. Regarding CD40L expression, no component cells of the cornea showed any immunoreactivity for CD40L by the immunoperoxidase staining technique (data not shown).

**Correlation between CD40 and CK19 Expression in Limbal and Corneal Epithelial Cells**

CK19 is a CK expressed by the basal cells of stratified squamous epithelial cells of various tissues such as exocervix, vagina, tongue, oral mucosa, and esophagus. These basal cells are considered to have high proliferative potential. CK19 has been shown to be expressed on the limbal and peripheral corneal epithelial cells and not to be expressed in the epithelial cells of the central cornea, which is similar to CD40 expression, as demonstrated in the foregoing section. Therefore, there was the possibility that CD40 and CK19 might be expressed in the same epithelial population. To test this possibility, serial OCT sections of the limbus and peripheral cornea were stained with anti-CD40 or anti-CK19 mAb. Positive staining for CK19 showed an irregular mosaic pattern in the limbal and peripheral corneal epithelial cells (Fig. 4). The positive staining for CD40 and for CK19 showed a quite similar pattern in the basal epithelial cell layer that often overlapped into the
suprabasal cell compartment. However, in the superficial cell layer, there was a great difference in staining. The superficial epithelial cells were uniformly positive for CK19. In contrast, these cells were mostly negative for CD40 (Table 1).

**CD40 Expression on Cultured HCE and HCS Cells**

Cultured HCE and HCS cells were stained with anti-CD40 mAb and examined by flow cytometry (Fig. 5). On primary-culture HCE cells (Fig. 5A), the percentage of CD40-positive cells was 21.6% ± 11.4% (mean ± SD of separate experiments with cultured HCE cells from 12 donor corneas) after the cultured cells had become confluent. Changes in the expression of CD40 during the culture period were examined, using subcultured HCE cells in 12-well plates transferred from confluent primary-culture cells. The cells in each well of the plates became confluent at 2 to 3 days. Shortly after confluence, the percentage of CD40-positive cells reached nearly 50%. CD40 expression then gradually decreased with lengthening culture period (Table 2).

In contrast, there were no or less than 5% CD40-positive HCS cells cultured with 10% FBS-DMEM (Fig. 5B; \( n = 12 \)). CD40 expression did not vary with the age of the primary culture or with the passage number of the culture. In addition, even though some HCS cells were cultured with modified SHEM, which contains a sufficient amount of such growth factors as EGF and insulin, there was no difference in CD40 expression. HCS cultures were performed using stromal explants from three donor corneas in which no CD40-positive stromal cells were observed by immunocytochemistry. On these cultured HCS cells, CD40 was never detected by flow cytometry. In addition, positive staining for CD40 was not found on cultured HCS cells grown on chamber slides by immunoperoxidase staining (data not shown). Consequently, the results of flow cytometry to determine CD40 expression on cultured HCS cells were not due to the effect of trypsin.

**Figure 5.** CD40 expression on confluent primary-culture HCE cells (A) and cultured HCS cells after four passages (B) were stained with anti-CD40 mAb or control IgG and analyzed by flow cytometry.

<table>
<thead>
<tr>
<th>Culture Period (days)</th>
<th>CD40-Positive Cells (%)</th>
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<tr>
<td>2-3</td>
<td>30.8 ± 5.4</td>
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<tr>
<td>4-5</td>
<td>46.2 ± 3.2</td>
</tr>
<tr>
<td>6-7</td>
<td>35.2 ± 1.7 ( * )</td>
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<tr>
<td>8-10</td>
<td>24.9 ± 4.1</td>
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* Cultured HCE cells were transferred from confluent primary cultures to 12-well plates, then incubated with culture medium for the indicated period. These cells were immunostained with anti-CD40 mAb and analyzed by flow cytometry. Data represent mean percentage and SD for four independent experiments, with cultured HCE cells obtained from four donor corneas.

\( * P < 0.0003 \), significantly different between the two groups; Student’s t-test.
Effect of IFN-γ/H9253 on CD40 Expression

Cultured HCE and HCS cells were treated with human recombinant IFN-γ/H9253 at various concentrations ranging from 250 to 1000 U/mL and for various incubation periods from 0 to 96 hours. The manner of induction by IFN-γ was basically the same in both cell types (Fig. 6), as follows: The increase of CD40 expression was apparent at after 2 days' incubation with IFN-γ/H9253 and reached a maximum at 3 days (Fig. 6A). The expression of CD40 showed a dose–response curve depending on the concentration of IFN-γ/H9253 (Fig. 6B). The maximum expression of CD40 reached nearly 70% positive cells by 3 days of treatment with 1000 U/mL IFN-γ/H9253. We performed the same experiments using cultured cells from four donor corneas, and we obtained consistent results in each experiment.

Effect of TNF-α/H9251 on CD40 Expression

We examined the effect of treatment with human recombinant TNF-α/H9251 on CD40 expression in cultured HCE and HCS cells used in the experiments with IFN-γ/H9253. A detectable increase in CD40 expression on cultured HCE cells by TNF-α required at least a 3-day incubation, in contrast to the requirement of a 2-day incubation with IFN-γ/H9253. The maximal expression of CD40 reached nearly 70% positive cells by 3 days of treatment with 1000 U/mL IFN-γ/H9253. We performed the same experiments using cultured cells from four donor corneas, and we obtained consistent results in each experiment.

Effect of TNF-α on CD40 Expression

We examined the effect of treatment with human recombinant TNF-α on CD40 expression in cultured human corneal epithelial cells and occasionally expressed on corneal stromal cells. CD40 was never expressed on corneal endothelium. In corneal epithelium, we found an intriguing topographical difference of CD40 expression as follows: CD40 was expressed predominantly on the limbal epithelial cells and the basal layer of peripheral corneal epithelium. The epithelium of the center of the cornea was totally negative for CD40, and CD40 expression was found most intensively on the basal cells of the limbal epithelial cells. Although we examined corneal tissues from 18 corneas, we found no large interindividual variation. Therefore, we concluded that this distribution of CD40 was found in cultured HCS cells after TNF-α treatment (Figs. 7A, 7B), even when the cells were treated with TNF-α at concentrations up to 4000 U/mL and for 96 hours. There was no variation in the effect of TNF-α on cultured HCS cells used in this study.

**DISCUSSION**

We demonstrated that CD40 is expressed on normal human corneal epithelial cells and occasionally expressed on corneal stromal cells. CD40 was never expressed on corneal endothelium. In corneal epithelium, we found an intriguing topographical difference of CD40 expression as follows: CD40 was expressed predominantly on the limbal epithelial cells and the basal layer of peripheral corneal epithelium. The epithelium of the center of the cornea was totally negative for CD40, and CD40 expression was found most intensively on the basal cells of the limbal epithelial cells. Although we examined corneal tissues from 18 corneas, we found no large interindividual variation. Therefore, we concluded that this distribution of CD40 was found in cultured HCS cells after TNF-α treatment (Figs. 7A, 7B), even when the cells were treated with TNF-α at concentrations up to 4000 U/mL and for 96 hours. There was no variation in the effect of TNF-α on cultured HCS cells used in this study.
CD40 expression was constitutive. In corneal stroma, stromal cells were negative for CD40 in the most of the donor tissues examined in this study. However, we found CD40 expression on stromal cells in 5 of the 18 donor corneas. We did not detect the regional difference found in the epithelial cells. No relationship was observed between CD40 expression and donor age, although we examined corneas from donors with a wide range of ages (7–92 years old). In three corneas, CD40 was expressed on both vascular endothelial cells and stromal cells. However, in no case examined in this study could cellular infiltration be found. Therefore, it is unlikely that the existence of subclinical inflammation caused production of proinflammatory cytokines, leading to CD40 expression on those cells. The reason for such interindividual variations in the CD40 expression on corneal stromal cells and limbal cells is now under investigation.

Dendritic cells observed in the limbus were positive for CD40, as has been demonstrated in other tissue, such as epidermis. However, CD40L expression was not detected on any component of normal cornea examined in the present study.

Regarding preferential expression of CD40 on limbal epithelial cells, there are plausible implications considered from two different points of view, the immunologic aspect and the proliferative potential.

First, the limbus is an entry zone for Langerhans cells and leukocytes to invade the diseased cornea. It has been shown that CD40 on thymic epithelial cells is capable of acting as a costimulatory molecule with IFN-γ and IL-1 for granulocyte-macrophage–colony-stimulating factor (GM-CSF) production. Also, ligation of CD40 has been shown to enhance the release of IL-8 on IFN-γ-stimulated keratinocytes and retinal pigment epithelial cells. GM-CSF is known to activate a variety of hematopoietic cells, such as neutrophils, macrophages, and Langerhans cells. In contrast, IL-8 is a powerful chemotactic factor for neutrophils and T lymphocytes in addition to its capability for activating neutrophils. On other cell types such as monocytes-macrophages, ligation of CD40 stimulates production of various proinflammatory cytokines, such as IL-1, -6, -8, and -12 and TNF-α. IL-12 is a critical cytokine for induction and maintenance of Th1-type cellular immune responses. In addition to cytokine production, the ligation of CD40 has been demonstrated to enhance the expression of surface molecules such as MHC class II, ICAM-1 (CD54), LFA-3 (CD58), and B7-2 (CD86) on several cell types. Taking all evidence together, there is the possibility that the CD40–CD40L interaction in corneal epithelium at the limbus may trigger production of proinflammatory cytokines by corneal epithelial cells and induction of the expression of surface molecules on these cells as occurs in other cell types, leading not only to enhancement of chemotaxis and activation of leukocytes but also to corneal epithelial cell–dependent Langerhans cell activation and migration into and out of the cornea. As we have demonstrated, proinflammatory cytokines such as IFN-γ and TNF-α induce marked CD40 expression on cultured HCE cells. These cytokines produced in the inflamed cornea could augment the reactions mentioned earlier, not only at the limbus, but also throughout the cornea. Regarding the effects of IFN-γ and TNF-α, the required incubation time for significant induction of CD40 by TNF-α was at least 3 days, much longer than that of ICAM-1 as previously demonstrated, also longer
than the induction of CD40 by IFN-γ (2 days). Therefore, it is suggested that CD40 may be indirectly induced by TNF-α, probably mediated through a second messenger. A difference in the behavior of IFN-γ and TNF-α in induction of CD40 expression has been shown in conjunctival epithelial cells. Twenty-four-hour treatment with IFN-γ significantly increased CD40 expression, whereas 48 hours but not 24 hours of treatment with TNF-α increased CD40 expression in a human conjunctival epithelial cell line. However, the significant upregulation of CD40 has been observed after 24 hours of stimulation by either IFN-γ or TNF-α on thymic epithelial cells and vascular endothelial cells. Although these dissimilarities may be due to differences in culture conditions, there is the possibility that regulation of CD40 expression by proinflammatory cytokines differs among cell types and origins.

Second, limbal basal epithelial cells have high proliferative potential. Because we found moderate expression of CD40 on the suprabasal cells of the limbal epithelial cells in addition to intense expression on the basal epithelial cells, CD40 expression cannot be restricted to corneal epithelial stem cells. CD40 was found on peripheral corneal epithelium, where the positive cells were primarily the basal cells. Based on previous studies in a variety of tissues, CD40 is preferentially expressed on cells forming the basal cell layer of normal stratified squamous epithelium such as nasopharynx, tonsils, and ectocervix. In these epithelia, the basal cells are considered to have as high a proliferative potential as in the limbal epithelial cells.

CK19, expressed on the regenerating basal cells of stratified squamous epithelial cells of various tissues, has also been shown to be expressed on the limbal and peripheral corneal epithelial cells. Therefore, we examined whether CD40 and CK19 may be expressed in the same epithelial population. Positive staining showed an overlapping pattern in the basal and the suprabasal epithelial cells. However, these patterns were quite different in the superficial epithelial cells. This result indicates that the corneal epithelial population expressing CD40 is not the same but somewhat overlaps with that expressing CK19. In addition to the immunohistochemical analysis of normal human corneal tissues, we studied CD40 expression by flow cytometry using cultured HCE cells. Shortly after the cultured cells became confluent, the percentage of cells with CD40 expression reached maximum, then gradually decreased with lengthening period of cell culture. Considering the in vivo and in vitro findings together suggests that CD40 is a novel physiological marker of epithelial proliferative potential in the cornea.

Regarding CD40 expression on corneal stromal cells, although stromal cells in the most of the donor corneas were negative for CD40, some samples (5/18 corneas) showed a positive reaction to CD40, as mentioned earlier. We also examined the expression of CD40 on cultured HCS cells from 12 donor corneas. None or less than 5% of the cultured HCS cells were positive for CD40, regardless of passage number and supplements to the basal medium. In addition, CD40 was never
detected on cultured HCS cells established from CD40-negative stromal explants. Over all, the expression of CD40 on corneal stromal cells is restricted to certain cell populations, which remains to be elucidated. However, IFN-γ-induced CD40 expression dramatically on all cultured HCS cells examined in this study. This finding implies that at the site of inflammation CD40–CD40L interaction between corneal stromal cells and infiltrated leukocytes may cause activation of stromal cells and production of cytokines, resulting in stromal opacity, as seen in corneal immunologic disorders such as herpetic stromal keratitis. Of note, TNF-α had little effect on CD40 expression on cultured HCS cells, in contrast to its marked induction of CD40 on HCE cells, as previously reported on keratinocytes and human retinal pigment epithelial cells. To better understand the role of CD40–CD40L interaction in the cornea, we are starting to investigate the mechanisms for differential cytokine regulation of CD40 between corneal epithelial and stromal cells.

Signals through CD40 have been demonstrated to trigger B-cell proliferation and inhibit Fas–Fas ligand (FasL)–induced apoptosis in B cells. Corneal epithelial cells have been shown to express both Fas and FasL. However, corneal stromal cells express only Fas. It has been demonstrated that a Fas-stimulating Ab triggers death of HCE and HCS cells in culture, characteristic of apoptosis. All evidence taken together, the signal mediated by CD40 may play an important role, not only in the escape of the epithelial stem cells and stromal cells from unfavorable cell death but also in the efficient epithelial regeneration for epithelial repair in corneal inflammatory diseases.

This study’s results together with the results of the previous study demonstrating CD40 expression on human conjunctival epithelial cell populations strongly suggest that CD40 plays an important role in both normal and inflammatory situations in the cornea and conjunctiva. We are now investigating what cellular reactions could be induced in corneal epithelial and stromal cells by signals through CD40. Furthermore, we are trying to determine whether ligands for CD40 comprising a soluble form have any clinical significance.

**References**


