

Expression Pattern of Connexins in the Corneal and Limbal Epithelium of a Primate

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Purpose: To detect the expression pattern of connexins in epithelial cells of the central cornea and limbus of the macaque.

Methods: Total RNA was extracted from the central corneal and limbal epithelia of *Macaca fascicularis* and processed by reverse transcriptase–polymerase chain reaction with isoform primers to detect the expression of 16 connexin (Cx). Immunofluorescent staining of frozen sections of corneal tissue confirmed and localized connexin proteins expression.

Results: Transcripts encoding 10 Cx isoforms (Cx26, Cx30, Cx30.3, Cx31, Cx31.1, Cx32, Cx43, Cx45, Cx50, and Cx58) were detected by reverse transcriptase–polymerase chain reaction in both central and peripheral corneal epithelium. Six (Cx26, Cx31, Cx32, Cx43, Cx45, and Cx58) were confirmed by laser scanning confocal microscopy. Cx26 was detected throughout the central corneal epithelium and in the mid and superficial layers of the limbal epithelium. Cx43 and Cx45 were localized to the basal and suprabasal epithelial cells. Cx58 was expressed in the superficial epithelium throughout the cornea, whereas Cx31 and Cx32 were mainly expressed in the central corneal epithelium and weakly in the limbal area.

Conclusions: The complex distribution pattern of the connexins suggests that selected isoforms play important roles in maintaining corneal homeostasis.

Key Words: macaque, cornea, connexins, limbus, epithelium

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Gap junctions are intercellular channels that allow low-molecular weight substances to pass between cells. These junctions are formed by connexin (Cx) that are encoded by a multigene family of more than 20 members. Protein structure analyses suggest that connexins share a common sequence of structural motifs, including 4 hydrophobic transmembrane, 2 extracellular domains, and 3 cytoplasmic domains.¹ Six

connexin molecules assemble to form a ring structure known as a connexon. Although the contributions of different Cx molecules to the mature gap junction is unclear, only a complete connexon forms functional channels.² Connexins such as Cx26, Cx30, Cx30.1, Cx43, and Cx50 mediate gap junction communication in the cornea of rats, rabbits, and humans.^{3–5}

Most cells express several Cx isoforms in a temporal-, spatial-, and differentiation-specific pattern. For example, epidermal cells express multiple connexins, including Cx26, Cx43, Cx30.3, and Cx31.1.^{6,7} Defects in Cx genes produce several genetic disorders. Mutations in Cx32 cause Charcot–Marie–Tooth disease,⁸ and germ line mutations in Cx43 may result in heart malformations.⁹ Connexin gene mutations have recently been associated with ocular disorders, including Cx46 and Cx50 mutations in congenital zonular pulverulent cataract,^{10–12} Cx43 mutations in oculodentodigital dysplasia,¹³ and Cx26 mutations in keratitis–ichthyosis–deafness syndrome.¹⁴

Little is known about the exact topographic distribution of connexins in the cornea, particularly the epithelium in the limbus. In this study, we systematically analyzed the expression patterns of different ocular-related Cx family members in the epithelial cells of the central cornea and limbal areas of a subhuman primate.

MATERIALS AND METHODS

Monkey Corneal and Limbal Tissue

Experiments were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine. We obtained 15 eyes freshly harvested from 8 male and 2 female 3- to 8-year-old *Macaca fascicularis*. The cornea and the limbal area were dissected and freed from surrounding tissues, such as the conjunctiva, Tenon capsule, iris, lens, and ciliary body. A 6-mm button of central cornea was dissected from the surrounding 2-mm-wide limbal area. From the first group of 10 eyes, we scraped epithelial cells from both these areas in the same animal separately (for a total of 10 corneas from the 5 monkeys) before putting the cells into RNA lysis buffer and storing them at -80°C until RNA extraction. A piece of skin and a lens from the first group were also harvested and placed into the lysis buffer as positive controls. Another 5 corneas and a piece of skin from the second group of macaques (as positive controls) were embedded in optimal

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TABLE 1. RT-PCR Primers and Conditions

| Connexin | GenBank Accession No. | Forward Primer (5'–3') | Reverse Primer (5'–3') | Size (bp) | AT (°C) |
|----------|-----------------------|------------------------|------------------------|-----------|---------|
| Cx26 | ENSMMUG00000010522 | CTAACGACATTGCAGCCTCA | CCATTTTGTCTCCCACTTA | 282 | 63.6 |
| Cx30 | ENSMMUG00000023295 | GCGAGGAGACAAGAGGAATG | AAGCAGCATGCAAATCACAG | 295 | 63.9 |
| Cx30.3 | ENSMMUG0000001860 | ATCTGCATCCTGCTCAACCT | GGGGGACCTGGTGATCTTAT | 252 | 63.8 |
| Cx31 | ENSMMUG00000022677 | CTAACCCAGGAAGGGGTCAT | TCCTCTGGAAGGAGTGGAGA | 178 | 64 |
| Cx31.1 | ENSBTAT00000007517 | GCCTACCTGGTGAGCAAGAG | AAAGATGAGGTGCGCTGAGA | 135 | 63.9 |
| Cx32 | ENSMMUG00000023295 | GCGAGGAGACAAGAGGAATG | AAGCAGCATGCAAATCACAG | 295 | 63.9 |
| Cx36 | XM001087723 | GCTATTGTCAATGGGGTGCT | ACATTCCACCTCCTTGATGC | 285 | 63.8 |
| Cx37 | ENSBTAT00000018428 | GACTCATCTCCCTGGTGCTC | GTTCTGCTCACTGGACGACA | 221 | 64 |
| Cx40 | AB046017 | CACCTGAGAGCCAGGAAGTC | CCACTGTGCCAGCTAATTT | 212 | 63.9 |
| Cx43 | AB169817 | AACTGGCATTCTGGGTTTG | CTCAGCATTTTCACCAGTCG | 258 | 63.5 |
| Cx45 | ENSMMUT00000007687 | GCACTGCCAGTAGCAAATCA | CCAACAGCATCCCTGAAGAT | 165 | 64 |
| Cx46 | ENSMMUT00000014722 | GCCGGCCAGTACTTTCTGTA | CCTGCTTGAGCTTCTCCAG | 205 | 64 |
| Cx47 | ENSMMUG00000023086 | CTGCTGTACGGCTTTGAGGT | CGCAGAGGTTGAGGAGTAGG | 157 | 64 |
| Cx50 | XM_001095298 | GGGCTACCAAGAGACTGTC | ACCTTCTCCTGCTCCTCCAT | 150 | 63.9 |
| Cx58 | ENSMMUG00000010135 | GGCAAAGGATGAAAGCTCAG | CAACCACAGAGCGAGTGAAA | 183 | 64 |
| Cx62 | XM_001092621 | GGCCAGCAGTGTATGATT | GCTGATTTCCAGCACTGTCA | 245 | 64 |
| GAPDH | AB171455 | ACCAAGATCATCCATGACAAC | GTCCACCACCCCGTTGTGTGA | 498 | 64 |

AT, annealing temperature; Cx, connexin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cutting temperature compound (Sakura Finetek USA Inc, Torrance, CA) and frozen in liquid nitrogen. Frozen tissues from the 5 corneas were cut into 10- μ m sections.

Histology

In histologic analysis, frozen sections were warmed to room temperature, stained in hematoxylin for 1 minute, rinsed in water, and then dipped in 95% ethanol for 1 minute. Eosin staining was performed for 30 seconds. Sections were rinsed in ethanol, dehydrated, cleared, and then mounted on slides with coverslips.

RNA Extraction and Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted according to the manufacturer's protocol (RNeasy Micro Kit; Qiagen Inc, Valencia, CA). DNase I (Qiagen Inc) was used to exclude DNA contamination. The total isolated RNA was quantified by its absorption at 260 nm and then stored at -80°C until use. With a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, as an internal control, we analyzed the messenger RNA (mRNA) expression of 16 Cx genes in corneal and limbal epithelia by semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR), as described previously.¹⁵ The first-strand complementary (cDNA) was synthesized from 1 μ g of total RNA with Omniscript RT Kit (Qiagen Inc). Polymerase chain reaction (PCR) amplification of the first-strand cDNA was performed using specific primer pairs designed from published macaque gene sequences (Table 1) for different Cx isoforms in a PCR system. Semiquantitative RT-PCR was established by terminating reactions at the intervals of 20, 24, 28, 32, 36, and 40 cycles for each primer pair to ensure that PCR products were within the linear portion of the amplification curve. All products were separated by 2% agarose gel electrophoresis and visualized with 0.5 μ g/mL ethidium bromide. We verified the fidelity of

the RT-PCR products by comparing their size with the size of cDNA bands and by sequencing the PCR products.

Immunofluorescent Staining

Immunofluorescent staining was performed by a previously reported method¹⁶ to evaluate the expression pattern of different Cx isoforms that had been detected by RT-PCR. Frozen corneal sections were thawed, dehydrated, and fixed in 2% paraformaldehyde at 4°C for 10 minutes or in acetone at -20°C for 10 minutes. Sections were blocked with 10% normal donkey serum in phosphate-buffered saline for 1 hour to decrease the nonspecific antibody reactions. Primary antibodies (monoclonal mouse antibodies or polyclonal rabbit antibodies) against Cx isoforms were applied and incubated overnight at 4°C . The dilution of the primary antibodies is listed in Table 2. Secondary antibodies (Alexa Fluor 488–conjugated donkey antimouse or antirabbit antibody;

TABLE 2. Primary Antibodies Against Connexins

| Protein | MC/PC | Dilution | Source (Catalog No.) |
|---------|-------|----------|---------------------------------|
| Cx26 | PC | 1:100 | Chemicon International (AB8143) |
| Cx30 | MC | 1:25 | Zymed (33-2500) |
| Cx30.3 | PC | 1:25 | Abgent (AP1544a) |
| Cx31 | PC | 1:200 | Abgent (AP1553c) |
| Cx31.1 | PC | 1:25 | Abgent (AP1545b) |
| Cx32 | PC | 1:50 | Zymed (71-0600) |
| Cx43 | MC | 1:125 | Chemicon International (05-763) |
| Cx45 | PC | 1:200 | Chemicon International (AB1745) |
| Cx50 | MC | 1:25 | Zymed (33-4300) |
| Cx58 | PC | 1:25 | Abgent (AP1550b) |
| K3 | MC | 1:100 | Chemicon International (CBL218) |

Cx, connexin; K, keratin; MC, monoclonal mouse antibody; PC, polyclonal rabbit antibody.

Invitrogen, Carlsbad, CA) were then applied, and sections were incubated in a dark chamber for 1 hour at room temperature, followed by counterstaining with propidium iodide in Fisher antifade Gel/Mount (Foster City, CA). A cover slide was applied, and the sections were examined with a laser scanning (krypton/argon and helium/neon) confocal microscope (LSM 510; Zeiss, Thornwood, NY) with 488- and 543-nm excitation and emission filters (LP 505 and LP 560, respectively). Images were acquired with a $\times 40$ oil-immersion objective and processed using Zeiss LSM-PC software and Adobe Photoshop 6.0 (San Jose, CA).

RESULTS

Histology

In the central area of the cornea, the epithelia were stratified, with 4–5 layers of cells and a total thickness of 15–20 μm . The superficial cells were flattened nucleated squamous cells, and the basal cells were tall columnar cells (Fig. 1A). In the limbal area, the epithelia were thicker and consisted of 10–15 layers of columnar cells that formed a single layer resting on a basement membrane (Fig. 1B). Melanocytes were scattered throughout the limbal epithelia, especially in the superficial layer. The limbal epithelium became thicker as the distance from the central cornea increased, blending with the bulbar conjunctiva.

Connexin mRNA Expressed in Central Corneal and Limbal Epithelia

RT-PCR was performed to detect Cx mRNA expression in the monkey's central corneal and limbal epithelia. Of the 16 cornea- and skin-related Cx candidates, 10 Cx transcripts (Cx26, Cx30, Cx30.3, Cx31, Cx31.1, Cx32, Cx43, Cx45, Cx50, and Cx58) were successfully amplified by RT-PCR in corneal and limbal epithelia (Fig. 2; Table 3). The mRNA levels of Cx30, Cx30.3, Cx31, and Cx32 were higher in the central corneal epithelia than in the limbal epithelia, whereas Cx45 and Cx31.1 were expressed more in limbal epithelia. Cx36, Cx37, Cx40, Cx46, Cx47, and Cx62 transcripts were not amplified in the epithelia after 40 cycles of PCR (repeated 3 times), although they were in the positive control (skin and lens) tissues (data not shown).

All the sequences of the PCR products were consistent with the sequences in gene bank. The sequence of the PCR product of Cx26 is shown as an example:

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GTCGAATGAGTTCCTAGTGATGGCTTATGATAGCAAA
TGGCCTCATGTCAAATATTTAGATGTAATTTTGTGTAA
GAAACACAGACTGGATGTACCACCACTACTACCTGT
AATGACAGACCTGTCCAACACATCTCCCTTTTCCGTG
ACTGTGTTGAATGGCAGCCAGCATCAGAAGGAACGC
TGATTTAAAGAGGTTCGCTTGAGAATTTTATTGACACA
GTACCATTTAATGGGGAGGACAAAATGGA.
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Connexin Proteins Expressed in Central Corneal and Limbal Epithelia

Of 10 connexins detected by RT-PCR, 6 (Cx26, Cx31, Cx32, Cx43, Cx45, and Cx58) were detected by immunofluorescent staining in the corneal and limbal epithelia (Fig. 3), and Cx30, Cx30.3, Cx31.1, and Cx50 were not detected in any epithelia (Table 4) but were expressed in skin epidermis under identical conditions. Keratin 3 (K3), a corneal-specific keratin gene used as another control for corneal-specific markers, was also detected (Fig. 3).

Cx26 antibody stained all the cell layers of the central corneal epithelia, most notably the intermediate cells. Cx26 antibody labeled the superficial and intermediate limbal epithelia but only weakly stained the basal epithelia. The expression of Cx26 was similar to that of K3, which was expressed by all layers of the central cornea and the superficial cells of the limbal area. Cx31 antibody stained the basal and suprabasal central corneal epithelia more strongly than the basal and suprabasal limbal epithelia. We confirmed Cx32 expression in the apical membrane region of the basal epithelia in the central cornea. Cx32 expression was weaker in the limbal area than in the central cornea.

The expression of Cx43 was confirmed in all but the most superficial layers of epithelia in macaques, most notably in most basal cells. The basal cells strongly expressed Cx45 protein, and all layers of the corneal epithelia were found to express this protein, as well. Cx58 was expressed on the membranes of all epithelia in the central and limbal areas, especially the superficial layers.

DISCUSSION

Gap junctions in multicellular organs maintain homeostasis, synchronize responses to stimuli, and control growth and development.⁶ In this study, we investigated the expression pattern of connexin proteins that contribute to gap junctions in epithelial cells of cornea. The aim of this study was to understand which connexins mediate intercellular communications

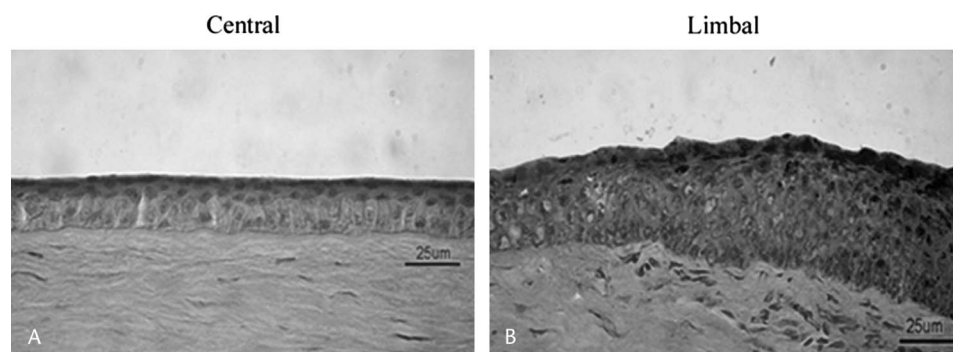


FIGURE 1. Macaque monkey's central corneal epithelia (A) and limbal epithelia (B) (hematoxylin and eosin staining, original magnification, $\times 400$).

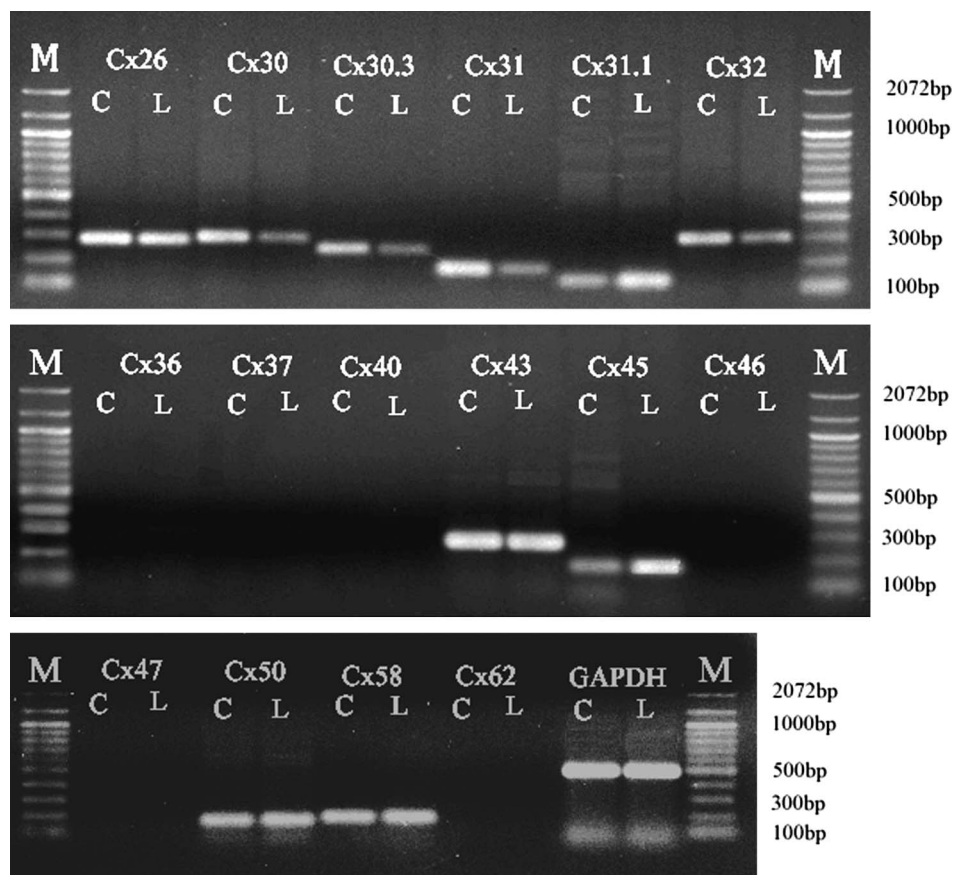


FIGURE 2. RT-PCR results of mRNA expression of connexin in central corneal epithelia (C) and limbal epithelia (L). M = 100 bp DNA ladder (100, 200, 300, 400, 500, ~1000, 1500, and 2072).

of the corneal and limbal epithelium. Our results indicate that the corneal and limbal epithelia express at least 6 connexin proteins: Cx26, Cx31, Cx32, Cx43, Cx45, and Cx58.

TABLE 3. mRNA Expression of Connexin Isoforms in Corneal Epithelium

| Connexin | Central Cornea | Limbus |
|----------|----------------|--------|
| Cx26 | ++ | ++ |
| Cx30 | + | ± |
| Cx30.3 | + | ± |
| Cx31 | ++ | + |
| Cx31.1 | ± | + |
| Cx32 | ++ | + |
| Cx36 | - | - |
| Cx37 | - | - |
| Cx40 | - | - |
| Cx43 | ++ | ++ |
| Cx45 | + | ++ |
| Cx46 | - | - |
| Cx47 | - | - |
| Cx50 | + | + |
| Cx58 | + | + |
| Cx62 | - | - |
| GAPDH | ++ | ++ |

-, negative; ±, weak positive; +, positive; ++, strong positive; Cx, connexin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Cx26, the only Cx known that causes a corneal epithelial disorder through mutations in its coding sequences, was detected in the basal and suprabasal central corneal epithelia and the superficial and intermediate epithelia in the limbal region, where it was much less evident in the basal cells. Our findings in monkey corneal epithelia generally agree with those reported in rabbit and human cornea.^{4,17}

The most prevalent Cx protein was Cx43. The absence of Cx43 has been used as evidence for identifying limbal stem cells,¹⁸ and Cx43 could be a negative marker to isolate the stem cell-containing population.¹⁹ Our results showed that Cx43 was expressed by almost all epithelial cells from the central cornea to the limbus, except for the most superficial layers and scattered single cells in the basal layers. An unexpected finding was that Cx43 expression by limbal basal cells was stronger than that in the more superficial epithelium. Cx43 may be involved in a range of non-gap-junction functions, such as suppression of cell and tumor growth, differentiation, and migration,^{20,21} and these possible functions may explain the Cx43 expression pattern detected in this study and others.²² Cx43-positive cells could be niche cells that support the division of stem cells because most cells surrounding these areas were strongly positive with some scattered negative ones below. Another possible explanation is that macaque monkeys are different in this respect from humans and that stem cells are not located among the basal epithelium of these primates.

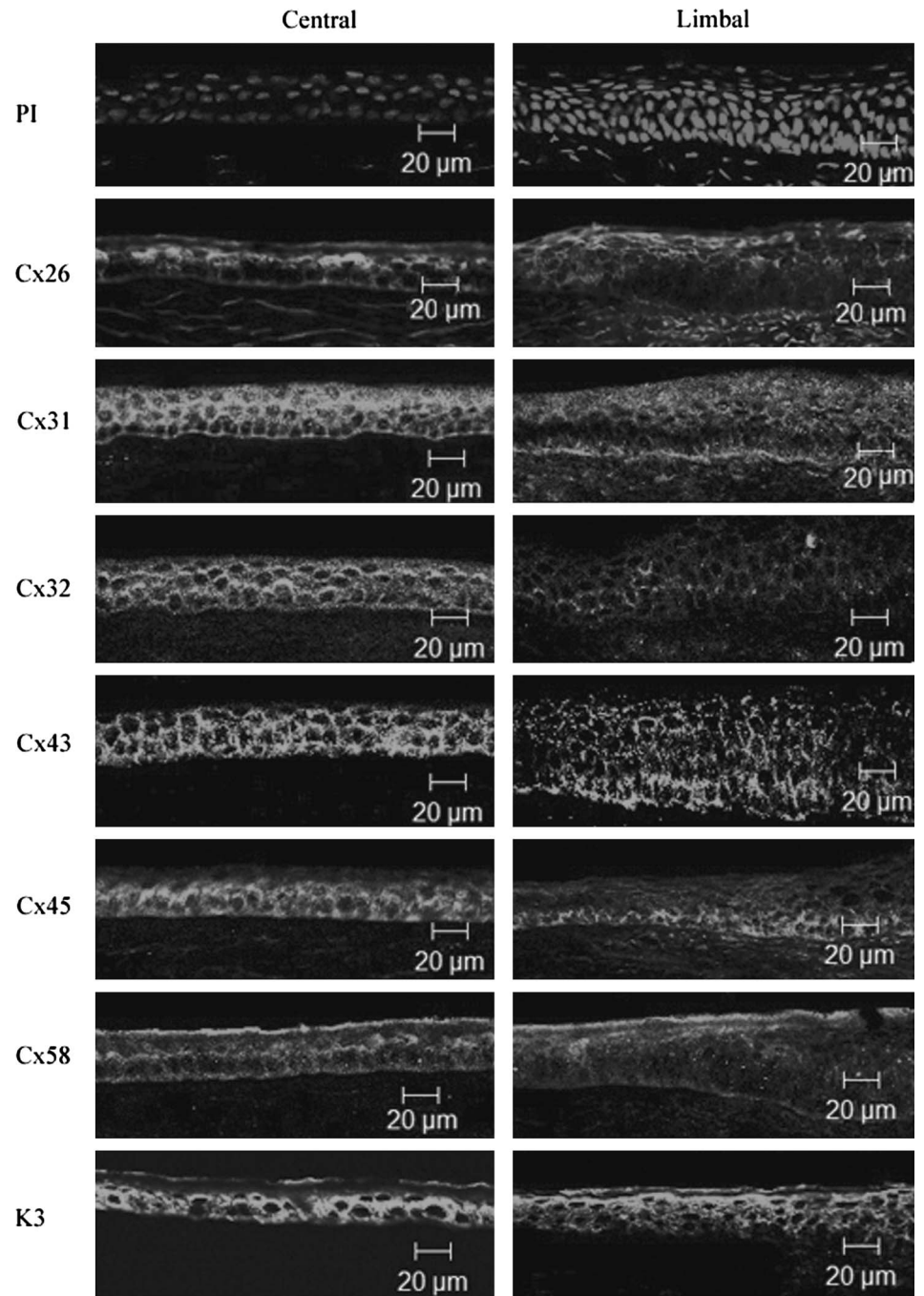


FIGURE 3. Immunofluorescent staining of connexin isoforms in macaque central corneal (left column) and limbal (right column) epithelia, with K3 as the positive control and PI as counterstain, using laser scanning confocal microscopy (original magnification, $\times 400$).

Cx45 shared a expression pattern similar to that of Cx43. The Cx45 mRNA level in limbal epithelia was greater than that in central corneal epithelia, and this was confirmed by immunofluorescent staining. Basal epithelial cells expressed Cx45 more strongly than did suprabasal cells in the limbal area. By contrast, in rat corneas, the Cx45 transcript could be detected only in the limbal area.⁵ In humans, Cx45 mRNA was found in cultured limbal epithelia, and the level could be changed by calcium.⁴ Cx58 is involved in neuronal gap junctions in the retina.^{23,24} We found that Cx58 was expressed

by corneal and limbal epithelia, both at the mRNA and protein levels, which may indicate that Cx58 has more function in the cornea of primates than other species.

Cx30, Cx30.3, and Cx31.1 were previously detected in human corneal epithelia.⁴ However, only Cx30 and Cx31.1 are present in rat corneas.⁵ Although control monkey skin tissue showed strong staining in our work, Cx30, 30.3, and 31.1 were not detected immunohistochemically. In our opinion, the differences in Cx expression patterns found among epithelia from humans, macaques, rats, and rabbits are

TABLE 4. Protein Expression of Connexin Isoforms in Corneal Epithelium

| Connexin | Central Cornea | | Limbus | |
|----------|----------------|------------|--------|------------|
| | Basal | Suprabasal | Basal | Suprabasal |
| Cx26 | ++ | ++ | ± | ++ |
| Cx30 | - | - | - | - |
| Cx30.3 | - | - | - | - |
| Cx31 | + | + | ± | ± |
| Cx31.1 | - | - | - | - |
| Cx32 | + | + | ± | ± |
| Cx43 | ++ | + | ++ | ++ |
| Cx45 | + | + | ++ | + |
| Cx50 | - | - | - | - |
| Cx58 | + | ++ | + | ++ |
| K3 | ++ | ++ | - | ++ |

-, negative; ±, weak positive; +, positive; ++, strong positive; Cx, connexin; K, keratin.

due to species-specific differences. We did not detect Cx50 protein in the central corneal and limbal epithelia, in accordance with findings in humans and rats.²⁵

In conclusion, our research sheds light on the Cx expression pattern in monkey corneal and limbal epithelia, especially the patterns of Cx43 and Cx45 in the basal epithelia; Cx26 and Cx58 in the suprabasal epithelia; and Cx31 and Cx32 in the epithelia of the central corneas. Cx43, Cx45, Cx26, Cx58, Cx31, and Cx32 seem to be involved in mediating the cell-cell communications that maintain homeostasis in the corneal epithelium.

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