In Vivo and In Vitro Expression of Connexins in the Human Corneal Epithelium

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Purpose. This study is designed to provide a comprehensive expression profile of connexins in the human corneal epithelium (in vivo) and in cultured primary corneal epithelial cells (PCECs) (in vitro). It also evaluates the pathologic effects of a pathogenic missense mutation in Cx26, which causes keratitis-ichthyosis-deafness syndrome (KIDS), a rare genetic disorder with corneal involvement.

Methods. RT-PCR analysis, immunohistochemistry, and fluorescent dye transfer assays were used to determine the expression pattern and gap junction intercellular communication in PCECs and human cornea. Differentiation-dependent differences in connexin expression of PCECs after a calcium switch were verified by real-time RT-PCR. The common KIDS mutation Cx26(D50N) was studied by determining transient expression in PCECs.

Results. In vivo immunostaining revealed widespread and overlapping expression of Cx43 and -30 in the basal and suprabasal layers. Cx26 staining was limited to the lower suprabasal cells, whereas Cx31.1 localized to the apical surface of basal cells in the central cornea and to the lower and middle suprabasal cells in the limbal region. Immunostaining for nine other connexins, including Cx50, was negative. In PCEC, nine connexin genes were detectable by RT-PCR, however, only Cx26, -30, and -43 formed visible gap junction plaques. High-Ca^{2+} culture conditions were accompanied by a 1.6- to 2.2-fold upregulation of expression of Cx26, -30, and -43 and a significant increase in gap-junction–mediated dye transfer. Transient expression of mutant Cx26(D50N) in PCECs resulted in cytoplasmic accumulation and lack of gap junction plaque formation and was not altered by coexpression of wild-type (wt)Cx26 or -30.

Conclusions. Gap junction communication in the human corneal epithelium is mediated by Cx26, -30, -31.1, and -43. Poorly differentiated PCECs are uncoupled, and Ca^{2+} induced differentiation is associated with an upregulation of connexin expression and intercellular communication. The transfection experiments suggest that KIDS Cx26(D50N) impairs intracellular formation and transport of connexons formed by Cx26 and -30, consistent with a dominant negative effect. (*Invest Ophthalmol Vis Sci.* 2005;46:1957–1965) DOI:10.1167/iovs.04-1364

Human connexins are a polygenic family of 21 transmembrane proteins that form aqueous gap junction channels. These channels permit the direct, cell-to-cell diffusional exchange of ions, secondary messengers, water, electrical impulses and low-molecular-weight (<1 kDa) metabolites and nutrients. Most cell types express several different connexin isoforms in a temporal-, spatial-, and differentiation-specific manner,[1,2] thus leading to a tremendous functional redundancy and diversity of the gap junction system. A striking example is the human epidermis, which utilizes up to 10 different connexins in an overlapping, cell-type- and differentiation-dependent pattern.[3–5] The epidermis has many characteristics and biological functions in common with the cornea, such as ectodermal origin, barrier function, and proliferation and differentiation potential. Both tissues are well coupled by gap junctions,[4,6,7] suggesting that they may share similar connexin expression patterns. Cx43 is widely expressed throughout all layers of rodent epidermis and the corneal epithelium, with exception of the most superficial corneal cells.[8] Matic et al.[9] reported Cx50 immunostaining of rabbit cornea, whereas Cx26 and -32 were not detected. However, when a different and specific monoclonal Cx26 antibody was used, Cx26 was observed in some but not all basal and lower suprabasal cells, especially in the cultured human limbal corneal epithelial cells on amniotic membranes.[10] This pattern is comparable with the focal basal expression of Cx26 in palmoplantar epidermis.[2] Although the distribution of other connexins, including Cx30, -30.3, -31, -31.1, -37, -40, and -45, have been extensively studied in human epidermis,[3] a comparable comprehensive evaluation of the connexin system in human cornea is still lacking.

The biological importance of gap junctions is vividly illustrated by the plethora of human genetic disorders that have been linked to inherited connexin gene defects in the past decade.[11,12] For example, missense mutations in the lens-specific connexins Cx46 and -50 cause zonular pulverulent cataracts and disturbed eye development. Mutations in Cx43 are responsible for oculo-dento-digital-dysplasia, which is associated with diverse ophthalmologic findings including microcornea, cataracts, and glaucoma.[13] Dominant mutations in four different epidermal connexin genes, Cx26, -30, -30.3, and -31, are responsible for an entire group of heritable skin disorders and ectodermal dysplasias characterized by impaired epidermal cornification and/or hair and nail abnormalities.[12] The phenotypic spectrum of Cx26 disorders is especially intriguing, ranging from nonsyndromic sensorineural hearing loss (SNHL) to keratitis-ichthyosis-deafness syndrome (KIDS). KIDS is a rare autosomal dominant ectodermal dysplasia characterized by superficial punctate keratitis; progressive corneal neovascularization; severe to profound SNHL; various skin, hair, and nail findings; and increased susceptibility to mucocutaneous infections and squamous cell carcinoma.[14] More than 90% of patients manifest photophobia during infancy or childhood

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TABLE 1. Donor Information

Donor	Age (y)	Time Postmortem (h)	Time until Culture† (d)	Specimen	Transport Condition
43	46	11	5.5	Rim	Preservative
44	59	6.5	5.8	Rim	Preservative
45	58	7.5	5.7	Rim	Preservative
46	31	7.8	3.5	Rim	Preservative
63	66	7.5	1.6	WC	MC
64	66	7.5	1.6	WC	MC

WC, whole cornea; MC, moist chamber.
* Hours from time of death until time of extraction.
† Days from death until culture.

and the progressive keratitis can eventually lead to blindness.[14–16]

Of all hereditary Cx26 disorders, KIDS is the only one involving corneal disease, which implies a functionally important role of Cx26 in the corneal epithelium and other ectoderm-derived epithelia. To date, four distinct pathogenic mutations of the Cx26 gene have been identified in KIDS.[17–19] Mutation D50N is most common, which accounts for 80% of KIDS cases and is located in a mutational hot spot in the Cx26 gene.[17] The individual Cx26 mutations, dependent on their nature and location, may interfere with different steps of connexin biogenesis, including connexin synthesis,[20] oligomerization and intracellular transport,[21] connexon–connexon interactions, and other functional properties of gap junction channels[22,23]. Several Cx26 mutations have been shown to exert a dominant negative effect on wild-type (wt)Cx26 and other coexpressed connexins, such as Cx30 or -43.[24,25] However, the specific pathologic consequences of KIDS mutations in the corneal epithelium remain elusive.

In this article, we report the expression profile of gap junction proteins in human cornea and in cultured primary corneal epithelial cells (PCECs) and demonstrate the upregulation of connexin expression when cultured in high-calcium medium in vitro. Moreover, expression studies of the KIDS mutation Cx26(D50N) in PCECs determined a failure in formation of visible gap junction plaques when expressed alone or together with wtCx26 or -30, suggesting a dominant inhibition of connexon hemichannel formation.

MATERIALS AND METHODS

Isolation and Culture of Human PCECs

Human corneal rims were obtained from the Wills Eye Hospital (Philadelphia, PA) and the Lions Eye Bank of the Delaware Valley. In addition, two cadaveric eyes were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA). All procedures conformed to the tenets of the Declaration of Helsinki for biomedical research involving human subjects. All corneal rims were stored in preservative (Optisol-GS; Bausch & Lomb, Rochester, NY) at 4°C, and whole eyes were shipped in a moist chamber wrapped in saline-soaked gauze at 4°C. Donor characteristics are summarized in Table 1.

PCECs were isolated from donor rims of four individuals and cultured by using established protocols.[26] In brief, corneal rims were trimmed of excess conjunctiva and washed several times in Hanks' balanced saline solution (HBSS) with 20 μg/mL gentamicin and 0.1% amphotericin B (Fungizone; Invitrogen-Gibco, Rockville, MD). The rims were then cut into six to eight pieces and treated in a 1:1 mixture of dispase (25 caseinolytic U/mL; BD Biosciences, Franklin Lakes, NJ) and HBSS with 5 μg/mL gentamicin and 0.1% amphotericin B overnight at 4°C to disrupt the basement membrane. Next, the epithelial sheets were peeled off and digested in 0.05% trypsin-EDTA at 37°C for 5 minutes. After trypsinization, cells were pelleted, resuspended in keratinocyte serum-free medium (KSFM), and plated on fibronectin-collagen–coated (FNC) T-25 flasks (AthenaES, Baltimore, MD). Cells were cultured at 37°C in 5% carbon dioxide in a humidified incubator. Experiments were performed on cells at passages 2 to 4. All cells demonstrated positive immunostaining for the corneal epithelial marker keratin 3 (ICN Biomedicals and Reagents, Aurora, OH). To induce differentiation, cells that were initially grown in low-calcium KSFM (0.09 mM $CaCl_2$; designated l-PCEC) were switched to high-calcium KSFM (1.64 mM $CaCl_2$ and 0.3% FBS) for a minimum of 48 hours (designated h-PCEC).

RT-PCR Analysis

PCECs were grown to 80% confluence, and total RNA was extracted (TRIzol reagent; Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. The RNA was further purified by twice passing the sample through a spin column at 8000 rpm for 1 minute (RNeasy; Qiagen Inc., Valencia, CA) and then treated with DNAse I according to the manufacturer's protocol (Invitrogen Corp.). As a positive control and to exclude any DNA contamination, RT-PCR was performed for each RNA sample using a balanced pair of intron-crossing primers for the pyruvate kinase gene. The primers were designed to produce a 475-bp product from genomic DNA and a 230-bp product from RNA. For RT-PCR, gene-specific primer pairs for 17 human connexin genes (Table 2) were either chosen from published sources[3] or newly created from published genomic gene sequences using BLASTn analysis, DIALGN for sequence comparisons, and the primer3 program for primer selection. RT-PCR analysis of connexin genes was performed with 800 ng of total RNA in a 15-μL first-strand synthesis cDNA reaction, with a 1:5 dilution of random hexamers (Amersham Inc., Piscataway, NJ). One microliter of the final cDNA reaction was then used in a 50-μL PCR reaction with *Taq* polymerase (Qiagen) and standard PCR conditions. All products were separated by 2% agarose gel electrophoresis and visualized with 0.05 mg/mL ethidium bromide. Gels were then analyzed with a geldoc reader utilizing Quantity One 4.5.0 software (Bio-Rad Laboratories, Inc., Hercules, CA).

Quantitative RT-PCR

To compare transcription levels of selected connexin genes between l-PCECs and h-PCECs, real-time RT-PCR was performed using ABI TaqMan MGB chemistries and an ABI 7000 light thermocycler. Briefly, primers and TaqMan probes were designed using the PrimerExpress software 4.0 (Applied Biosystems, Foster City, CA) and RT-PCR reactions were optimized to ensure high amplification efficiency (Table 2). Fifty-microliters RT-PCR reactions were performed with 2× master mix reagent, 40× reverse transcriptase (Multiscribe; ABI), 120 ng of each primer, 1 μL of 1:10 dilution of probe (*Taq*Man), and 35 ng of RNA (ABI). Cycling conditions were as follows: 48°C for 10 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. No-template control analyses were run for each primer set and 18S rRNA control analyses were run for each sample. All reactions were performed in triplicate, and expression levels were

TABLE 2. RT-PCR Primers and Conditions

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	AT °C
Cx26	TCTTTTCCAGAGCAAACCGC	GACACGAAGATCAGCTGCAG	58
Cx30	GCTACCTGCTGCTGAAAGTG	CGTTGTGTATGAATGGAGCA	58*
Cx30.3	TACCCACCTGCATCCACTGG	GGTGGACGTACTTGCTGAGC	60
Cx31	AATTCTCGCAGGTAGGCAC	CCAGAGAGTGTGCAGCAGGT	60*
Cx31.1	GTGGACATATGTCTGCAGCC	CTATGAGAGATGCTAGAGC	58
Cx31.9	GCTGCTACCTGCTGAGCGT	TTCTGCGCCTCTTCGTGT	60
Cx32	GACAGGTTTGTACACCTTGC	CGTCGCACTTGACCAGCCGC	58*
Cx37	GTTGCTGGACCAGGTCCAGG	GGATGCGCAGGCCACCATCT	60
Cx40	GTACACAAGCACTCGACCGT	GCAGGGTGGTCAGGAAGATT	58
Cx40.1	AGCGAAAAGCTGGGCAGAC	TCACACCCACTCAGACTTCCT	60*
Cx43	CAATCACTTGGCGTGACTTC	GTTTGGGCAACCTTGAGTTC	58
Cx45	GGAGCTTTCTGACTCGCCTG	CGGCCATCATGCTTAGGTTT	58
Cx46	TTCAAACTGCTAGCCCTGAC	CTCCCCTCCAGACTGCTG	58*
Cx47	AGAGAGGCCTACGAGCCAGA	CAAGCTGCAAGGCATATCAA	56
Cx50	TCCACTCCATTGCTGTCTC	GTGGTCAGCCTCTCTGCTTC	69
Cx58	GAAATGCCTAGGGATCGGA	AACCTAGGTGGAAAATTTCAAGA	56
Cx62	AAGGATGTCTGCTGCGTACTTA	GCCTGTTTCATCCTCAATGC	56
PK	TCGTGGTGATCTAGGCATTG	AGTCCCCTTTGGCTGTTTCT	58*

AT, annealing temperature.
* Use of 10× Q-solution (Qiagen, Valencia, CA).

normalized to the average level of 18S ribosomal rRNA in each sample. ΔΔCT was calculated to compare the expression of Cx26, -30, and -43 in h-PCECs with expression levels in l-PCECs.

Immunohistochemistry

PCECs were grown on four-chamber glass slides for 24 to 48 hours (80% confluence) and fixed in ice-cold methanol for 30 minutes. For in vivo studies, human corneas were harvested from eyes obtained through NDRI within 36 hours after death. The corneas were cut in half and snap frozen in optimal cutting temperature compound (OCT), and 8- to 10-μm radial sections were created as previously described.[27] Slides were rehydrated and fixed in ice-cold methanol for 30 minutes. Immunohistochemistry [IHC] techniques, primary and secondary antibodies, and optimized IHC conditions are outlined in Table 3. Biotinylated secondary antibodies visualized with streptavidin-bound Texas red or FITC were used to confirm any negative results for all 13 connexin antibodies tested. Slides were sealed with mounting medium (Fluoromount-g; Vector Laboratories, Burlingame, CA) and the results were analyzed on an inverted fluorescence microscope (TE 2000U; Nikon, Tokyo, Japan) equipped with a digital camera (ORCA 100 Coolpix; Hamamatsu Corp., Bridgewater, NJ).

Dye-Transfer Assays

PCECs were grown to 80% confluence and maintained in their respective media. Using a semiautomatic microinjection workstation for adherent cells (Brinkman Eppendorf AG, Westbury, NY) the cytoplasm of a single cell surrounded by neighboring cells was continuously microinjected under low pressure (10–20 hPa) with 5% aqueous Lucifer yellow (Sigma-Aldrich Co., St. Louis, MO). Each injection lasted 2 minutes, and the experiment was visualized with the inverted fluorescence microscope (TE 2000U; Nikon) with a heated stage (37°C). After 2 minutes, the number of fluorescently labeled cells surrounding the injected cell was scored. Results are reported as the average number of cells that received the dye ± SE. To determine quantitative differences in gap junction communication, the percentage of injected cells that transferred dye to two or more neighboring cells was calculated. Student's *t*-test was used for statistical analysis.

EYFP and ECFP Expression Constructs

Plasmids vectors were created to allow the expression of Cx26(D50N) fused at the carboxyl terminus with EYFP and the expression of wtCx26 and -30 fused at the carboxyl terminus with ECFP, as previ-

TABLE 3. Immunocytochemistry Techniques

Antigen	PC/MC	Dilution	Source
Cx26	MC	1:100	Zymed (cat no. 33–5800)
Cx30.3	PC	1:100	Richard (unpublished)
Cx30	MC	1:100	Zymed (cat no. 71–2200)
Cx31	PC	1:100	Di et al.[3]
Cx31.1	PC	1:50	Goliger and Paul[4]
Cx32	PC	1:100	Zymed (cat no. 34–5700)
Cx36	PC	1:100	Zymed (cat no. 36–4600)
Cx37	PC	1:250	Yeh et al.[28]
Cx40	PC	1:500	Yeh et al.[28]
Cx43	MC	1:100	Chemicon (cat no. MAB 3068)
Cx45	PC	1:1000	Koval et al.[29]
Cx46	PC	1:2000	Koval et al.[30]
Cx50	PC	1:100	White et al.[31]
DSG-1	MC	1:100	Chemicon (cat no. CLB 176)
Involucrin	MC	1:250	Peter Steinert (Biomedical Technologies, Stoughton, MA)
Profilaggrin	MC	1:100	Yuspa et al.[32]
K3	MC	1:100	ICN (cat no. 691431)

MC, monoclonal mouse antibody; PC, polyclonal rabbit antibody.

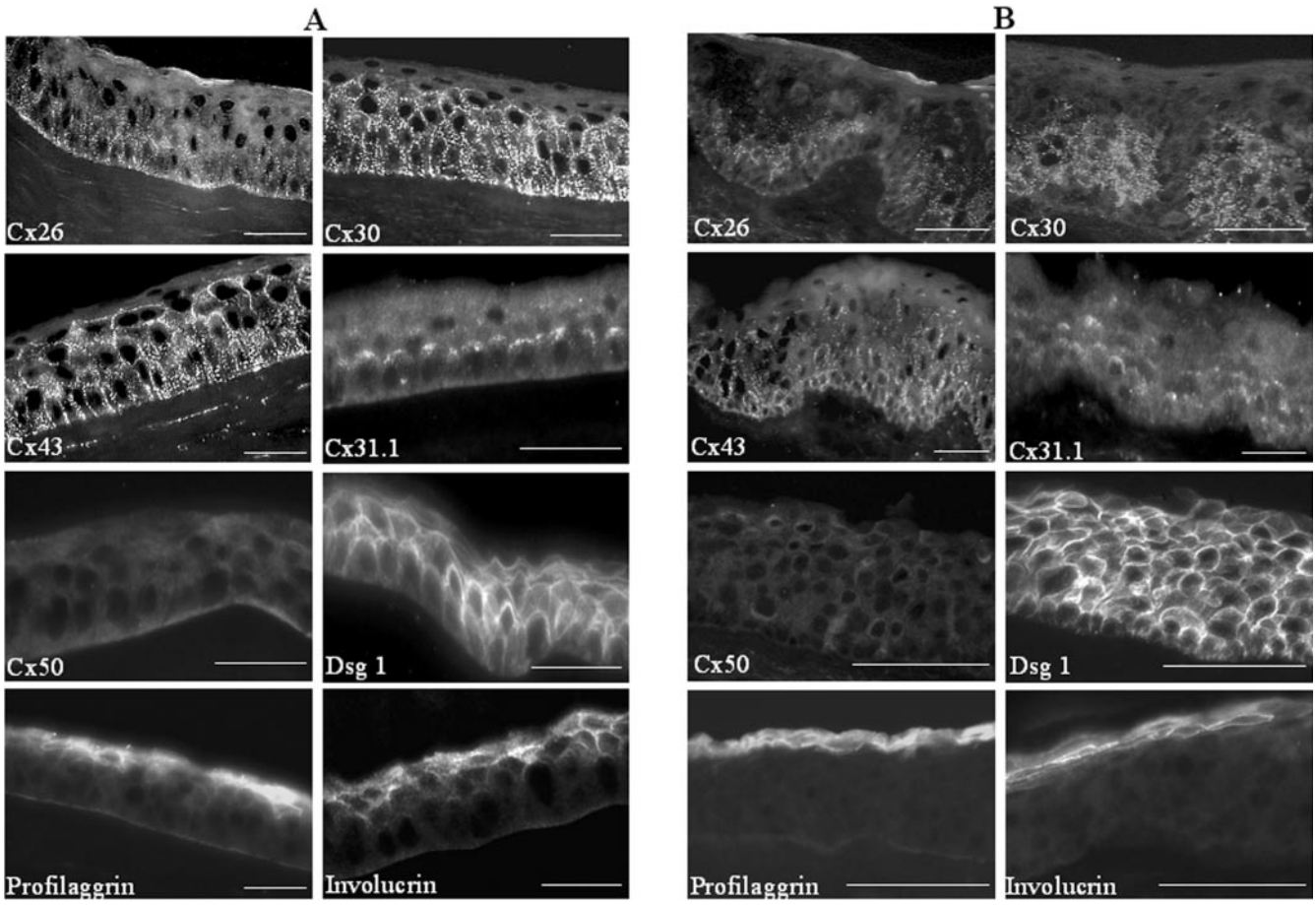

FIGURE 1. Overlapping yet distinct expression patterns of Cx26, -30, -31.1, and -43 in the human epithelium of the CC (**A**) and LR (**B**). No immunostaining was observed for Cx50 and eight other human gap junction proteins (not shown). For comparison, the desmosomal plaque protein Dsg 1 and the differentiation markers involucrin and profilaggrin are also shown. Scale bars: (**A**) 30 μm; (**B**) 60 μm.

ously described (BD-Clontech, Palo Alto, CA).[33] The presence of EYFP/ECFP does not interfere with the formation and function of gap junction channels, as demonstrated in various studies.[34,35] Competent DH5α *Escherichia coli* cells (Invitrogen Corp.) were transformed with these plasmid constructs, positive clones were identified by culture selection with kanamycin, DNA was extracted and purified (Lambda Mini Kit; Qiagen) and screened for correctness by direct DNA sequencing analysis.

Cell Culture and Transfection

Subconfluent h-PCECs were electroporated with 3 to 4 μg of either Cx26(D50N)-pEYFP DNA or wtCx-pECFP DNA, using a kit (EP42) according to the manufacturer's protocol (Amaxa Biosystems, Gaithersburg, MD). Briefly, 2 million h-PCECs were resuspended in 100 μL of transfection reagent (EP42 Nucleofector) and 2 to 3 μL of cloned DNA. The solution was electroporated in a cuvette by a commercial apparatus (Nucleofector; Amaxa; program selection, T-23). For coexpression studies, a 1:1 mixture of wtCx-pECFP and mtCx26-pEYFP was used in a single electroporation experiment. Expression was analyzed at 16, 24, and 48 hours after transfection, using live microscopy on the inverted microscope (TE 2000U; Nikon) using YFP- and CFP-specific filters (41028 Yellow GFP BP and 31044V2 CFP BP filters; Chroma Technology Corp., Rockingham, VT). Fluorescent and phase-contrast images were documented with a digital camera (Orca 100 Coolpix; Hamamatsu) and an imaging system (Phase3 Imaging, Glen Mills, PA).

Electronic Resources

On-line resources used in the study were BLASTn analysis, www.ncbi.nlm.nih.gov/blast (National Center for Biotechnology Information, Bethesda, MD); sequence alignments: www.genomatix.de/cgibin/dialign/dialign.pl (Genomatix, Munich, Germany); primer analysis, frodo.wi.mit.edu/cgibin/primer3/primer3 (Massachusetts Institute of Technology, Cambridge, MA); and quantitative RT-PCR analyses: www.appliedbiosystems.com (ABI).

RESULTS

Gap Junction Proteins Expressed in Human Corneal Epithelium

Immunostaining of radial frozen cornea sections for connexins Cx26, -30, -31.1, and -43 revealed a characteristic punctate plasma membrane staining indicative of gap junction plaques (Fig. 1). The membrane localization of these connexins corresponded with that of other known membrane proteins, such as desmoglein (Dsg) 1, a major desmosomal plaque component (Fig. 1). Cx26 immunostaining was limited to the basal cells and the first layer of suprabasal cells across the central cornea (CC). In the limbus region (LR), basal cells were negative for Cx26, whereas staining was detected in the lower one to three suprabasal layers. A similar suprabasal distribution was found for Cx30, although Cx30 immunostaining was much more intense and spread across several lower wing-cell layers in the CC and LR. The most prevalent gap junction protein was Cx43, which was expressed throughout all epithelial layers, with the exception of the most superficial cells and individual basal cells in the LR. Cx31.1 immunostaining was weak compared with other connexins. In the CC, Cx31.1 staining was most pro-

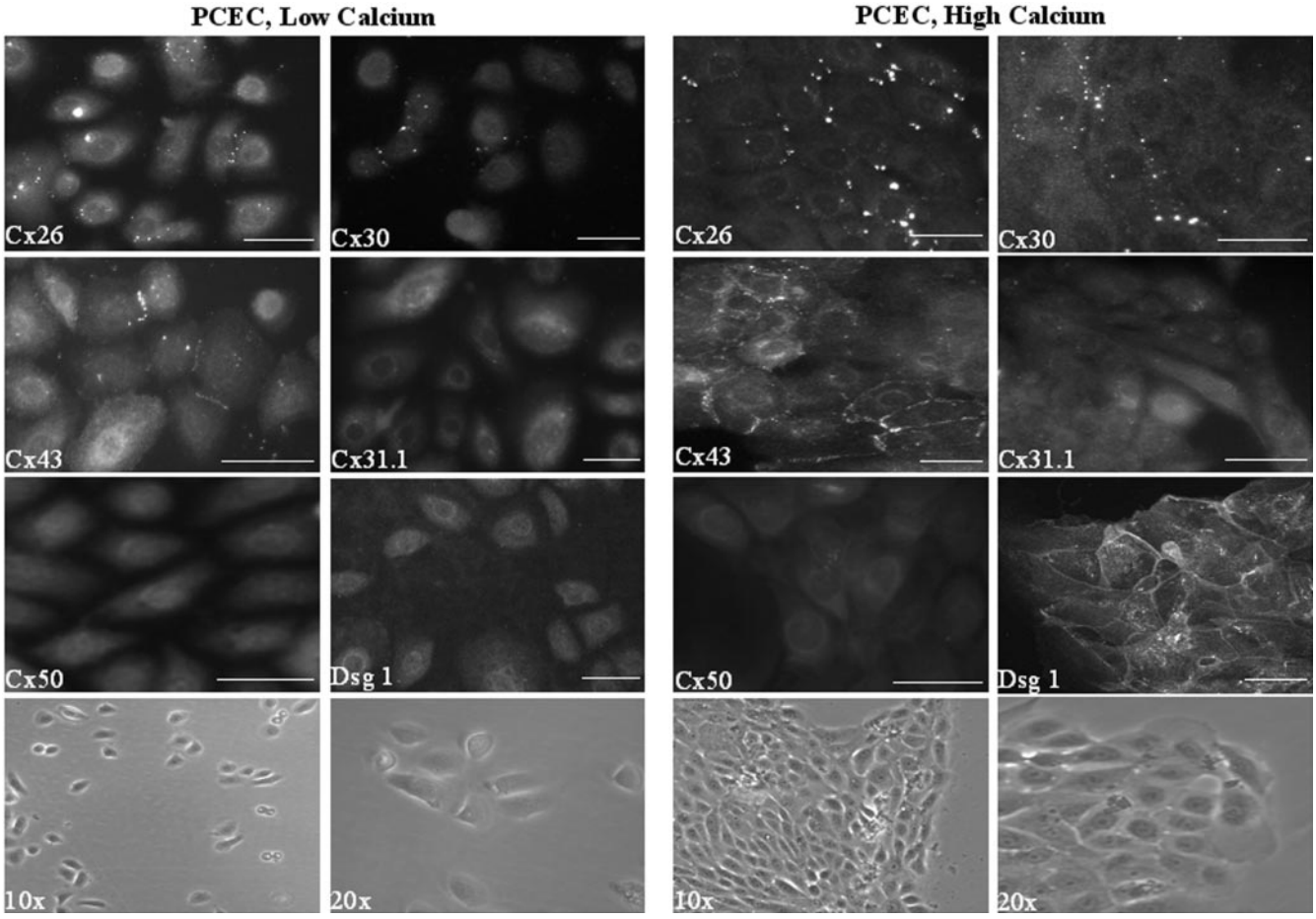

FIGURE 2. Connexin expression in cultured corneal epithelial cells in low-calcium (0.09 mM) KFSM (l-PCECs; *left*) and, after calcium exchange (1.6 mM), in high-calcium KFSM (h-PCECs; *right*). Note the punctate plasma membrane staining for Cx26, -30, and -43, which was much more intense and widespread in h-PCECs. No immunoreactivity was detected for Cx31.1 or -50. l-PCECs lacked expression of the desmosomal adhesion protein Dsg 1, but calcium-induced plasma membrane staining for Dsg 1 in h-PCECs was strong. Scale bars: 10 μm.

nounced on the apical cell surfaces of basal cells, completely sparing the basolateral cell membranes. In the LR, Cx31.1 extended into the suprabasal layers. Neither Cx26, -30, -31.1, nor -43 was expressed by the most differentiated superficial corneal cells, which highly expressed the late differentiation markers profilaggrin and involucrin (Fig. 1). Immunostaining for all other connexins tested, including Cx50, was negative, even when using a biotin-streptavidin immunohistochemistry system to enhance test sensitivity.

Connexin Expression in PCECs and Calcium-Induced Upregulation of Cx26, -30, and -43

To evaluate how cell culture influences the requirements for gap-junction–mediated intercellular communication, RT-PCR was performed from tRNA of PCECs obtained from four donors for 17 human connexin genes. l-PCECs grew as individual, roundish cells forming very little contact among neighboring cells. The switch to high-calcium KFSM (1.6 mM Ca^{2+} and 0.3% FBS) resulted in flattening and formation of large epithelial sheets within 24 to 48 hours (h-PCECs; Fig. 2). A substantial increase in cell adhesion for h-PCECs was also evident from the bright plasma membrane staining for the desmosomal plaque protein Dsg 1 (Fig. 2). Using RT-PCR, we consistently identified transcripts of Cx26, -30, -30.3, -31.1, -40, -43, -45, and -58 in all samples tested (Table 4). All positive controls for pyruvate kinase validated that the positive results originated from tRNA and not from contamination with gDNA (Table 4). Cx31 tran-

TABLE 4. Calcium-Induced Changes in Connexin RNA Levels in PCEC

	Low-Ca^{2+} PCECs	High-Ca^{2+} PCECs
Cx26	+	+
Cx30	+	+
Cx30.3	+	+
Cx31	(+)	+
Cx31.1	+	+
Cx40	+	+
Cx43	+	+
Cx45	+	+
Cx58	+	+
Cx31.9	Ø	Ø
Cx32	Ø	Ø
Cx37	Ø	Ø
Cx40.1	Ø	Ø
Cx46	Ø	Ø
Cx47	Ø	Ø
Cx50	Ø	Ø
Cx62	Ø	Ø
PK	+	+

+, Transcript detected by RT-PCR in all four donors; Ø, no transcript detected in all four donors; (+), transcript detected in two of four donors; PK, pyruvate kinase housekeeping gene positive control.

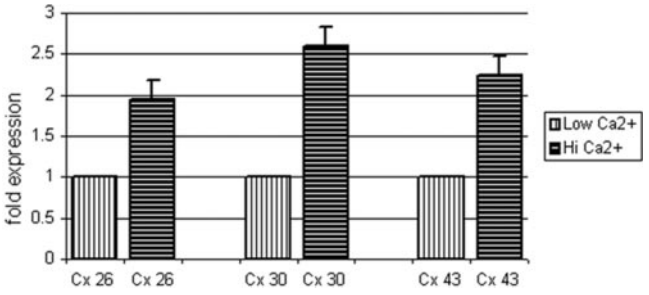

FIGURE 3. Real-time (quantitative) RT-PCR revealed calcium-induced upregulation of gene transcription for Cx26, -30, and -43 in PCECs.

scripts were detectable in only two of 4 l-PCEC cultures, whereas Cx31 was expressed in all four h-PCEC cultures (Table 4). On visual inspection of the RT-PCR results the transcription of Cx26, -30, and -43 appeared to be upregulated in h-PCECs. This observation was backed by a semiquantitative analysis of the band density of RT-PCR amplicons, which approached a significance level of 0.1 (χ^2 2.15; $df = 1$; data not shown).

Subsequent quantitative RT-PCR demonstrated that transcription of Cx26, -30, and -43 was increased 48 hours after calcium exchange (Fig. 3). The upregulation of the Cx30 gene was most pronounced, yielding a 2.6-fold increase (+0.88), followed by Cx43 and -26 with 2.2-fold (+1.20) and 2-fold (+0.14) increases, respectively.

Although nine different connexin genes were found to be transcribed in PCECs, connexin immunostaining demonstrated the expression of only three gap junction components on a protein level: Cx26, -30 and -43. As shown in Figure 2, scarce punctate plasma membrane staining was observed for Cx26, -30, and -43 in l-PCECs. Immunoreactivity was considerably increased after a calcium exchange, most notably for Cx43, which yielded comparable results obtained for desmoglein 1. These data correspond well with the detected transcriptional activation for these connexin genes in PCECs. Despite the presence of Cx31.1 staining in the human corneal epithelium, there was no evidence of gap junction plaques containing Cx31.1 in the PCECs. This evidence suggests that the connexin expression pattern in PCECs (in vitro) is similar but not identical with corneal cells in vivo.

Calcium-Induced PCEC Differentiation and Gap Junction Coupling

Although Cx26, -30, and -43 were detectable in a punctate pattern at the plasma membranes of some l-PCECs grown to high confluence (>85%; Fig. 2), cells were poorly coupled in dye-transfer assays. After microinjection of Lucifer yellow in the cytoplasm of a cell, a dye spread through gap junction channels was observed to an average of 0.86 ± 1.03 neighboring cells ($n = 46$). In l-PCECs, the maximum dye transfer was one neighboring cell and the switch to h-PCECs resulted in a significant increase (fivefold) in gap-junction–mediated dye transfer, with dye spread to an average of 5.72 ± 3.14 cells ($n = 30$; $P < 0.00001$).

Expression of Fluorescently Tagged Cx26 Harboring a Pathogenic Missense Mutation Responsible for KIDS

Genetic studies have demonstrated that approximately 80% of KIDS patients harbor the D50N mutation in Cx26.[36] Therefore, we studied the pathologic consequences of KIDS mutations in transiently transfected PCECs. Expression of wild-type connexins in PCECs, including constructs pCx26-EYFP, pCx26-ECFP, and pCx30-ECFP, resulted in punctate or small linear fluorescent signals at points of cell–cell contacts, characteristic of gap junction plaques, within 16 hours of electroporation. There was also some perinuclear accumulation of protein visible in all experiments (data not shown). A similar outcome was obtained for the coexpression of equal amounts of cDNA of plasmids pCx26-EYFP and pCx26-ECFP as well as pCx26-EYFP and pCx30-ECFP as shown in Figure 4, where EYFP fusion proteins were pseudocolored in green and ECFP-fusion protein in red. The yellow color indicated that most of the transfected cells expressed both wild-type connexins. In contrast, the mutant Cx26(D50N)-EYFP protein by itself failed to accumulate at the plasma membrane and showed primarily cytoplasmic protein localization (Fig. 4). The protein formed clumps around the nucleus, indicating that it might be trapped in the endoplasmic reticulum (ER)-Golgi compartment similar to previous studies for Cx32 mutants, which were shown to be rapidly degraded and cleared from the cells.[21] Thus, the accumulation of Cx26(D50N) probably has no toxic effect but does prevent the formation of gap junction plaques.[21]

To simulate the in vivo situation in KIDS, where mutant and wtCx26 subunits are coexpressed due to the heterozygous nature of the mutations, we coexpressed pCx26(D50N)-EYFP with equal amounts of pCx26-ECFP. The results, however, were comparable to the expression of pCx26(D50N)-EYFP alone (Fig. 4). Both mutant and wtCx26 formed cytoplasmic clumps and were not targeted to the plasma membrane, indicating that Cx26(D50N) traps wtCx26 and results in a trafficking defect. Considering recent in vitro and in vivo evidence for co-oligomerization of Cx26 with Cx30 into mixed (heteromeric) connexin hemichannels in HeLa cells[24] and cochlea,[37,38] respectively, we also studied the effect of Cx26(D50N) on wtCx30 in cotransfected PCECs. As shown in Figure 4, the presence of Cx26(D50N; green) also prevented the accumulation of wtCx30 into gap junction plaques at plasma membranes. These data indicate that the mutant Cx26(D50N) may elicit a dominant negative effect on wtCx26 and a trans-dominant-negative effect on wtCx30. Together with the observed coexpression of Cx26 and -30 in the lower layers of the corneal epithelium, our results suggest that the corneal disease in KIDS results from a functional disruption of the gap junction system of corneal epithelial cells.

DISCUSSION

Gap junctions are known to control a multitude of physiological processes and serve diverse functions tailored to the specific needs of the tissues in which they are expressed.[39–41] Therefore, determining the constituents of the corneal gap junction system and their specific expression patterns is essential for understanding the physiology and pathology of the corneal avascular stratified epithelium. We demonstrated in this study that four gap junction proteins, Cx26, -30, -31.1, and -43, mediate intercellular communication of the human corneal epithelium. Each connexin has a distinct expression pattern, although they overlap widely. Our findings in human cornea are consistent with those reported in rabbit cornea by Ratkay-Traub et al.,[42] who detected Cx26 and -43, but not Cx32, -37, and -40. In central cornea, Cx26 was found to stain the basolateral plasma membranes of basal cells and, occasionally, lower wing cells. In contrast, Cx43 localized to the apical surface in the basal layer and was found at all plasma membranes throughout the suprabasal layers. Recently, Laux-Fenton et al.[43] studied 14 connexins in the rat cornea and identified eight connexin transcripts (Cx26, -30.3, -31, -31.1, -33, -37, -43, and -50) in the central rat corneal epithelium and four more (Cx30, -40, -45, and -46) in the peripheral epithelium. Of the 12 connexins expressed by RT-PCR, only five, Cx26, -30,

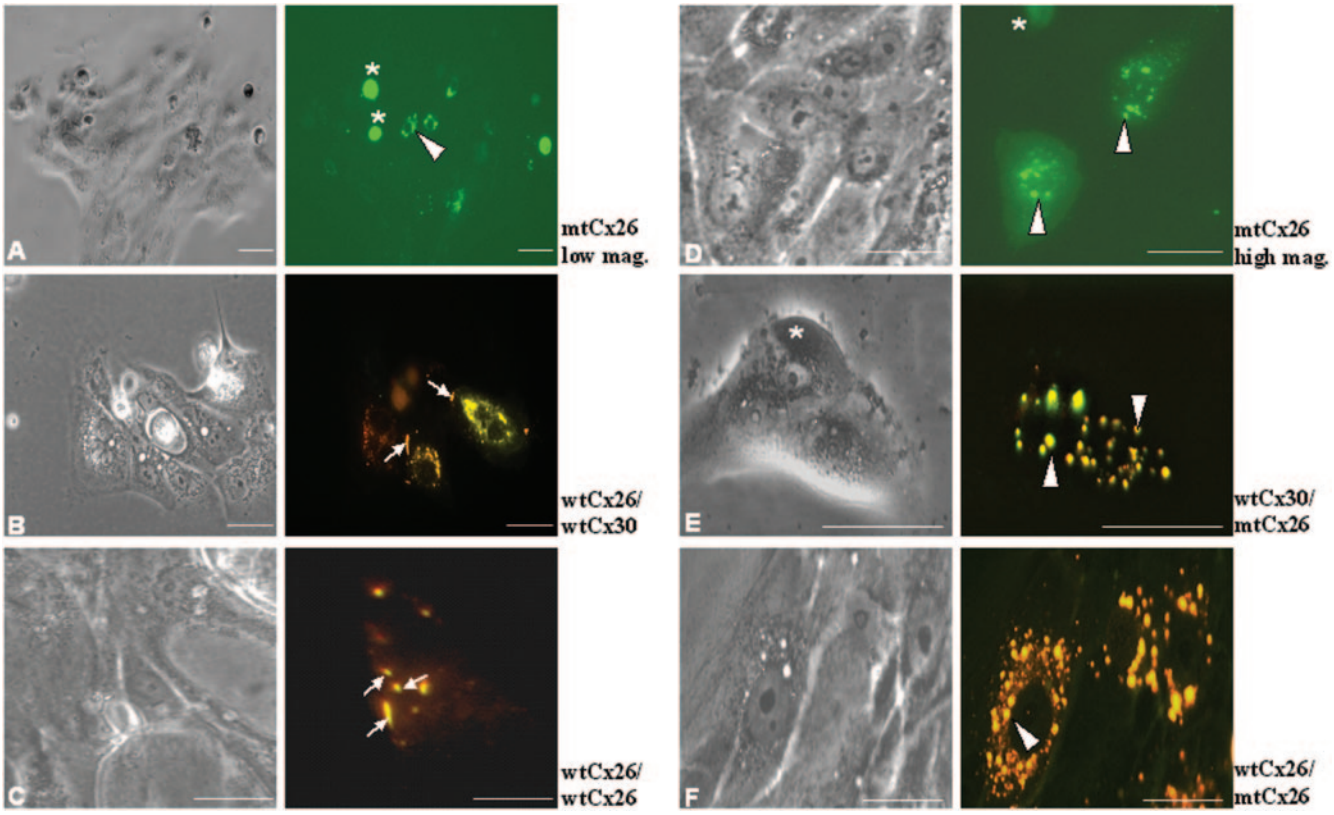

FIGURE 4. Coexpression of KIDS mutant Cx26(D50N)-EYFP (*green*) with wtCx26-ECFP (*red*) or Cx30-ECFP (*red*) in PCECs. *Left images*: phase-contrast; *right images*: corresponding area by epifluorescence. Expression of wtCx26 alone (**A**, **D**) or together with wtCx30 (**B**, **E**) led to formation of multiple gap junction plaques at points of cell–cell contact (*arrows*). Most of the proteins colocalized at plasma membranes (*yellow*). In contrast, coexpression of mutant Cx26(D50N)-EYFP (*green*) with wtCx26-ECFP (*red*) or wtCx30-ECFP (*red*) (**F**) resulted in formation of large, perinuclear cytoplasmic clumps (*arrowheaads*) and lack of visible gap junction plaques. Note also the rounding (✶) and increased vacuolization of cells expressing Cx26(D50N)-EYFP, indicative of apoptosis. (**C**) A control experiment for cotransfection of wtCx26 and mtCx26. mt, mitochondrial; low mag., low magnification; high mag., high magnification. Scale bars: 30 μm.

-31.1, -37 and -43, were confirmed on a protein level.[43] Our in vitro studies of PCECs revealed comparable results. We found transcripts of nine connexin genes, yet only three gap junction proteins (Cx26, -30, and -43) were detectable in PCECs and four connexins (Cx26, -30, -31.1, and -43) in human frozen cornea sections, by immunohistochemistry. This discrepancy probably reflects the much greater sensitivity of RT-PCR than immunostaining. It is, however, also possible that connexins undergo posttranslational modifications or transcriptional regulation, or that small amounts of some connexins are synthesized but do not form detectable gap junction plaques at cell membranes.

It is worthwhile to mention that there is also evidence of a few species-specific differences in corneal connexin expression. In rat cornea, Cx30 immunostaining was localized to the peripheral corneal epithelium and disappeared toward the central cornea, whereas Cx30 was consistently expressed across the CC and LR in basal and lower suprabasal layers of the human cornea. Cx31.1 expression in rat cornea was limited to the most superficial cells, but we observed Cx31.1 signals at the apical surfaces of basal cells in CC and more widespread signal throughout the suprabasal layers in the LR. Similar to other studies,[8,9,43] we also found no evidence of expression of Cx37 or -50 in the human corneal epithelium. The polyclonal Cx50 antibody used in this study was shown to be Cx50-specific and showed no immunoreactivity in Cx50-null mice.[44] Therefore, it seems likely that previous positive results were artifacts due to antibody cross-reactivity with other connexins.[44]

The gap junction expression of cultured PCECs was very similar to the in vivo findings. However, cells in culture did not express Cx31.1 at detectable levels, and Cx31 was found in h-PCECs and not on the protein level, despite having positive epidermal control cultures for both a monoclonal and a polyclonal Cx31 antibody. Our results in calcium exchange experiments suggest that calcium-induced epithelial differentiation is associated with an increase in connexin expression, both on a transcript and protein level. It remains to be determined whether calcium has a direct effect on connexin expression or an indirect effect via differentiation. Our observations reflect the greater need for gap-junction–mediated cell–cell coupling in high-calcium conditions, as illustrated by the significant increase in dye transfer between h-PCECs, which has also been demonstrated in rabbit corneal epithelial cells.[45] Wolosin et al.[45] studied the effects of clonal progression and calcium concentration on Cx43 expression in these rabbit cells and observed an increase in Cx43 protein levels in high-calcium cultures, paired with a slight decrease in RNA levels determined by semiquantitative RT-PCR.

Because our results demonstrated coexpression of four connexins including Cx26 in the basal layer of the corneal epithelium and Cx26 has been shown to co-oligomerize with Cx30 into heteromeric hemichannels,[24,38] we sought to determine whether a bona fide mutation resulting in KIDS exerts a dominant negative effect on other coexpressed corneal connexins in vitro. Our expression studies showed formation of gap junction plaques at the membrane planes when wtCx26 and

-30 were coexpressed. In the presence of mutant Cx26(D50N), however, all newly synthesized fluorescently tagged proteins formed aggregates in perinuclear localization and failed to traffic to the membrane. Similar observations were reported for a mutant Cx30.3 variant when coexpressed with wtCx30.3 or -31 in coupling-deficient HeLa cells.[46] Other functional studies of selected missense mutations in the first extracellular domain of Cx26 (W44S, R75W) revealed an intracellular trafficking defect of mutant Cx26, which could be rescued, in contrast to our results, by coexpression of wtCx26 or -30.[47] Whereas these mutations result in SNHL, other mutations within the same protein domain but with additional skin manifestations selectively alter the function of Cx26 but not of Cx30 (D66H), or visa versa (G59A).[24,47] These results underscore that different mutant Cx26 variants have distinct structural and functional implications, which perhaps explains the different tissue manifestations of individual mutations and the corneal involvement of D50N not seen in other Cx26 mutations. It remains to be determined whether Cx26(D50N) has a trans–dominant-negative effect on Cx43 or -31.1, which are also expressed in human cornea.

In summary, we demonstrated that four connexins, Cx26, -30, -31.1 and -43, mediate gap junction intercellular communication in the human corneal epithelium. These connexins were expressed in distinct yet overlapping patterns. Cultured corneal epithelial cells showed a similar connexin expression profile except for Cx31.1, which was not detectable. h-PCECs resulted in an approximately twofold induction of connexin gene transcription of Cx26, -30, and -43, a striking increase in gap junction plaque formation by immunostaining and significant increase in dye coupling between neighboring cells. Expression of a pathogenic Cx26(D50N) mutant, characteristic for the corneal disease of KIDS, revealed intracellular trafficking defects as previously observed for other connexin mutants with skin manifestations and hearing loss. However, the mutant Cx26 also dominantly interfered with the formation of gap junction plaques when incorporated into hemichannels with wtCx26 and -30. This dominant negative effect may alter the gap junction coupling in the corneal epithelium and other affected tissues in the skin and inner ear or, alternatively, may change the permeability and other functional parameters of gap junction channels or hemichannels, as seen with certain Cx30 mutations.[48]

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