

# Disruption of Zonula Occludens-1 Localization in the Rabbit Corneal Epithelium by Contact Lens–Induced Hypoxia

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**PURPOSE.** Hypoxia impairs the barrier function of the corneal epithelium. This function depends on tight junctions, of which zonula occludens (ZO)-1 is a major component. The authors have investigated the effects of hypoxia on ZO-1 localization and expression in the rabbit corneal epithelium in vivo.

**METHODS.** A polymethylmethacrylate (PMMA) or rigid gas-permeable (RGP) lens was applied to one eye each of albino rabbits for 24 hours. The structure of the corneal epithelium was examined by in vivo confocal microscopy, and epithelial barrier function was evaluated by measurement of central corneal thickness. The distribution and expression of ZO-1 in the corneal epithelium were examined by immunofluorescence analysis and by immunoblot and reverse transcription-polymerase chain reaction analyses, respectively.

**RESULTS.** Application of a PMMA lens, but not that of an RGP lens, resulted in a reduction in cell size at the surface of the corneal epithelium, compared with that in control eyes, and an increase in central corneal thickness. Immunofluorescence analysis revealed a continuous pattern of ZO-1 immunoreactivity around the perimeter of superficial corneal epithelial cells in control eyes or in eyes treated with an RGP lens. In contrast, the pattern of ZO-1 staining was discontinuous and patchy in eyes treated with a PMMA lens. Amounts of ZO-1 mRNA and protein in the corneal epithelium were reduced by application of a PMMA lens but not by that of an RGP lens.

**CONCLUSIONS.** Hypoxia at the ocular surface induced the disruption of tight junctions between superficial cells in the rabbit corneal epithelium in vivo. (*Invest Ophthalmol Vis Sci.* 2009; 50:4605–4610) DOI:10.1167/iovs.09-3407

The corneal epithelium plays important roles in the refraction of light at the ocular surface, stabilization of the tear film, and provision of a renewable protective barrier for the eye.<sup>1,2</sup> The rapid turnover of corneal epithelial cells at the ocular surface is associated with a high metabolic activity that requires a consistent supply of oxygen. When the eye is open,

a substantial portion of the oxygen consumed by the corneal epithelium is derived by diffusion from the atmosphere through the tear film.<sup>3</sup> During eye closure, oxygen is derived by diffusion from the aqueous humor and the blood vessels of the overlying palpebral conjunctiva. Hypoxia affects corneal glucose metabolism by inducing a switch from aerobic to anaerobic respiration, the latter of which yields less energy and generates lactic acid as a waste product.<sup>4–6</sup>

Tight junctions play an important role in the establishment and maintenance of barrier function.<sup>7–10</sup> The tight junction complex includes transmembrane proteins such as claudin and occludin; membrane-associated proteins such as zonula occludens (ZO)-1, ZO-2, and ZO-3; and actin filaments.<sup>11,12</sup> Tight junctions in the corneal epithelium contribute to the barrier function at the ocular surface that protects the inner tissues of the eye.<sup>13,14</sup> Hypoxia has been shown to disturb the distribution of the tight junction protein ZO-1 in vascular endothelial cells.<sup>15,16</sup> The tight junctions formed between adjacent cells in the superficial cell layer of the corneal epithelium prevent the movement of microorganisms, debris, and various biological agents from tear fluid into the intercellular space.<sup>17</sup> ZO-1 is expressed in superficial and sub-superficial cell layers of the corneal epithelium and contributes to its barrier function.<sup>18,19</sup> We have previously shown that exposure of a cultured monolayer of human corneal epithelial cells to 1% oxygen results in the redistribution and degradation of ZO-1 and in the disruption of barrier function.<sup>20</sup>

The wearing of contact lenses results in a substantial reduction in the oxygen supply to the cornea. The extent of hypoxia depends on the gas permeability of the lens material; greater oxygen permeability results in higher oxygen tension under the lens.<sup>21,22</sup> Polymethylmethacrylate (PMMA) lens wear in rabbits reduces oxygen tension at the corneal surface to zero in vivo.<sup>21</sup> To investigate the effects of hypoxia induced by PMMA lens wear on the rabbit corneal epithelium in vivo, we examined cell morphology by in vivo confocal microscopy, the localization of ZO-1 by immunofluorescence analysis, and the expression of ZO-1 at the mRNA and protein levels by reverse transcription (RT)-polymerase chain reaction (PCR) analysis and immunoblot analysis, respectively. To assess the barrier function of the epithelium, we also measured central corneal thickness in vivo by ultrasound pachymetry.

## METHODS

### Antibodies and Reagents

Rabbit polyclonal antibodies to ZO-1 were obtained from Zymed (Carlsbad, CA), mouse monoclonal antibodies to  $\alpha$ -tubulin and a protease inhibitor cocktail were from Sigma (St. Louis, MO), and horseradish peroxidase-conjugated secondary antibodies were obtained from Promega (Madison, WI). Dulbecco modified Eagle medium (DMEM)-F12 (50:50, vol/vol) and Alexa Fluor 488-labeled goat antibodies to rabbit immunoglobulin G were obtained from Invitrogen-Gibco (Rockville, MD), red fluorescent nucleic acid for probing DNA

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was from Molecular Probes (SYTO 59; Eugene, OR), and bovine serum albumin (BSA) was from Nacalai Tesque (Kyoto, Japan). Nitrocellulose membranes and an enhanced chemiluminescence (ECL) kit were obtained from GE Healthcare UK (Chalfont, UK).

### Animals

Eighteen albino rabbits (body weight, 2.0–2.5 kg) were obtained from Kitayama Labes (Kyoto, Japan). Animals were cared for and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the study was approved by the animal ethics committee of Yamaguchi University Graduate School of Medicine. All animals were healthy and free of apparent ocular disease. The rabbits were maintained in individual cages at 22°C to 24°C and relative humidity of 50% to 70%, with a 12-hour-light/12-hour-dark cycle (lights on from 7 AM to 7 PM). Animals were anesthetized by administration of 0.4% oxybuprocaine eyedrops (Santen Pharmaceuticals, Osaka, Japan) for removal of the nictitating membrane and cilia of each eye 1 week before lens wear. The corneal surface was examined by slit lamp microscopy 24 hours after the initiation of lens wear and before experimental evaluation by *in vivo* confocal microscopy. All animals manifested a stable ocular surface, without hyperemia, discharge, or epithelial defects, and they retained the applied lens. In addition, no substantial fluorescein staining was apparent in any of the eyes tested.

### Lenses

Conventional PMMA contact lenses and RGP lenses with an oxygen transmissibility (Dk/L) of  $125 \times 10^{-9}$  (cm/s)/mL · O<sub>2</sub>/mL mm Hg were obtained from Menicon (Nagoya, Japan). The lenses were specifically designed for rabbit eyes, with a uniform thickness of 0.15 mm (parallel) and a diameter of 14.0 mm, and they were selected from a fitting with four available base curves (7.60, 7.80, 8.00, or 8.20 for PMMA lenses; 7.75, 7.95, 8.15, or 8.20 for RGP lenses). A PMMA or RGP lens was applied to one eye of each rabbit for 24 hours, with the second eye of each animal serving as a lens-free control.

### In Vivo Confocal Microscopy

After lens wear for 24 hours, animals were anesthetized by intramuscular injection of xylazine (1 mg/kg body weight; Bayer, Shawnee Mission, KS) and sodium pentobarbital (20 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan) and by administration of 0.4% oxybuprocaine eyedrops. The lens was gently removed from each eye, and the central cornea was immediately observed with an *in vivo* confocal microscope (ConfoScan 1; Nidek, Fremont, CA). High-resolution images of the cornea were viewed in real time on the digital video monitor at a rate of 25 frames per second. Images of all corneal layers were obtained and stored on S-VHS videotape. Digital still pictures were obtained from the videotape by conversion of analog to digital information with a computer. Individual cells at the corneal surface were outlined on such images with reference to the moving images on the videotape, and the cell area was measured with the use of cellular imaging software (MetaMorph; Universal Imaging, Downingtown, PA). All measurements were performed by a single investigator masked to the specific experimental conditions. Analysis of endothelial cell density was performed with the use of dedicated software (ConfoCommander 2.7.1; TOMÉY, Nagoya, Japan) in a semiautomated manner. When possible, at least three images of each corneal layer were analyzed, and an average was obtained for epithelial cell size and endothelial cell density.

### Ultrasound Pachymetry

Central corneal thickness was measured with a handheld ultrasound pachymeter (SP-2000; TOMÉY) calibrated by the manufacturer. The instrument used an ultrasound velocity (acoustic index) of 1640 m/s. The cornea was anesthetized by topical application of 0.4% oxybuprocaine, and the pachymeter probe was brought in light contact with the

cornea centrally and perpendicularly. Central corneal thickness was recorded as the average of a minimum of three individual acquisitions.

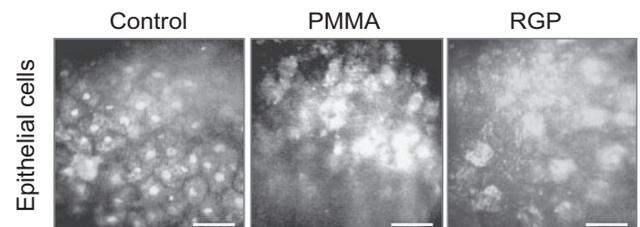
### Immunofluorescence Microscopy

After *in vivo* examinations, rabbits were killed with an overdose of sodium pentobarbital (100 mg/kg) injected intravenously. Each cornea was fixed *in situ* with 3% paraformaldehyde in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline [PBS<sup>-</sup>] for 3 minutes and was then cut into two or three blocks (2 × 3 mm). Tissue blocks were washed with PBS<sup>-</sup>, permeabilized with acetone for 5 minutes at -20°C, and incubated for 1 hour at room temperature in PBS<sup>-</sup> containing 1% BSA. They were then incubated for 2 hours at 4°C with antibodies to ZO-1 [1:100 dilution in PBS<sup>-</sup> containing 1% BSA], washed with PBS<sup>-</sup>, and incubated for 1 hour at 4°C with Alexa Fluor 488-conjugated secondary antibodies (1:2000 dilution) and red fluorescent nucleic acid (1:1000 dilution; Syto59; Molecular Probes) in PBS<sup>-</sup> containing 1% BSA before examination with a laser confocal microscope (LSM Pascal; Zeiss, Jena, Germany).

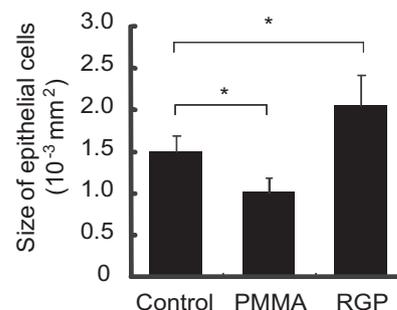
### Immunoblot Analysis

Excised corneas were washed several times with PBS<sup>-</sup>, the endothelial layer was removed mechanically, and the remaining tissue was incubated with dispase (2 mg/mL) in DMEM-F12 for 1 hour at 37°C. The epithelial cell layer was then harvested and washed several times with PBS<sup>-</sup>. Corneal epithelial cells were lysed in a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 2 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 2% SDS, and 1% protease inhibitor cocktail. The lysates (equal amounts of protein) were subjected to SDS-PAGE, and the separated proteins were transferred electrophoretically to a nitrocellulose membrane and exposed consecutively to primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Immune complexes were detected with ECL reagents. Band intensities were measured by image analysis software (Multi Gauge version 3; Fuji Film, Tokyo,

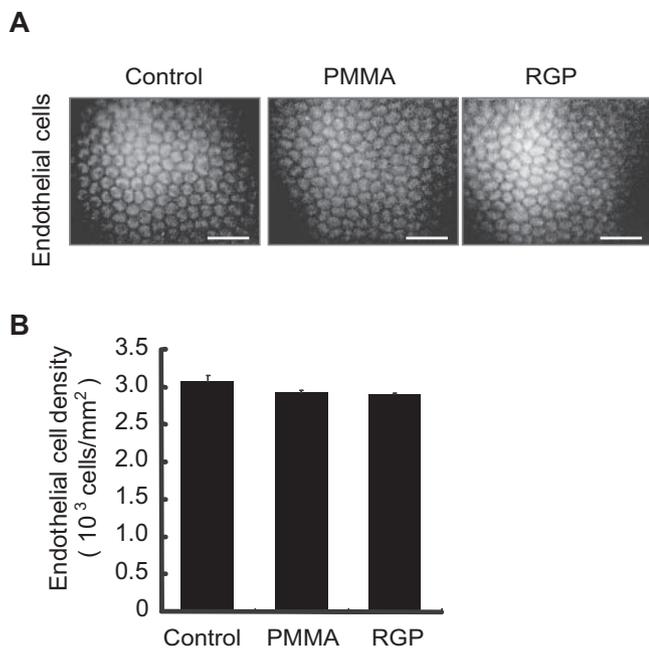
A



B



**FIGURE 1.** Effect of contact lens-induced hypoxia on the morphology of rabbit corneal epithelial cells. (A) Eyes treated with a PMMA or an RGP lens or left untreated (control) for 24 hours were subjected to *in vivo* confocal microscopy of the corneal epithelium. Scale bars, 60  $\mu$ m. (B) Cell size at the epithelial surface in images similar to those shown in (A) was determined. Data are mean  $\pm$  SE of values from three eyes per group. \* $P < 0.05$  (Dunnett test).



**FIGURE 2.** Effect of contact lens-induced hypoxia on the morphology of rabbit corneal endothelial cells. (A) Eyes treated with a PMMA or an RGP lens or left untreated (control) for 24 hours were subjected to *in vivo* confocal microscopy of the corneal endothelium. Scale bars, 60  $\mu$ m. (B) Endothelial cell density in images similar to those shown in (A) was determined. Data are mean  $\pm$  SE of values from three eyes per group.

Japan), and those for ZO-1 were normalized by the corresponding value for  $\alpha$ -tubulin.

### RT-PCR Analysis

Corneal epithelial cells were harvested as described for immunoblot analysis, and total RNA was isolated from the cells with the use of a purification kit (RNeasy; Qiagen, Valencia, CA). Portions (0.5  $\mu$ g) of the RNA were subjected to RT-PCR analysis (One-Step RT-PCR kit) based on the DNA polymerase high fidelity (Platinum *Taq*; Invitrogen, Carlsbad, CA) system. The PCR protocol was designed to maintain amplification in the exponential phase. Sequences of the PCR primers were as follows: ZO-1 sense, 5'-TGCCATTACAGGTCCTCTG-3'; ZO-1 antisense, 5'-GGTTCTGCCTCATCATTTCCCTC-3'; glyceraldehyde-3-phosphate dehydrogenase (G3PDH; internal control) sense, 5'-ACCA-CAGTCCACGCCATCAC-3'; and G3PDH antisense, 5'-TCCACCACCCT-GTTGCTGTA-3'. RT and PCR incubations were performed with a PCR system (GeneAmp 2400-R; Perkin-Elmer, Foster City, CA). RT was performed at 50°C for 30 minutes, and the PCR cycle comprised incubations at 94°C for 2 minutes, 58°C for 30 seconds, and 72°C for 1 minute. The reaction mixture was finally cooled to 4°C, and the products of amplification were fractionated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. Band intensities were measured by image analysis software (Multi Gauge version 3; Fuji Film),<sup>2</sup> and those for ZO-1 were normalized by the corresponding value for G3PDH.

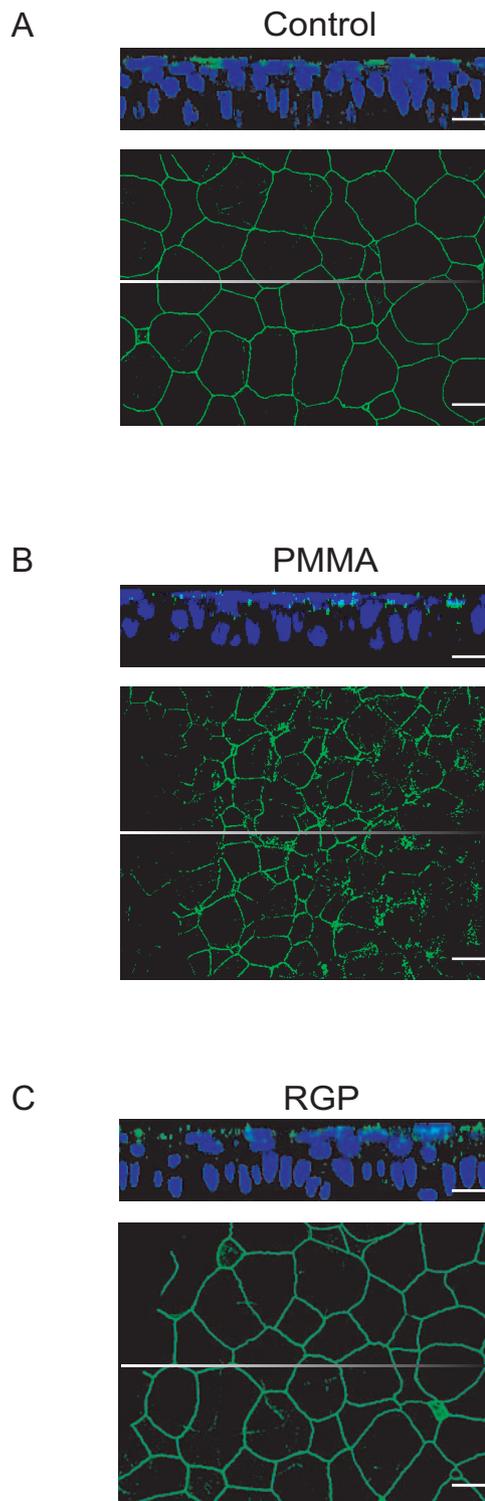
### Statistical Analysis

Quantitative data are presented as mean + SE and were analyzed by Dunnett multiple comparison test.  $P < 0.05$  was considered statistically significant.

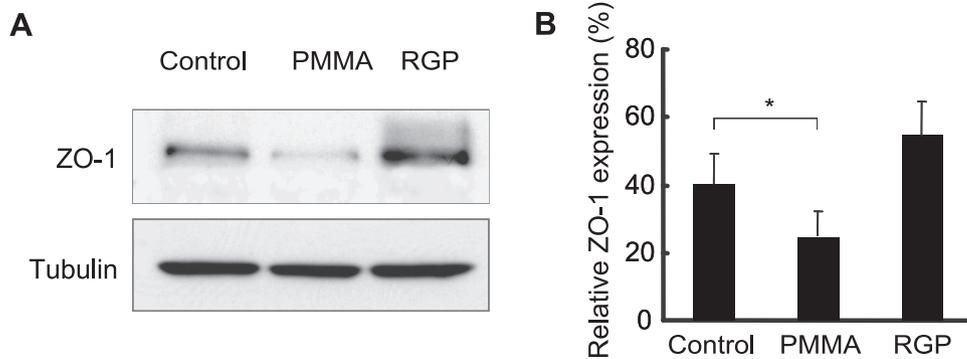
## RESULTS

We used *in vivo* confocal microscopy to examine the corneal epithelia of rabbits after they wore contact lenses for 24 hours.

Corneal epithelial cells of eyes not subjected to lens wear (control) had a polygonal mosaic appearance with brightly reflective nuclei (Fig. 1A). In contrast, those of eyes that re-



**FIGURE 3.** Effect of contact lens-induced hypoxia on localization of ZO-1 in the rabbit corneal epithelium. Corneal tissue blocks prepared from a control eye (A) or from eyes treated with a PMMA (B) or an RGP (C) lens for 24 hours were subjected to immunofluorescence staining with antibodies to ZO-1 (green) and to staining of nuclei with red fluorescent nucleic acid (blue). Lower: images of the superficial layer of the corneal epithelium in the  $xy$  plane and (upper) three-dimensional reconstructions in the  $xz$  plane. Scale bars, 20  $\mu$ m.



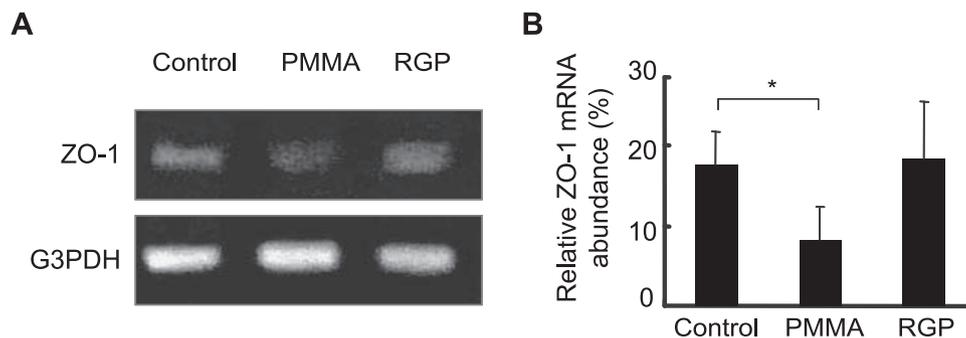
**FIGURE 4.** Effect of contact lens-induced hypoxia on the expression of ZO-1 protein in the rabbit corneal epithelium. (A) Corneal epithelial cells isolated from a control eye or from eyes treated with a PMMA or an RGP lens for 24 hours were subjected to immunoblot analysis with antibodies to ZO-1 or to  $\alpha$ -tubulin (loading control). (B) Quantitative analysis of ZO-1 band intensity in blots similar to those shown in (A). Data were normalized by the corresponding  $\alpha$ -tubulin band intensity and are mean  $\pm$  SE of values from three eyes per group. \* $P < 0.05$  (Dunnett test).

ceived a PMMA lens showed a polygonal mosaic appearance with dark areas in the focal plane. Wing epithelial cells were clearly observed at the corneal surface of the eyes treated with the PMMA lens, and the size of the surface cells was significantly smaller, by 32.6%, than that of control eyes (Fig. 1B). These observations suggested that hypoxia induced by the wearing of a PMMA lens resulted in an increase in the extent of desquamation, with the loss of superficial epithelial cells giving rise to the appearance of wing cells at the corneal surface. In contrast, the size of surface cells in the corneal epithelium of eyes treated with an RGP lens was significantly larger, by 36.6%, than that of control eyes (Fig. 1B). These larger cells had a brightly reflective polygonal mosaic appearance without obvious nuclei (Fig. 1A). Whereas individual corneal epithelial cells were distinct in eyes not subjected to lens wear, the boundaries of surface cells in eyes treated with PMMA or RGP lenses were not readily apparent and the confocal images were blurry (Fig. 1A). These findings suggest that both types of lens may cause mechanical damage to the surface cells of the corneal epithelium.

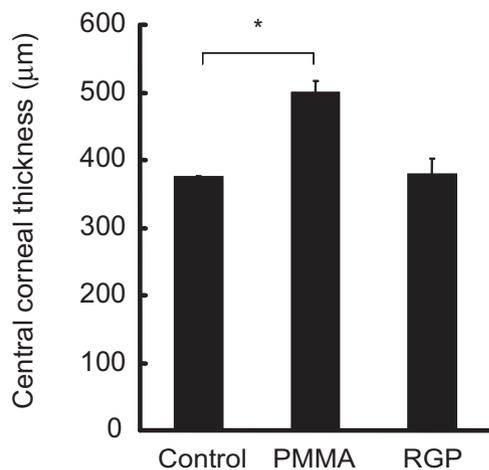
In contrast to these effects of lens wear on the corneal epithelium, the morphology of corneal endothelial cells did not appear to differ among eyes treated with PMMA or RGP lenses or control eyes (Fig. 2A). The endothelial cells of all eyes had a polygonal mosaic appearance, with some larger hexagonal cells also apparent. Cell density in the endothelium was not significantly affected by the wearing of either type of lens (Fig. 2B).

We investigated the possible effect of hypoxia induced by PMMA lens wear on the localization of ZO-1 in the rabbit corneal epithelium. Immunofluorescence microscopy revealed a continuous linear pattern of ZO-1 staining at the boundaries of adjacent superficial cells in the  $x$ - $y$  plane of control eyes (Fig. 3A). Reconstruction of three-dimensional data sets along the  $x$ - $z$  plane revealed ZO-1 to be localized at apical cell-cell junctions of superficial cells (Fig. 3A). In contrast, ZO-1 staining was patchy and discontinuous in the  $x$ - $y$  plane of eyes treated with a PMMA lens (Fig. 3B). It was also more broadly distributed from the apical side to the middle layers of the epithelium in the  $x$ - $z$  plane of such eyes (Fig. 3B). The pattern of ZO-1 staining in eyes treated with an RGP lens was similar to that in control eyes, though it was also more broadly distributed from the apical side to the middle layers of the epithelium in the  $x$ - $z$  plane (Fig. 3C). These observations thus suggested that hypoxia induced by PMMA lens wear disrupted the localization of ZO-1 in the rabbit corneal epithelium in vivo. Mechanical effects of lens wear might also influence the distribution of ZO-1 through the induction of apoptosis or the desquamation of surface cells in the corneal epithelium.

The expression of ZO-1 protein and the abundance of ZO-1 mRNA in the rabbit corneal epithelium were examined by immunoblot and RT-PCR analyses, respectively. Treatment of eyes with a PMMA lens resulted in significant reductions in the amounts of ZO-1 protein (Fig. 4) and ZO-1 mRNA (Fig. 5) in the corneal epithelium compared with amounts in control eyes. In contrast, application of an RGP lens had no significant effect on



**FIGURE 5.** Effect of contact lens-induced hypoxia on the abundance of ZO-1 mRNA in the rabbit corneal epithelium. (A) Corneal epithelial cells isolated from a control eye or from eyes treated with a PMMA or an RGP lens for 24 hours were subjected to RT-PCR analysis of ZO-1 mRNA. (B) Quantitative analysis of ZO-1 band intensity in gels similar to that shown in (A). Data were normalized by the corresponding G3PDH band intensity and are mean  $\pm$  SE of values from three eyes per group. \* $P < 0.05$  (Dunnett test).



**FIGURE 6.** Effect of contact lens-induced hypoxia on central corneal thickness in the rabbit. Central corneal thickness was measured by ultrasound pachymetry in control eyes or in eyes treated with a PMMA or an RGP lens for 24 hours. Data are mean  $\pm$  SE of values from three eyes per group. \* $P < 0.05$  (Dunnett test).

the amounts of ZO-1 protein (Fig. 4) and ZO-1 mRNA (Fig. 5). These results thus indicated that hypoxia induced by PMMA lens wear triggered downregulation of the expression of ZO-1 at the mRNA and protein levels in the rabbit corneal epithelium *in vivo*.

Finally, to assess the influence of hypoxia on epithelial barrier function, we measured central corneal thickness in the three groups of eyes. Central corneal thickness was significantly increased by 33.3% in eyes that received a PMMA lens compared with that in control eyes, but it was not affected by treatment with an RGP lens (Fig. 6). These data thus suggested that hypoxia induced by PMMA lens wear leads to corneal edema as a result of a loss of epithelial barrier function.

## DISCUSSION

We have now shown that hypoxia disturbs the localization and downregulates the expression of the tight junction component ZO-1 in the rabbit corneal epithelium. These *in vivo* effects of hypoxia were associated with the development of corneal edema. We found that hypoxia resulted in a reduction in the size of cells at the surface of the corneal epithelium but that it did not affect cell density in the corneal endothelium, as revealed by *in vivo* confocal microscopy. These observations suggest that hypoxia promotes desquamation of superficial cells of the corneal epithelium and adversely affects the structure of tight junctions.

An important function of the corneal epithelium is to provide a barrier to the external environment. Tight junctions of the corneal epithelium prevent fluid loss from the cornea and the invasion of potential pathogens. Tight junctions can be disrupted as a result of mechanical trauma,<sup>1</sup> drug toxicity,<sup>23–25</sup> or exposure to lens care products.<sup>26</sup> In addition, hypoxia has been shown to disrupt corneal epithelial barrier function *in vitro*<sup>20</sup> and *in vivo*,<sup>27</sup> especially as a result of the wearing of contact lenses.<sup>28,29</sup> The pattern of ZO-1 staining has been used as an index of tight junction integrity in the corneal epithelium.<sup>13,14,20,30–35</sup> We have now shown that control eyes and eyes treated with an RGP lens exhibited a continuous linear pattern of ZO-1 staining at cell-cell boundaries at the surface of the rabbit corneal epithelium *in vivo*. In contrast, in eyes subjected to hypoxia by the application of a PMMA lens, ZO-1 immunoreactivity was patchy and discontinuous, suggestive of the disruption of tight junctions and the loss of

superficial cells in many areas of the epithelium. Furthermore, hypoxia induced by PMMA lens wear resulted in the downregulation of ZO-1 expression at the mRNA and protein levels. The wearing of a PMMA lens was previously shown to impair the barrier function of the rabbit corneal epithelium.<sup>27</sup> Moreover, changes in the distribution and expression of ZO-1 have been found to affect barrier function in the corneal epithelium.<sup>13,14,30–33</sup> Our observations thus suggest that hypoxia induced by application of a PMMA lens results in an increase in permeability of the corneal epithelium as a consequence of changes in ZO-1 distribution and expression.

Hypoxia-induced changes in permeability in an *in vitro* model of the blood-brain barrier have been shown to correlate with changes in the distribution of tight junction proteins.<sup>36</sup> The patterns of ZO-1, ZO-2, and occludin staining at the cell membrane changed from continuous to discontinuous in response to the switch from normoxia to hypoxia. These findings are consistent with our present results showing that hypoxia induced by the wearing of a PMMA lens disrupted the continuous linear pattern of ZO-1 immunoreactivity at cell-cell boundaries in the rabbit corneal epithelium.

*In vivo* confocal microscopy allows noninvasive assessment of the cornea at the cellular level. Different lesions can be examined during the same examination, and the same lesion can be examined at different time points. Images are available within seconds, and no tissue processing is necessary. Images of healthy and pathologic rabbit corneal epithelia obtained by *in vivo* confocal microscopy in the present study correlated well with results obtained by immunohistologic staining for ZO-1. *In vivo* confocal microscopy thus revealed a significant decrease in the size of cells at the surface of the corneal epithelium in eyes exposed to hypoxia, suggestive of a loss of superficial epithelial cells and the consequent exposure of the smaller, underlying wing cells at the ocular surface. This change was associated with hypoxia-induced disruption of the pattern of ZO-1 staining at the epithelial surface. Others<sup>37</sup> and we<sup>38</sup> previously found that eyes subjected to PMMA lens wear showed a decrease in the size of corneal epithelial cells at the ocular surface compared with control eyes on examination by tandem scanning microscopy. We have now confirmed this finding by *in vivo* confocal microscopy. Furthermore, this confirmation supports the validity of the experimental conditions of the present study.

Contact lenses protect the surface of the cornea from normal shearing forces of the eyelids.<sup>39</sup> Previous studies have found that the size of superficial corneal epithelial cells is increased as a result of contact lens wear in rabbits<sup>38,40</sup> and humans.<sup>41–45</sup> The extent of this increase was found to depend on the oxygen transmissibility of the lens material,<sup>42</sup> as was a delay in lens-induced desquamation of superficial cells.<sup>40</sup> Consistent with these observations, we have now shown that the size of cells at the surface of the rabbit corneal epithelium was decreased after wear of a PMMA lens (low oxygen transmissibility) and was increased after that of an RGP lens (high oxygen transmissibility). Further confirmation of a good correlation between the results of *in vivo* confocal microscopy and those of conventional histology will support the use of the former approach in both the clinical setting and experimental studies.

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