Upregulation of Tight-Junctional Proteins in Corneal Epithelial Cells by Corneal Fibroblasts in Collagen Vitrigel Cultures

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Purpose. To investigate the effects of corneal fibroblasts on the differentiation of corneal epithelial cells in a coculture system based on a collagen vitrigel membrane.

Methods. Simian virus 40–transformed human corneal epithelial (HCE) cells and human corneal fibroblasts were cultured on opposite sides of a collagen vitrigel membrane. The distribution of HCE cells and corneal fibroblasts on the collagen membrane was determined by immunofluorescence staining and immunoblot analysis of marker proteins. Expression of the tight-junctional proteins ZO-1, occludin, and claudin and of the adherens-junctional proteins E- and N-cadherin in HCE cells was determined at the mRNA and protein levels by reverse transcription–polymerase chain reaction analysis and immunoblot analysis, respectively.

Results. The abundance of ZO-1, occludin, and claudin mRNA and proteins in HCE cells was markedly increased by coculture with corneal fibroblasts. The expression of E- or N-cadherin did not differ between HCE cells cultured with corneal fibroblasts and those cultured without them. PD98059, a specific inhibitor of signaling by extracellular signal regulated kinase (ERK), prevented the upregulation of tight-junctional proteins in HCE cells by corneal fibroblasts.

Conclusions. Human corneal fibroblasts regulated the expression of tight-junctional proteins in HCE cells, suggesting that corneal fibroblasts may play an important role in the differentiation of corneal epithelial cells. (*Invest Ophthalmol Vis Sci.* 2008;49:113–119) DOI:10.1167/iovs.07-0353

The transparency of the cornea is determined in large part by uniformity in the alignment of collagen fibers in the stroma. Such uniformity is affected by the amount of water in the stroma, which, in turn, is determined by the barrier function of the corneal epithelium and the pump activity of the corneal endothelium. The activation of corneal fibroblasts in pathologic conditions is often accompanied by disturbance of the corneal epithelium and loss of its barrier function, suggesting that corneal fibroblasts affect the functions of the corneal epithelium. The corneal epithelium forms a physical barrier that isolates the eye from the outside environment and regulates the passive movement of fluid, electrolytes, and macromolecules through the paracellular pathway.

Tight junctions between epithelial cells primarily serve as a barrier to the diffusion of water and solutes through the paracellular pathway by sealing the intercellular space. Tight junctions are thus the morphologic counterpart of the localized diffusion barrier[1] and are the major components of the epithelial barrier in the cornea.[2,3] The expression and distribution of tight junction-related proteins have been examined in the corneal epithelium of various species, including rats, rabbits, and humans.[4] Furthermore, simian virus 40 (SV40)-transformed human corneal epithelial cells have been shown to express the tight-junctional proteins ZO-1, occludin, and claudin in culture.[5,6] The tight-junctional complex is composed of the transmembrane proteins claudin and occludin and the membrane-associated proteins ZO-1, ZO-2, and ZO-3.[7,8] Both occludin and claudin have been detected in the corneal epithelium,[9] as has ZO-1.[4] Furthermore, activation of the extracellular signal regulated kinase (ERK) signaling pathway in human corneal epithelial cells has been shown to result in the disruption of tight junctions,[10] suggesting that tight junctions are regulated by this pathway in these cells.

The interaction between cells and the extracellular matrix (ECM) plays an important role in the regulation of cellular activities such as migration, proliferation, and differentiation.[11] ECM proteins thus induce changes in the differentiation state of a variety of cell types, including epithelial cells,[12] endothelial cells,[13] and myoblasts.[14] Type I collagen is the major component of the corneal stroma and provides structural support to the stromal cells. Corneal fibroblasts thus form an interconnected network of cells interspersed between lamellae composed of collagen fibers of uniform size and spatial distribution.[15–17] In addition, the basal layer of corneal epithelial cells is attached to a basement membrane.[18] The functions of both corneal fibroblasts and epithelial cells are thus likely continuously modulated by interaction of the cells with the ECM.

A collagen vitrigel system for cell culture was recently developed.[19] The vitrigel is produced from type I collagen and is molded into a membrane. We have now used this system to examine whether corneal fibroblasts regulate the differentiation of corneal epithelial cells grown on a shared collagen substrate. Culture of SV40-transformed human corneal epithelial cells and human corneal fibroblasts on opposite sides of the same collagen vitrigel membrane revealed that the fibroblasts upregulated the tight-junctional proteins occludin, claudin, and ZO-1 in the epithelial cells and that this effect was mediated by activation of the ERK signaling pathway. Corneal fibroblasts did not affect expression of E- or N-cadherin, which are components of adherens junctions, in the epithelial cells. These results thus suggest that the formation and maintenance

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of tight junctions, but not those of adherens junctions, in the corneal epithelium may be regulated by corneal fibroblasts.

METHODS

Antibodies and Reagents

Rabbit polyclonal antibodies to ZO-1, to occludin, or to claudin were obtained from Zymed (Carlsbad, CA), and those to E-cadherin or to N-cadherin were obtained from Transduction Laboratories (Lexington, KY). Rabbit polyclonal antibodies to type I collagen were from LSL (Tokyo, Japan). Mouse monoclonal antibodies to α-tubulin were obtained from Sigma (St. Louis, MO), and those to K12 were kindly provided by Winston W. Kao (University of Cincinnati). Guinea pig polyclonal antibodies to vimentin were from Progen (Heidelberg, Germany). Mouse monoclonal antibodies to ERK or to phosphorylated ERK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies were from Promega (Madison, WI), and fluorescent dye (Alexa Fluor 488- or Alexa Fluor 555)-conjugated secondary antibodies and (Alexa Fluor 568)-conjugated phalloidin and iodide (TOTO-3) were from Molecular Probes (Carlsbad, CA). PD98059 was obtained from Calbiochem (San Diego, CA).

HCE Cell Culture

SV40-transformed human corneal epithelial (HCE) cells, originally established and characterized by Araki-Sasaki et al.,[20] were obtained from RIKEN Biosource Center (Tsukuba, Japan). The cells were maintained in supplemented hormonal epithelial medium (SHEM), which consists of Dulbecco modified Eagle medium (DMEM)-F12 (50:50, vol/vol) supplemented with 15% heat-inactivated fetal bovine serum (FBS), bovine insulin (5 μg/mL), cholera toxin (0.1 μg/mL), recombinant human epidermal growth factor (10 ng/mL), and gentamicin (40 μg/mL).

Corneal Fibroblast Culture

Human corneal fibroblasts were prepared from the tissue remaining after corneal transplantation surgery and were cultured as described previously.[21] In brief, human corneas were obtained from Mid-America Transplant Service (St. Louis, MO), Northwest Lions Eye Bank (Seattle, WA), or the Eye Bank of Wisconsin (Madison, WI). The donors were white adults and children of both sexes ranging in age from 4 to 65 years. The cells prepared from each cornea were maintained separately in DMEM supplemented with 10% heat-inactivated FBS until they had achieved approximately 90% confluence. Cells in the third to seventh passages were used for the experiments described in the present study. The purity of the cell cultures was assessed on the basis of both the distinctive morphology of human corneal fibroblasts and their reactivity with antibodies to vimentin in immunofluorescence analysis. All cells were positive for vimentin and negative for cytokeratin, suggesting the absence of contamination by epithelial cells. The human tissue was used in strict accordance with the Declaration of Helsinki.

Coculture of HCE Cells and Corneal Fibroblasts on a Collagen Membrane

HCE cells (2×10^5) were seeded on a collagen vitrigel membrane (Asahi Technoglass, Tokyo, Japan) in DMEM supplemented with 10% heat-inactivated FBS (Fig. 1).[19] After 24 hours, the membrane was turned upside down in another dish, and corneal fibroblasts (1×10^5) were seeded on the empty side of the membrane, also in DMEM supplemented with 10% heat-inactivated FBS. After 6 to 8 hours, the membrane was again turned upside down in another dish containing DMEM supplemented with 0.5% heat-inactivated FBS. The cells were then cultured at 37°C in a humidified incubator containing 5% CO_2 and 95% air. As a control, HCE cells were seeded on a vitrigel membrane without corneal fibroblasts.

RT-PCR Analysis

Total RNA was isolated from HCE cells with the use of a purification kit (RNeasy; Qiagen, Valencia CA), and portions (0.5 μg) were subjected to reverse transcription (RT) and polymerase chain reaction (PCR) analysis with an RT-PCR kit (One-Step; Invitrogen, Carlsbad, CA) based on a high-fidelity, high-yield polymerase system (Platinum *Taq*; Invitrogen). The PCR protocol was designed to maintain amplification in the exponential phase. Sequences of the PCR primers were as follows: ZO-1 sense, 5′-TGCCATTACACGGTCCTCTG-3′; ZO-1 antisense, 5′-GGTTCTGCCTCATCATTTCCTC-3′; occludin sense, 5′-AGTGTGATAATAGTGAGTGCTATCC-3′; occludin antisense, 5′-TGTCATACCTGTCCATCTTTCTTC-3′; claudin sense, 5′-TTCTCGCCTTCCTGGGATG-3′; claudin antisense, 5′-CTTGAACGATTCTATTGCCATACC-3′; glyceraldehyde-3-phosphate dehydrogenase (G3PDH, internal control) sense, 5′-ACCACAGTCCACGCCATCAC-3′; G3PDH antisense, 5′-TCCACCACCCTGTTGCTGTA-3′. RT and PCR incubations were performed with a PCR system (GeneAmp PCR System 2400-R; Perkin-Elmer, Wellesley, MA). RT was performed at 50°C for 30 minutes, and the PCR cycle consisted of incubations at 94°C for 2 minutes, 58°C for 30 seconds, and 72°C for 1 minute; the reaction mixture was then cooled to 4°C. Amplification products were fractionated by electrophoresis on a 4% agarose gel and stained with ethidium bromide. Band intensities were measured by image analysis (Multi Gauge V3(2) software; Fuji Film, Tokyo, Japan), and those for ZO-1, occludin, and claudin were normalized by the corresponding value for G3PDH.

For RT and real-time PCR analysis, the total RNA was subjected to RT with a kit (Promega), and the resultant cDNA was subjected to

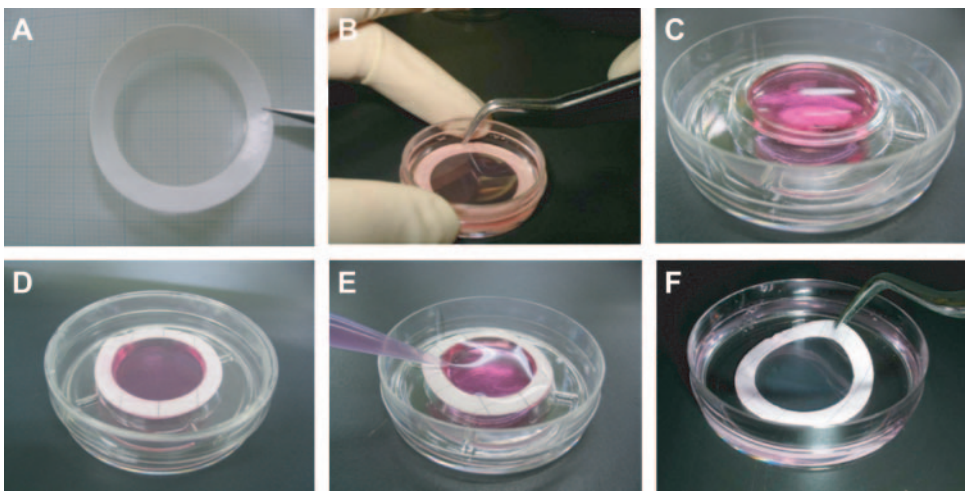

FIGURE 1. Coculture of HCE cells and human corneal fibroblasts on a collagen vitrigel membrane. (**A**) Collagen vitrigel membrane. (**B**) Seeding of HCE cells on the membrane. (**C**, **D**) Membrane was turned upside down. (**E**) Seeding of corneal fibroblasts on the membrane. (**F**) Medium was changed to DMEM supplemented with 0.5% heat-inactivated FBS, and cells were cultured for various times.

real-time PCR analysis by rapid cycling in glass capillaries with a thermocycler (Light-Cycler; Roche Molecular Biochemicals).

Immunoblot Analysis

HCE cells on a collagen vitrigel membrane (with or without human corneal fibroblasts) were washed twice with phosphate-buffered saline (PBS) and lysed in 200 μL solution containing 150 mM NaCl, 2% SDS, 5 mM EDTA, and 20 mM Tris-HCl (pH 7.5). Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a nitrocellulose membrane and exposed consecutively to primary antibodies and horseradish peroxidase–conjugated secondary antibodies. Immune complexes were detected with enhanced chemiluminescence reagents (GE Healthcare UK, Little Chalfont, UK).

Immunofluorescence Analysis

Collagen vitrigel membranes with their attached cells were embedded in OCT compound, frozen with liquid nitrogen, and stored at −80°C. Sections of each membrane were cut at a thickness of 8 μm with a cryostat (HM505N; Microm, Walldorf, Germany), and the sections were transferred to silane-treated slides and allowed to dry in air at room temperature. The sections were then fixed with 100% methanol for 10 minutes at −20°C, washed with PBS, and incubated for 30 minutes at room temperature with 1% bovine serum albumin (BSA) in PBS. For double staining of K12 and vimentin, sections were incubated for 1 hour at room temperature with antibodies to K12 (1:100 dilution in PBS containing 1% BSA), washed three times with PBS, and incubated for 30 minutes at room temperature with fluorescent dye (Alexa Fluor 488; Molecular Probes)-conjugated goat antibodies to mouse immunoglobulin G (1:1000 dilution in PBS containing 1% BSA). The sections were then washed with PBS, incubated for 1 hour at room temperature with guinea pig antibodies to vimentin (1:200 dilution in PBS containing 1% BSA), washed again with PBS, and incubated for 30 minutes at room temperature with fluorescent dye (Alexa Fluor 555; Molecular Probes)-conjugated goat antibodies to guinea pig immunoglobulin G (1:1000 dilution in PBS containing 1% BSA). For double staining of type I collagen and F-actin, sections were incubated consecutively for 1 hour at room temperature with rabbit antibodies to type I collagen (1:1000 dilution in PBS containing 1% BSA), for 30 minutes at room temperature with fluorescent dye (Alexa Fluor 488; Molecular Probes)-conjugated goat antibodies to rabbit immunoglobulin G (1:1000 dilution in PBS containing 1% BSA), and for 30 minutes at room temperature with fluorescent dye (Alexa Fluor 568; Molecular Probes)-conjugated phalloidin (1:200 dilution in PBS containing 1% BSA). Finally, all sections were incubated for 10 minutes with iodide (TOTO-3 [Molecular Probes]; 1:2000 dilution in PBS containing 1% BSA of a 1 mM solution in dimethyl sulfoxide) to stain nuclei. Sections were examined with a laser-scanning confocal microscope (Axiovert 200M; Carl Zeiss, Oberkochen, Germany).

Statistical Analysis

Data are presented as mean ± SE from three independent experiments and were analyzed by Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Coculture of HCE Cells and Corneal Fibroblasts on a Collagen Vitrigel Membrane

We examined collagen vitrigel membranes by immunostaining after culture of HCE cells in the absence or presence of human corneal fibroblasts (Fig. 2A). Staining with antibodies to type I collagen and with phalloidin (for F-actin) and TOTO-3 iodide (for nuclei) revealed the presence of cells on both sides of the membrane for cocultures and on one side of the membrane for control cultures (HCE cells alone). Furthermore, staining with antibodies to K12 (for epithelial cells) and those to vimentin (for fibroblasts) revealed that HCE cells were restricted to one side of the membrane, as were corneal fibroblasts when present. We also subjected lysates of HCE cells or corneal fibroblasts prepared from cocultures to immunoblot analysis with antibodies to K12 and to vimentin (Fig. 2B). The HCE cell lysates were positive for K12 but not vimentin, whereas the fibroblast lysates were positive for vimentin but not K12. These results thus showed that there was no cross-contamination of HCE cells and fibroblasts in the coculture system.

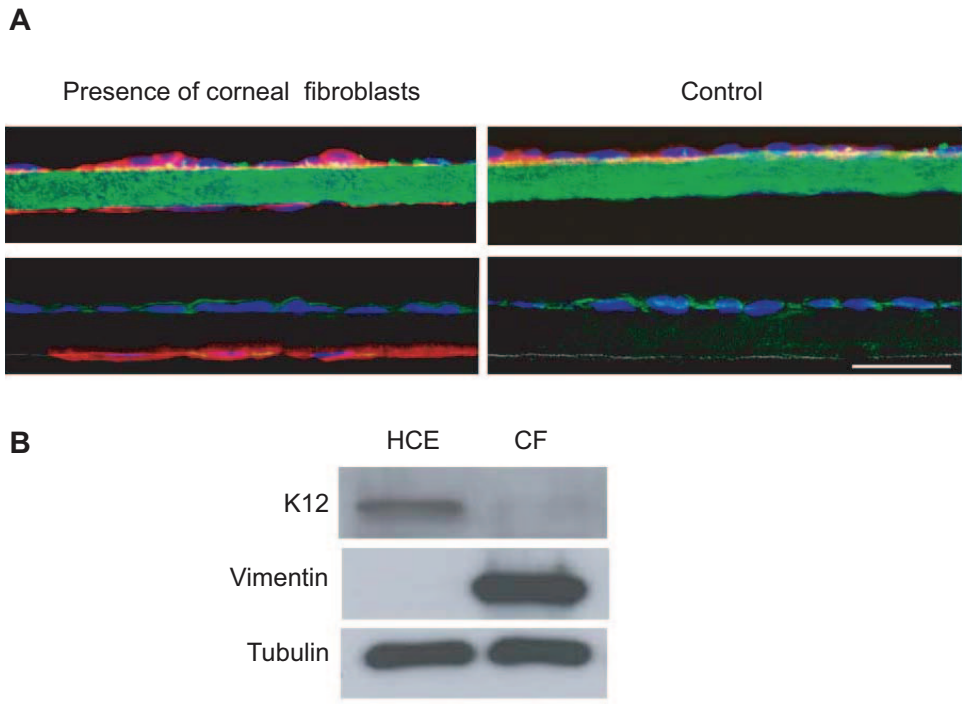

Figure 2. Lack of cross-contamination of HCE cells and corneal fibroblasts in cocultures. (**A**) Immunostaining of cells attached to a collagen vitrigel membrane. Membranes were cultured for 1 day with HCE cells and corneal fibroblasts or with HCE cells alone (control). Sections of each membrane were then stained with antibodies to type I collagen (*green*) and phalloidin (*red*) in the *upper panels* or with antibodies to K12 (*green*) and those to vimentin (*red*) in the *lower panels*. All sections were also stained with TOTO-3 iodide (*blue*). Scale bar, 50 μm. (**B**) Lysates of HCE cells or corneal fibroblasts (CF) cultured on a collagen vitrigel membrane for 1 day were subjected to immunoblot analysis with antibodies to K12, vimentin, or α-tubulin (loading control).

Effects of Corneal Fibroblasts on Expression of Junctional Proteins in HCE Cells

We next investigated the effect of the presence of corneal fibroblasts on expression of the tight-junctional proteins ZO-1, occludin, and claudin in HCE cells. Immunoblot analysis revealed that the amounts of ZO-1, occludin, and claudin in HCE cells cultured in the presence of corneal fibroblasts for 24, 48, or 72 hours were markedly increased compared with those in HCE cells cultured alone (Figs. 3A, 3B). The amounts of these proteins in HCE cells cocultured for 96 hours did not differ significantly from those in HCE cells cultured alone. Cadherins contribute to large protein complexes that couple cell adhesion to cell morphology, cell motility, and intracellular signaling events.[22–24] Given that expression of ZO-1 has been shown to be affected by that of cadherins,[25,26] we investigated the expression of E- and N-cadherin in HCE cells. The expression of these cadherins in HCE cells was not significantly affected by the presence of corneal fibroblasts (Figs. 3C, 3D). These results thus indicated that the presence of corneal fibroblasts resulted in specific upregulation of tight-junctional proteins in HCE cells, without affecting the expression of adherens-junctional proteins.

We then examined whether this effect of corneal fibroblasts on the expression of tight-junctional proteins in HCE cells might be mediated at the transcriptional level. RT-PCR analysis revealed that the amounts of ZO-1, occludin, and claudin mRNAs in HCE cells cultured with corneal fibroblasts were increased compared with those in HCE cells cultured alone (Figs. 4A, 4B). This effect of corneal fibroblasts was significant at 12 to 48 hours for ZO-1 and claudin mRNAs and at 24 to 48 hours for occludin mRNA. We further examined by RT and real-time PCR analysis the observed tendency for the abundance of occludin and claudin mRNAs to be increased in HCE cells cultured with corneal fibroblasts for 6 hours. This analysis revealed that the amounts of occludin and claudin mRNA at 2, 4, or 6 hours did not differ between HCE cells cultured with corneal fibroblasts and those cultured alone (Fig. 4C).

Effect of ERK Inhibition on Upregulation of Tight-Junctional Proteins in Cocultures of HCE Cells and Corneal Fibroblasts

Finally, we examined whether the upregulation of tight-junctional proteins in HCE cells by corneal fibroblasts might be mediated by the ERK signaling pathway. PD98059, which inhibits the activation of ERK, inhibited the basal level of ERK phosphorylation but did not affect the expression of ZO-1, occludin, or claudin in control cultures of HCE cells (Fig. 5A). The presence of corneal fibroblasts increased the phosphorylation of ERK in HCE cells, and both this effect and the upregulation of the tight-junctional proteins in HCE cells by corneal fibroblasts were inhibited by PD98059. We also examined by RT and real-time PCR analysis the effects of PD98059 on the amounts of tight-junctional protein mRNAs in HCE cells or corneal fibroblasts cultured together for 12 hours. Although PD98059 had no effect on the abundance of ZO-1, occludin, or

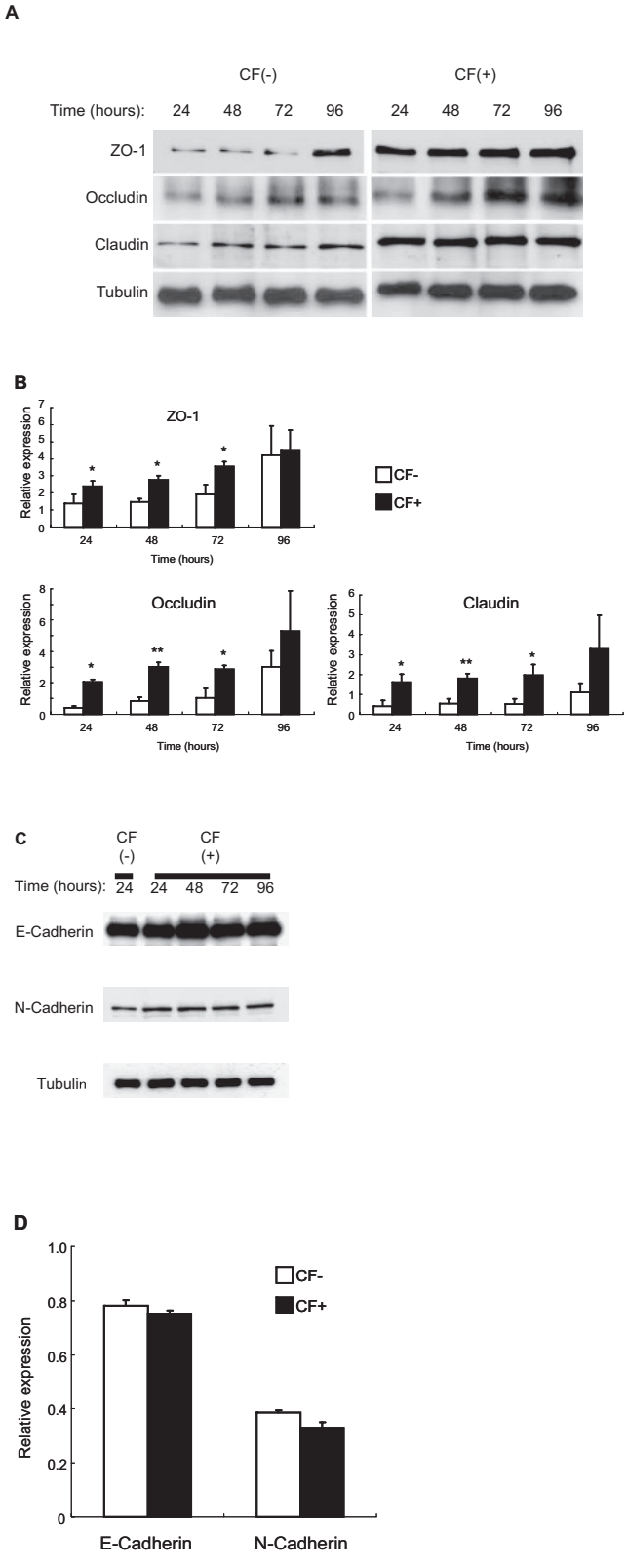

FIGURE 3. Expression of tight-junctional and adherens-junctional proteins in HCE cells cultured with or without corneal fibroblasts. (**A**) Lysates of HCE cells cultured for 24 to 96 hours on a collagen vitrigel membrane in the absence or presence of corneal fibroblasts (CF) were subjected to immunoblot analysis with antibodies to ZO-1, occludin, claudin, or α-tubulin. (**B**) The expression of each tight-junctional protein was quantified by scanning of immunoblots similar to those shown in (**A**). Data were normalized by the abundance of α-tubulin and are mean + SE of values from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the corresponding value for HCE cells incubated in the absence of corneal fibroblasts. (**C**) Lysates of HCE cells cultured as in (**A**) were subjected to immunoblot analysis with antibodies to E-cadherin, N-cadherin, or α-tubulin. (**D**) The expression of each adherens-junctional protein after culture for 24 hours was quantified by scanning of immunoblots similar to those shown in (**C**). Data are mean + SE of values from three separate experiments.

claudin mRNAs in corneal fibroblasts, it markedly reduced the amounts of each of these mRNAs in HCE cells (Fig. 5B).

Expression of Tight-Junctional Protein Genes in Corneal Fibroblasts

As a final control, we examined the expression of tight-junctional protein genes in corneal fibroblasts cultured alone or together with HCE cells. RT and real-time PCR analysis revealed that the amounts of ZO-1, occludin, and claudin mRNAs did not differ between corneal fibroblasts cultured with HCE cells and those cultured alone (Fig. 6).

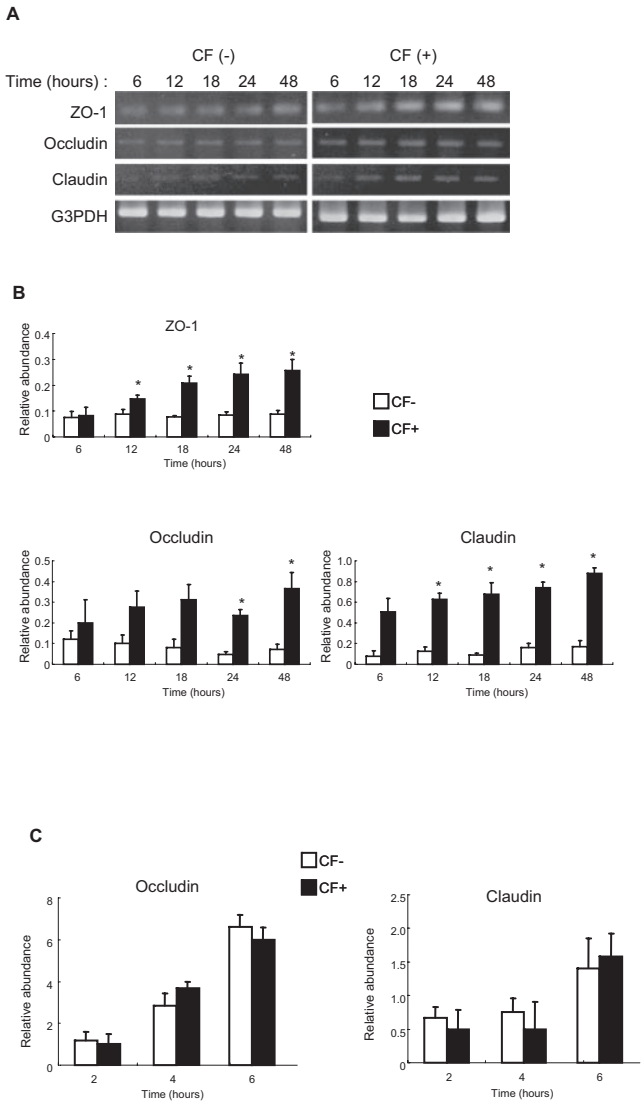

FIGURE 4. Abundance of mRNAs for tight-junctional proteins in HCE cells cultured with or without corneal fibroblasts. (**A**) Total RNA prepared from HCE cells cultured for the indicated times on a collagen vitrigel membrane in the absence or presence of corneal fibroblasts was subjected to RT-PCR analysis of ZO-1, occludin, claudin, and G3PDH mRNA. (**B**) The abundance of mRNAs for the tight-junctional proteins was quantified by scanning gels similar to those shown in (**A**). (**C**) The abundance of mRNAs for occludin and claudin in HCE cells cultured in the absence or presence of corneal fibroblasts was also quantified by RT and real-time PCR analysis at early time points (2, 4, and 6 hours). Data in (**B**) and (**C**) were normalized by the abundance of G3PDH mRNA and are mean + SE of values from three separate experiments. $^*P < 0.05$ versus the corresponding value for HCE cells incubated in the absence of corneal fibroblasts.

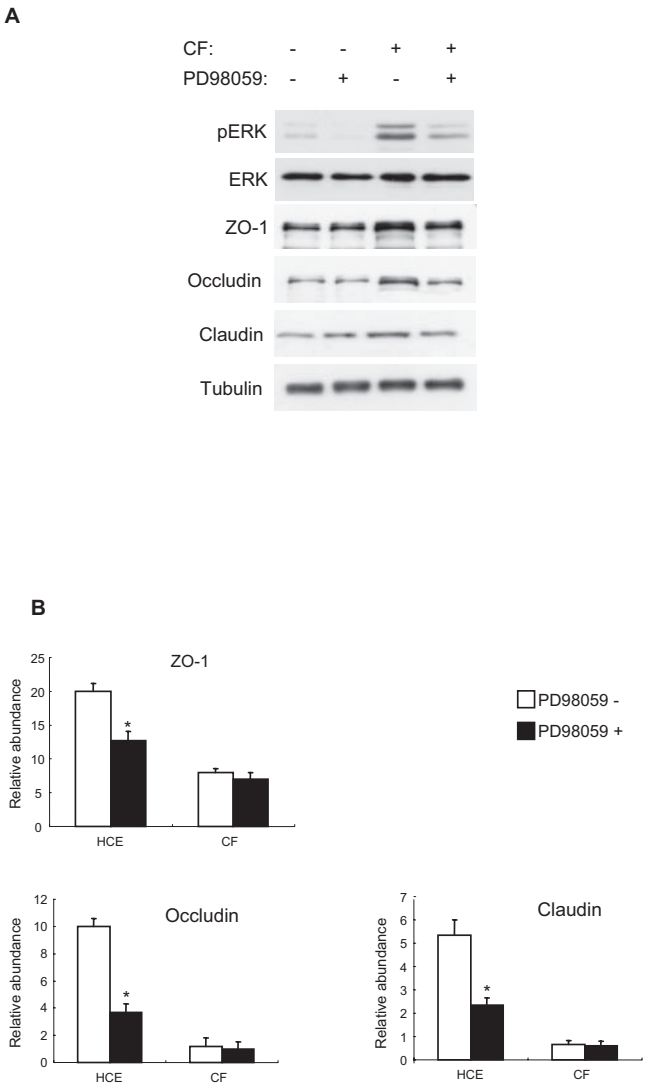

FIGURE 5. Effects of corneal fibroblasts on ERK phosphorylation in HCE cells and of PD98059 on corneal fibroblast-induced upregulation of tight-junctional proteins in HCE cells. (**A**) Lysates of HCE cells cultured for 24 hours in the absence or presence of corneal fibroblasts and 10 μM PD98059, as indicated, were subjected to immunoblot analysis with antibodies to phosphorylated ERK (pERK), ERK, ZO-1, occludin, claudin, or α-tubulin. (**B**) The abundance of mRNAs for ZO-1, occludin, and claudin in HCE cells or corneal fibroblasts cocultured for 12 hours in the absence or presence of 10 μM PD98059 was quantified by RT and real-time PCR analysis. Data were normalized by the abundance of G3PDH mRNA and are mean + SE of values from three separate experiments. $^*P < 0.05$ versus the corresponding value for cells incubated in the absence of PD98059.

DISCUSSION

We have shown that the presence of corneal fibroblasts upregulated expression of the tight-junctional proteins ZO-1, occludin, and claudin, but not that of the adherens-junctional proteins E- and N-cadherin, in HCE cells cultured on a collagen membrane. Furthermore, this effect of corneal fibroblasts was apparent at both the protein and the mRNA levels and appeared to be mediated by the ERK signaling pathway. These results thus suggest that corneal fibroblasts influence the differentiation of corneal epithelial cells and the establishment or maintenance of the barrier function of the corneal epithelium.

The corneal epithelium is a stratified squamous epithelium, with the basal cells differentiating into intermediate (wing)

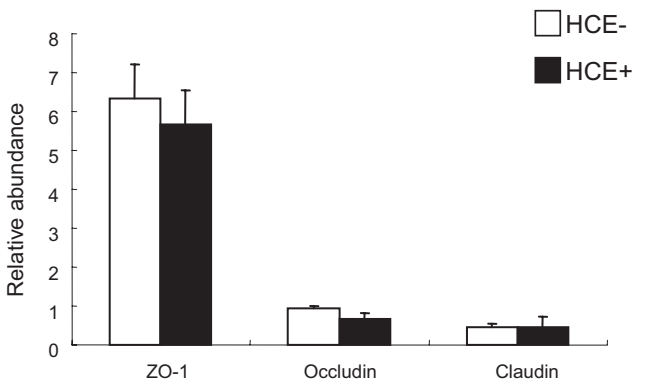

FIGURE 6. Abundance of mRNAs for tight-junctional proteins in corneal fibroblasts cultured with or without HCE cells. Total RNA prepared from corneal fibroblasts cultured for 12 hours on a collagen vitrigel membrane in the absence or presence of HCE cells was subjected to RT and real-time PCR analysis of ZO-1, occludin, and claudin mRNA. Data were normalized by the abundance of G3PDH mRNA and are mean + SE of values from three separate experiments.

cells and superficial cells. Localization of various types of junctional proteins has been characterized in the corneal epithelium. Basal cells express gap-junctional proteins such as connexin 43, whereas superficial cells express the tight-junctional proteins ZO-1, occludin, and claudin. Other types of junctional proteins, including the adherens-junctional proteins E- and N-cadherin, are expressed throughout all layers of the corneal epithelium. The formation of tight junctions is important for the barrier function of the corneal epithelium. We previously showed that the formation of tight junctions at the superficial layer of the corneal epithelium constitutes the final stage of corneal epithelial wound healing.[24] We have now shown that the presence of corneal fibroblasts induces the upregulation of ZO-1, occludin, and claudin in HCE cells and may therefore be required for the formation of tight junctions by corneal epithelial cells during normal maintenance of the corneal epithelium and during the healing of corneal epithelial wounds. The expression of adherens-junctional proteins in HCE cells was not affected by the presence of corneal fibroblasts. Adherens junctions are present in all layers of the corneal epithelium and are not substantially changed during the process of corneal epithelial wound healing. We were unable to examine the effects of corneal fibroblasts on the expression of gap-junctional proteins in HCE cells in the present study because these cells do not express connexin 43 as a result of transformation by SV40.

HCE cells and corneal fibroblasts were cultured separately on the two sides of a collagen vitrigel membrane and did not come into direct contact with each other. It is therefore likely that a factor (or factors) released by corneal fibroblasts is responsible for the upregulation of tight-junctional protein expression in HCE cells. We showed that HCE cells did not affect the expression of tight-junctional protein genes in corneal fibroblasts. The identity of the factor (or factors) released from corneal fibroblasts remains to be determined. Ex vivo expansion of corneal limbal epithelial cells has been used to provide cells for the treatment of patients with limbal stem cell deficiency.[27,28] For this purpose, corneal limbal epithelial cells from donor corneas are cultivated on a denuded amniotic membrane carrier or temperature-sensitive polymers,[29] and a feeder layer of mouse 3T3 fibroblasts is required. Identification of the factor (or factors) released from corneal fibroblasts that promotes the differentiation of corneal epithelial cells might allow the preparation of corneal epithelial sheets for human treatment without the use of mouse 3T3 cells.

ERK is a member of the mitogen-activated protein kinase family of serine-threonine kinases that play an important role in the transduction of externally derived signals that regulate cell growth and differentiation.[30–33] We have now shown that the presence of corneal fibroblasts induced ERK phosphorylation in HCE cells and that inhibition of ERK signaling by PD98059 blocked the upregulation of tight-junctional proteins in HCE cells by corneal fibroblasts. Inhibition of ERK signaling by PD98059 did not affect the expression of tight-junctional protein genes in corneal fibroblasts cocultured with HCE cells. The signal derived from corneal fibroblasts that is responsible for the upregulation of tight-junctional proteins in HCE cells thus appears to be transmitted through the ERK signaling pathway.

Although we have shown that corneal fibroblasts induce the upregulation of tight-junctional proteins in HCE cells, it remains to be determined whether this effect is accompanied by the formation of functional tight junctions. Transepithelial resistance is an indicator of barrier function, but measurement of this parameter is not possible with the coculture system used in the present study. Our coculture system should prove useful, however, for further investigations into the role of corneal fibroblasts in the differentiation of the corneal epithelium and in corneal epithelial wound healing.

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