# Interleukin-1β and Barrier Function of Retinal Pigment Epithelial Cells (ARPE-19): Aberrant Expression of Junctional Complex Molecules

*Toshiaki Abe, Eriko Sugano, Yoko Saigo, and Makoto Tamai*

**Purpose.** To examine the effects of interleukin (IL)-1β on the resistance and permeability of the tight junctions of cultured retinal pigment epithelial cells.

**Methods.** A human RPE cell line (ARPE-19) cultured on microporous filter-supports was used. IL-1β and monoclonal anti-IL-1β antibody–treated IL-1β (mAbIL-1β) were added to the standard culture medium. Transepithelial resistance (TER) of confluent RPE cells was measured by epithelial voltmeter. The permeability of the RPE cells to sodium fluorescein, horseradish peroxidase, and inulin was measured. The expression of the occludin and claudin was determined by real-time polymerase chain reaction (PCR), immunohistochemistry, and Western blot analysis.

**Results.** A significantly greater decrease of TER occurred in IL-1β–supplemented medium than in standard medium plus mAbIL-1β after several days of stimulation. A significantly greater increase of sodium fluorescein, horseradish peroxidase, and inulin permeability occurred in IL-1β–supplemented medium than in standard medium. The expression of the occludin gene and some types of claudin genes was observed. The expression of occludin was downregulated and that of claudin-1 upregulated more in IL-1β–supplemented medium than in standard medium by real-time PCR, immunohistochemistry, and Western blot analysis.

**Conclusions.** The tight junctions of ARPE-19 cells are altered by IL-1β supplementation either directly or through other factors activated by IL-1β. The downregulation of occludin and upregulation of claudin-1 may have participated in the dysfunction of the RPE tight junctions in these in vitro experiments. (*Invest Ophthalmol Vis Sci.* 2003;44:4097–4104) DOI:10.1167/iovs.02-0867

Tight junctions are the most apical structures of the junctional complex of epithelial cells.[1–4] They serve as barriers regulating the passage of ions and small molecules through the paracellular pathway. They also serve as a fence to restrict the diffusion of membrane lipids and proteins between the apical and basolateral compartments to maintain cellular polarity.[5,6]

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Retinal pigment epithelial (RPE) cells form an important permeability barrier, the blood–retinal barrier (BRB), between the sensory retina and the choriocapillaris, and they play an important role in maintaining the microenvironment of the sensory retina and the choriocapillaris. Many reversible and irreversible retinal changes result from the disruption of the BRB, such as the retinal thickness, cystoid macular edema, and bullous retinal detachment. The mechanisms for these changes are still undetermined,[7,8] but any dysfunction induced by lesions associated with diseases may lead to photoreceptor degeneration and blindness.

Cytokines are chemical mediators that play important roles in maintaining the physiological condition of an organism and are activated under different pathologic conditions. Many cytokines are present in ocular tissues and in ocular proliferative membranes. Interleukin (IL)-1β has been shown to stimulate several proinflammatory cytokines, such as IL-6 or -8[9] or monocyte chemotactic protein (MCP)-1,[10] and to play an important role during retinal inflammation. IL-1β may also participate in the breakdown of the BRB, especially the endothelial cell barrier of the retinal blood vessels.[11–14]

We hypothesized that IL-1β participates, not only in the induction of several inflammatory cytokines but also in maintaining the integrity of RPE tight junctions. To test this hypothesis, we have examined the effects of IL-1β on the tight junctions of cultured RPE cells. In our experiments, addition of IL-1β to the standard growth medium altered the resistance and permeability of the tight junctions in cultured RPE cells.

## Materials and Methods

### Human RPE Cell Cultures

Human ARPE-19 cells were kindly given to us by Leonard Hjelmeland (Department of Ophthalmology, Section of Molecular and Cellular Biology, University of California, Davis, CA). The cells were passaged and propagated by a slight modification of the technique of Dunn et al.[15] In brief, the cells were cultured in medium (DMEM/F12; Gibco BRL, Grand Island, NY) with 1% (standard medium) or 10% fetal bovine serum (FBS; ThermoTrace, Melbourne, Australia) in a humidified incubator at 37°C in 5% $CO_2$. The medium was changed every 2 to 3 days.

### Measurement of Transepithelial Electrical Resistance

Measurements of transepithelial electrical resistance (TER) were performed by a slight modification of the method reported by Dunn et al.[15] RPE cultures were grown on a microporous filter (Transwell; Costar, Corning Inc., Corning, NY), a permeable membrane treated with an equimolar mixture of type I and III collagen. The membrane was supported by 24-well culture plates. The microporous filter had 0.4 μm pore size and was 6.5 mm in diameter. The cells used in these experiments were between passages 18 and 22. Initially, the culture was started at a concentration of 80,000 RPE cells/well ($4 \times 10^5$ cells/mL) in a medium with 10% FBS at 37°C in 5% $CO_2$. The volume on the apical side was 0.2 mL (inside of the membrane) and 1.0 mL

(outside of the membrane) on the basolateral side. The fluid volume in both chambers was equal.

The measurement of TER was performed daily with the same wells, and the time course of TER development was monitored. Seven days after the culture was started, the medium was changed to medium supplemented with 10 ng/mL recombinant human IL-1$\beta$, which corresponds to approximately $1 \times 10^3$ U/mL (PepcoTech House, London, UK) for the TER experiments. The concentration of the IL-1$\beta$ was based on the report of Bamforth et al.,[16] who used a single dose of 1 to $2 \times 10^3$ U of murine recombinant IL-1 $\beta$ in the vitreous cavity of rats.

We also measured the TER in the IL-1$\beta$–supplemented medium after treating the medium with monoclonal anti-IL-1$\beta$ antibody (mAbIL-1$\beta$; 10 $\mu$g/mL, immunized with purified *Escherichia coli*–derived recombinant human IL-1$\beta$; R&D System, Inc., Minneapolis, MN). When the anti-IL-1$\beta$ antibody was used, the medium supplemented with IL-1$\beta$ was incubated with mAbIL-1$\beta$ (10 $\mu$g/mL) before the experiments were started.

The TER was measured using an epithelial voltmeter (EVOM; World Precision Instruments, Sarasota, FL) according to the manufacturer's instructions. The cells were taken from the incubator and placed at room temperature (RT) for 30 minutes of equilibration before the experiments. The TER (in ohms per square centimeter) of the filter alone was measured as background and subtracted from the TERs obtained with the filters and the RPE cells. Measurements were repeated at least six times for each well, and each experiment was repeated on at least four different wells.

## Permeability Assay

The permeability of the RPE cells was determined by measuring the apical-to-basolateral movements of sodium fluorescein (fluorescein dye; 376 Da), horseradish peroxidase (HRP; 40 kDa), and HRP-labeled inulin (5 kDa; Sigma-Aldrich, Poole, UK). The test molecules were added to the apical compartment of the cells and the concentrations of the added tracers were 25 $\mu$g/mL (fluorescein dye), 50 $\mu$g/mL (HRP), and 1000 $\mu$g/mL (inulin). Sixteen days after culturing began, the tracers were added to the media, and 20 $\mu$L of the sodium fluorescein-treated wells or 50 $\mu$L from the HRP-treated and the HRP-labeled inulin-treated wells were collected from the basolateral side at 5, 10, and 30 minutes after adding the molecules. The same volume of the appropriate medium was added to replace the medium removed.

A minimum of four cultures was used for each time measurement. The concentration of sodium fluorescein was quantified by spectrophotometry (SpectraMax Gemini $V_{max}$; Molecular Devices, Sunnyvale, CA) using a standard curve. The concentrations of HRP and HRP-labeled inulin were determined in 20-$\mu$L aliquots added to 150 $\mu$L of freshly made substrate (*o*-phenylenediamine, 400 $\mu$g/mL in 0.05 M citric acid and 0.1 M phosphate, with 0.012% hydrogen peroxidase [pH 5.0]) by spectrophotometry (SpectraMax Gemini $UV_{max}$; Molecular Devices) using a standard curve. The concentration in the standard medium was used as the background concentration in each experiment. Each experiment was repeated at least three times.

## Cell Viability Test and Apoptotic Cell Staining

We performed a cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) proliferation assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI) after 8 and 16 days.[17] For this, 40 $\mu$L of one solution reagent was added to the medium (200 $\mu$L), followed for 1 hour at 37°C in humidified 5% $CO_2$ atmosphere. We recorded the absorbance at 490 nm by spectrophotometer (SpectraMax Gemini$UV_{max}$; Molecular Devices).

We also used Hoechst 33552 (bisbenzimide H33342 fluorochrome, trihydrochloride (HOE33342:2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2, 5′-bi-1H-benzimidazole HCl; Calbiochem, San Diego, CA) for apoptotic cell staining, using the same cell condition as for the MTS assay. On the indicated days, the membrane was washed with phosphate-buffered saline (PBS) and incubated with 5 $\mu$g/mL Hoechst 33552 for 15 minutes at room temperature. After the membrane was washed, the cells were fixed with 2% paraformaldehyde and photographed by fluorescein microscopy (DMIRE; Leica Microsystems Imaging Solutions, Ltd., Cambridge, UK).

## Extraction of mRNA, cDNA Generation, Reverse Transcriptase–Polymerase Chain Reaction, Sequencing, and Real-Time PCR

mRNA was extracted from each cell culture with oligo dT cellulose, and cDNA was generated according to the manufacturer's instruction (Pharmacia Biotech Inc., Uppsala, Sweden). PCR was performed in 50 $\mu$L of reaction mixture as reported[18] (reaction cycles were 30 or 35). The annealing temperatures depended on each primer sets for 2 minutes (Table 1).

Some of the amplified PCR products were subsequently subcloned into a T-vector[19] (Promega). Subcloned PCR products were then sequenced with an automatic DNA sequencer (LKB ALF; Pharmacia) using a dideoxy chain termination protocol.[20] Real-time PCR was also performed according to the manufacturer's instructions. The PCR products were quantified by green nucleic acid gel stain (SYBR Green I; BMA, Rockland, ME)[21,22] and a fluorescein detection–specific thermal cycler (Smart Cycler; TaKaRa, Kyoto, Japan). We examined the gene expression of occludin; claudin-1, -11, and -12; and $\beta$-actin. Optimal conditions for each gene amplification were: 3.5 mM $MgCl_2$, 94°C for 30 seconds, 1 cycle; 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds, 86°C for 10 seconds, 35 cycles (for $\beta$-actin), 4.0 mM $MgCl_2$, 94°C for 30 seconds, 1 cycle; 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds, 84°C for 10 seconds, 35 cycles (for occludin and claudin-11 and -12), and 3.5 mM $MgCl_2$, 94°C for 30 seconds, 1 cycle; 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and 84°C for 10 seconds, 35 cycles (for claudin-1). After amplification, the PCR products were confirmed by melting-curve analysis and gel electrophoresis.[23]

## Primer Preparations

The primers used for occludin,[24] claudin-1 to -18,[25–32] and $\beta$-actin[18] are listed in Table 1. We did not perform RT-PCR on claudin-13.

## Immunohistochemistry

The formation of tight junctions in ARPE-19 cells was also examined by immunohistochemistry by a slight modification of the methods of Holtkamp et al.[9] On the indicated days, filters were washed with PBS two times and fixed with 4% paraformaldehyde for 10 minutes, followed two washes with PBS. The filters were permeabilized by 0.2% Triton X-100 in PBS for 20 minutes and blocked with 10% goat serum at RT for 30 minutes. Rabbit anti-occludin or claudin-1 antibodies (Zymed Laboratories Inc., San Francisco, CA) were incubated overnight at 4°C. The filters were washed with PBS and further incubated with rhodamine-conjugated anti-rabbit IgG (Sigma-Aldrich) for 30 minutes at RT. Photographs were taken by fluorescein microscopy (DMIRE2; Leica Microsystems Imaging Solutions, Ltd.).

## Western Blot Analysis

Western blot analysis was performed for occludin and claudin-1. In brief, the cells cultured on the microporous membranes were harvested by the methods described earlier, and 20 $\mu$g of each sample (SDS sample buffer) was applied to an SDS-10% polyacrylamide gel. Incubation with rabbit anti-occludin antibody (2 $\mu$g/mL) or rabbit anti-claudin-1 antibody (2 $\mu$g/mL) was performed at 4°C overnight. After gels were washing with Tween-PBS, alkaline phosphatase–conjugated goat anti-rabbit IgG was applied and processed according to the manufacturer's instruction for the chemiluminescent substrate kit (Bio-Rad, Herts, UK) and exposed to autoradiograph film (XAR; Eastman Kodak, Rochester, NY).

**TABLE 1.** Primer Sequences

| Gene | Sequence | Size (bp) | Annealing Temperature |
|---|---|---|---|
| Occludin | 5′-TGCATGTTCGACCAATGC-3′<br>5′-AAGCCACTTCCTCCATAAGG-3′ | 235 | 55 |
| Claudin-1 | 5′-GATGAGGTGCAGAAGATGAGG-3′<br>5′-AGAAGGCAGAGAGAAGCAGC-3′ | 200 | 57 |
| Claudin-2 | 5′-AAGACTGTGCATCTCATGCC-3′<br>5′-AGCATTGTGACAGCAGTTGG-3′ | 206 | 55 |
| Claudin-3 | 5′-TCACGTCGCAGAACATCTGG-3′<br>5′-GATGGTGATCTTGGCCTTGG-3′ | 236 | 57 |
| Claudin-4 | 5′-GATGCAGTGCAAGGTGTACG-3′<br>5′-CACTATCACCATAAGGCCGG-3′ | 220 | 57 |
| Claudin-5 | 5′-TTCGCCAACATTGTCGTCC-3′<br>5′-TCTTCTTGTCGTAGTCGCCG-3′ | 232 | 56 |
| Claudin-6 | 5′-GATGCAGTGCAAGGTGTACG-3′<br>5′-CCTTGGAATCCTTCTCCTCC-3′ | 161 | 57 |
| Claudin-7 | 5′-AATGTACGACTCGGTGCTCG-3′<br>5′-AATCTGATGGCCATACCAGG-3′ | 238 | 56 |
| Claudin-8 | 5′-GAAGGACTGTGGATGAATTGC-3′<br>5′-GATGAAGATGATTCCAGCCG-3′ | 240 | 55 |
| Claudin-9 | 5′-ACTATGTGTGAGGCTGAGGC-3′<br>5′-GGTTGCAACCACTACAGAGG-3′ | 182 | 57 |
| Claudin-10 | 5′-CTCCAACTGCAAGGACTTCC-3′<br>5′-ATCCAGTCATTGAGCACAGC-3′ | 224 | 55 |
| Claudin-11 | 5′-TGGTGGACATCCTCATCC-3′<br>5′-AGAGAGCCAGCAGAATGAGC-3′ | 195 | 55 |
| Claudin-12 | 5′-TTCCTTCCTGTGTGGAATCG-3′<br>5′-GTTGCACATTCCAATCAGGC-3′ | 295 | 55 |
| Claudin-14 | 5′-TTGATCACCACCATCCTGC-3′<br>5′-ACACACACTCCATCCAGAGC-3′ | 103 | 55 |
| Claudin-15 | 5′-GCTCATTCCTCTGACCTGCC-3′<br>5′-ACAGTTGCCATGAAGAAGCC-3′ | 270 | 57 |
| Claudin-16 | 5′-CATGTTGCCATCCTGATGG-3′<br>5′-TCGTACTCATCACAGGTGCG-3′ | 226 | 55 |
| Claudin-17 | 5′-TGCTTATTGGCATCTGTGGC-3′<br>5′-TTCTGACCTATGTGGATGGC-3′ | 191 | 55 |
| Claudin-18 | 5′-GATGATCGTAGGCATCGTCC-3′<br>5′-ATGCCGGTGTACATGTTAGC-3′ | 228 | 55 |
| β-Actin | 5′-CTACAATGAGCTGCGTGTGG-3′<br>5′-CGGTGAGGATCTTCATGAGG-3′ | 313 | 55 |

## Statistical Analysis

Multivariate analysis was performed using the Kruskal-Wallis and post hoc tests. $P < 0.05$ was considered significant.

## RESULTS

The TER of cells grown in the standard medium, medium with mAbIL-1β, and medium with 10% FBS increased with longer culture times (Fig. 1). However, the TERs fluctuated somewhat on the first 2 to 3 days after changing the medium from 10% FBS to each of the test media. The TER appeared to be somewhat affected by the serum, as reported previously.[33]

The TER in the medium with 10% FBS (Fig. 1, open circles) were significantly lower than the TER in mAbIL-1β (Fig. 1, *) and standard medium (Fig. 1, +) on the indicated days. In contrast, there was a gradual decrease of the TER in cells grown in standard medium supplemented with IL-1β. The TER in the IL-1β–supplemented medium became significantly lower than the TERs of cells grown in mAbIL-1β, medium with 10% FBS, and in standard medium on the indicated days (Fig. 1, §; $n = 12$ in each experiment).

The permeability assays were performed with cells cultured for 16 days under the same conditions as those used for the TER measurements (Fig. 2). The concentration of fluorescein dye in the basal compartment increased linearly. The concentration of fluorescein dye in the IL-1β–supplemented medium at 10 minutes after adding the test molecules was significantly higher than in the standard medium. Similar findings were made at 30 minutes (Fig. 2a).

The concentration of HRP in the basolateral compartment was also significantly higher in the IL-1β–supplemented medium than in the standard medium at 10 minutes after adding the test molecules. At 30 minutes, the concentration was significantly higher in the IL-1β–supplemented medium than in the standard medium (Fig. 2b). A statistically significant increase of inulin was also observed in the basolateral chamber in the IL-1β–supplemented medium than in standard medium at 30 minutes after adding the molecule (Fig. 2c). The concentration of fluorescein dye recovered to the same level as that in the standard medium 24 hours after replacing the IL-1β–supplemented medium with the standard medium (Fig. 2d).

The MTS assay showed that the proliferation and viability of cells in the standard medium did not differ significantly in the IL-1β–supplemented medium on the indicated days. No significant difference was observed on the average absorbance at 490 nm between standard medium and IL-1β–supplemented medium on days 8 and 16 (Fig. 3a; $n = 4$). We also found no obvious apoptotic cells in both the standard medium (Fig. 3b) and in the IL-1β–supplemented medium (Fig. 3c), when using Hoechst 33552. The positive control for apoptotic RPE cells (Fig. 3d, arrows) cultured on normal culture plate exposed to hypoxia (1% $O_2$) and no serum for 7 days are shown in Figure 3d.

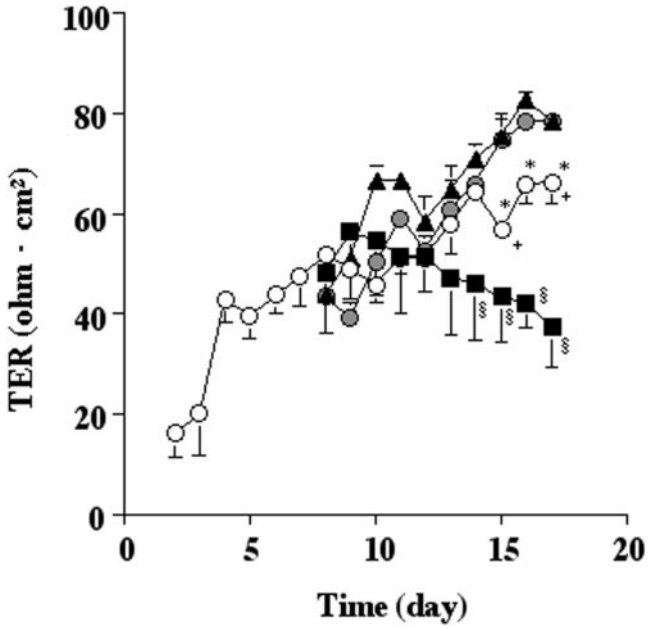


**FIGURE 1.** TERs in media with or without IL-1$\beta$ supplementation. Vertical axis is the TER, and the horizontal axis is the time. Significant differences are shown by *, +, and §. Error bars, SD. (■) IL-1 $\beta$; (▲) mAbIL-1 $\beta$; (●) standard medium; and (○) standard medium with 10% FBS.

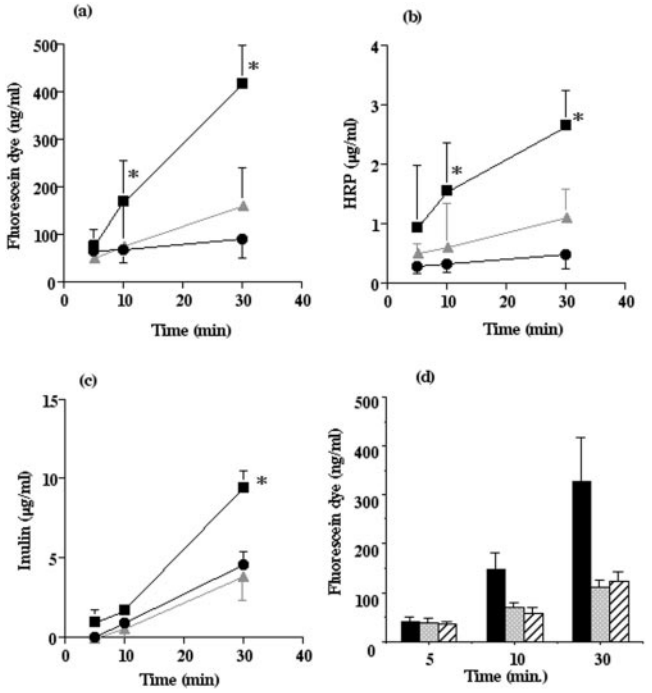


**FIGURE 2.** Results of the permeability assay showing the permeability of sodium fluorescein (**a**), HRP (**b**), and inulin (**c**). Vertical axes are concentrations of each molecule, and horizontal axes are time after addition of the molecules. Symbols for each medium are the same as in Figure 1. (**d**) Permeability of fluorescein dye after the medium was changed from IL-1$\beta$ to standard medium 24 hours before addition of the molecule. Vertical axis shows the concentration of fluorescein dye, and horizontal axis shows the time after addition of the molecule. (■) Permeability in IL-1$\beta$'3b ('3c1138'3e) permeability after change of medium from IL-1$\beta$–supplemented to standard medium; (▨) standard medium. *Statistically significant differences.

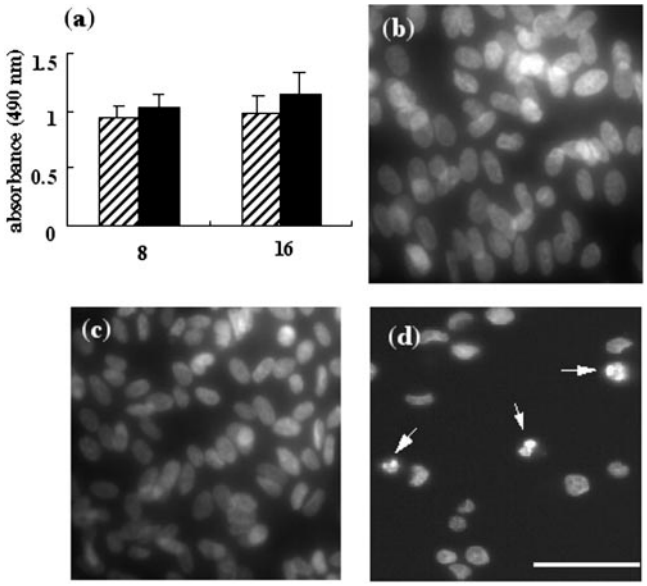


**FIGURE 3.** Results of proliferation assay (**a**) and apoptotic cell staining (**b**, **c**, **d**). No statistical significance was observed between standard medium and standard medium with IL-1$\beta$ on days 8 and 16 by proliferation assay. No obvious apoptotic cell was observed in both the standard medium (**b**) and in standard medium with IL-1$\beta$ (**c**). The data in (**d**) represent the positive control of apoptotic RPE cells. Bar, 50 $\mu$m.

RT-PCR demonstrated that both the occludin and at least seven claudin genes were expressed in the ARPE-19 cells when they were cultured on microporous filter membranes (Fig. 4).

Sequence analysis of the PCR products of occludin and the claudins (claudin-1, -11, and -12) revealed that the nucleotides in the PCR products completely matched the originally reported ones.[24,25]

The expression levels of occludin and claudin-1, -11, and -12 in the IL-1$\beta$–supplemented medium are presented as the ratio of the density of the gene to that of the internal standard ($\beta$-actin) in standard medium (mean ± SD; $n = 4$). The claudin-1 gene expression was upregulated in the IL-1$\beta$ medium, and conversely, the expression of occludin gene was downregulated (Fig. 5). The expression of claudin-11 and -12 genes was slightly lower in the IL-1$\beta$–supplemented medium than in the standard medium (Fig. 5a).

The results of RT-PCR on day 12 and on day 16 for the occludin (Fig. 5b) and claudin-1 (Fig. 5c) genes are shown in Figure 5. The lower panel for each gene shows the band of $\beta$-actin. These results demonstrated that the expression of occludin gene was lower in the IL-1$\beta$–supplemented medium than in the standard medium at 12 and 16 days. Conversely, the expression of claudin-1 gene was upregulated in the IL-1$\beta$ medium than in the standard medium at 12 and 16 days.

The results of immunohistochemistry after 16 days of culture on both occludin and claudin-1 are shown in Figure 6. Junctional staining of each peptide was observed in media with or without IL-1$\beta$. However, the staining of occludin in the medium with IL-1$\beta$ (Fig. 6b) appeared to be somewhat less

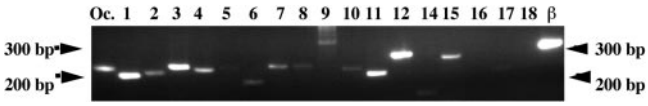


**FIGURE 4.** Results of RT-PCR for occludin and claudin genes in ARPE-19 cells. Oc, occludin gene, the number *above* the lane is the number of the claudin gene (claudin-13 was not tested). The size of each product is shown in Table 1.

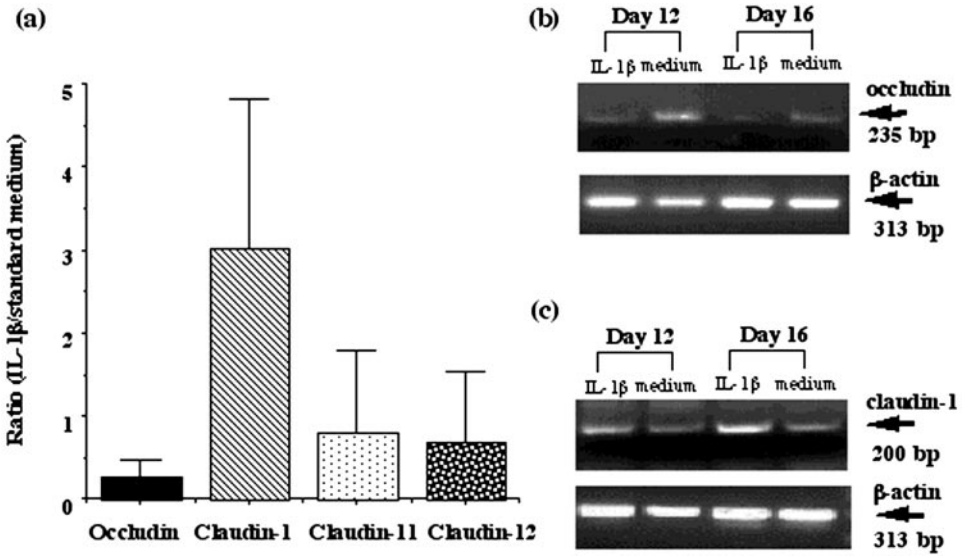


**FIGURE 5.** Results of real-time PCR (**a**) and RT-PCR (**b**, **c**). Vertical axis (**a**) is the relative expression level of each gene in the IL-1β medium against the standard medium. The expression level of each gene in the medium with IL-1β is indicated as the ratio to the internal standard (β-actin). The results are the mean ± SD. The genes examined are shown on the *x*-axis. The data shown in (**b**) and (**c**) are the results at 12 and 16 days, respectively, after the start of the experiments in medium with or without IL-1β; M, marker of 100 base pairs. *Arrows*: size of the PCR products of occludin (**b**), claudin-1 (**c**), and β-actin (**b**, **c**).

than that in the standard medium (Fig. 6a). Conversely, the staining of caludin-1 in the medium with IL-1β (Fig. 6d) appeared to be stronger than that in the standard medium (Fig. 6c).

For Western blot analysis, samples were taken from 12- and 16-day-old cultures with or without IL-1β supplementation. Bands at approximately 60-kDa (Fig. 7a; occludin) and at approximately 22-kDa (Fig. 7b; claudin-1) were found. The expression of occludin was lower in the IL-1β–supplemented medium than in the standard medium (Fig. 7a). Conversely, the claudin-1 expression in the IL-1β medium was upregulated when compared with that in standard medium (Fig. 7b). These results agree with the observations made with real-time PCR and RT-PCR.

## DISCUSSION

ARPE-19 is a human RPE cell line with differentiated properties and exhibits morphologic polarization when cultured as a monolayer on microporous filter supports.[15] Under these conditions, the tight junctions of the RPE cells lead to a maximum TER of 50 to 100 [ohm]/cm$^2$. Zech et al.[34] reported a mean TER of 67.8 ± 18.8 [ohm]/cm$^2$ in rat RPE cells, and the TER of our ARPE-19 cells grown on microporous membranes was slightly higher at 70 to 80 [ohm]/cm$^2$. This TER was somewhat lower than that originally reported by Dunn et al.,[15] but they reported that lower-passage cells have lower TERs. Our observations agree with their findings, as we found that the TER of ARPE-19 cells at lower passages (18–22 passages) was higher

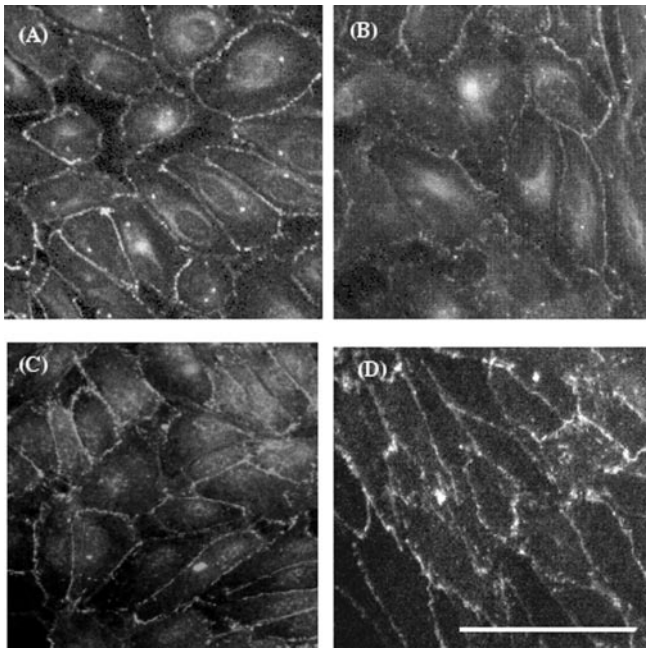


**FIGURE 6.** The results of immunohistochemistry for occludin and claudin-1 16 days after start of culturing. (**a**) Results of anti-occludin in ARPE-19 cells in the standard medium. (**b**) Anti-occludin in the IL-1β–supplemented medium. (**c**) anti-claudin-1 in the standard medium; (**d**) anti-claudin-1 in IL-1β–supplemented medium. Bar, 50 μm.

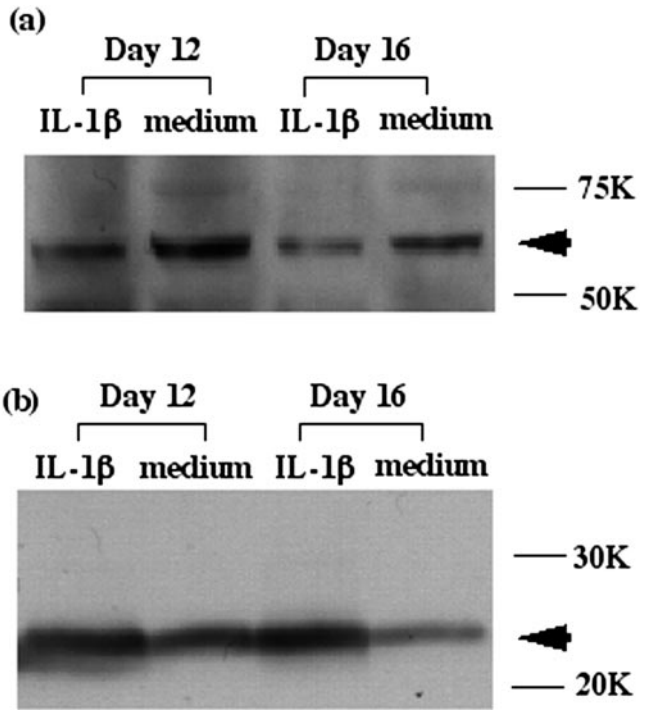


**FIGURE 7.** Results of Western blot analysis. (**a**) Results of occludin and (**b**) results of claudin-1. *Arrows*: predicted size of each molecule.

than that of cells of more than 34 passages (data not shown). The decrease of TER in IL-1β–supplemented medium was also greater in lower-passage ARPE-19 cells.

The cell proliferation rate was not significantly different in standard medium and in IL-1β–supplemented medium. We also used Hoechst 33552 to test for viable and apoptotic cell staining and found no significant differences between the cells cultured in medium with or without IL-1β. These results suggest that the significant difference of the TER in the medium with IL-1β was not due to changes in cell proliferation or damage but was due to the effect of the IL-1β on the cells, although the changes did not occur until several days after stimulation.

IL-1β is a proinflammatory cytokine stimulating the expression of IL-6 and -8[9] and MPC-1[10] and may play an important protective role against retinal inflammation.[14] IL-1β may also be involved in the breakdown of the BRB. Retinal photocoagulation[35] and experimental autoimmune uveitis (EAU)[12] also cause a breakdown of the BRB through cytokines such as IL-1β. EAU, induced in rats by immunization with S-antigen, is accompanied by a breakdown of the BRB and albumin extravasation from both the retinal vessel endothelial and RPE cells.[12] This inflammatory response is reversible.[13] In our in vitro model, the TER gradually decreased in IL-1β–supplemented medium, suggesting a breakdown of the tight junctions of the RPE as was observed in the endothelial cells of the retina during EAU. The higher permeability to different size tracer molecules in IL-1β–supplemented medium also strongly suggests a breakdown of the tight junctions by IL-1β. As in EAU, the change in permeability to fluorescein dye was reversible.

Rapid reversible changes of TER have been reported in cultured rat RPE cells.[33] When the medium was changed from serum-containing to serum-free, hormonally defined medium (HDM), the TER increased rapidly. Conversely, when the medium was changed from HDM to HDM with serum, the flux of both fluorescein dye and HRP also increased significantly in rat RPE cultures.[33] The early change in TER was thought to be due to some molecules in the serum. These results also mirrored our results, in that the increased permeability of fluorescein dye was returned to almost the control level when the medium was returned to standard medium. The molecules stimulated by IL-1β, such as MCP-1, may mediate these effects. Investigation of the conditioned medium may reveal the mechanism of the effect.

It has been reported that HRP does not leak across the RPE when IL-1β is injected into the vitreous.[36] However, we found an increase in the permeability to HRP in cells cultured with standard medium supplemented with IL-1β. Although our in vitro system used a human RPE cell line with partially differentiated properties, the TER was lower than that in vivo.[15] The TERs of cultured RPE reported previously were also fairly low compared with other epithelial cell lines.[3] These properties may have influenced the effects of IL-1β on the TER of cultured ARPE-19 cells. We also used relatively early-passage cells than Dunn et al.[15] used. The younger passaged cells may show higher TER and greater resistance to IL-1β. However, as discussed by Bamforth et al., the concentration of the IL-1β in the vitreous in their in vivo experiments may not have been high enough to break down the tight junctions of the RPE.[36] Also, the stimulation by IL-1β in their experiment was not continuous as in our in vitro model.

Zech et al.[34] reported that injection of lipopolysaccharides with interferon-γ and tumor necrosis factor-α decreased the TER of rat RPE tight junctions in vitro. Thus, RPE cells may respond to several cytokines and produce leaky RPE tight junctions and may be related to several ocular diseases.[37] However, the events we report herein occurred several days after IL-1β treatment. Thus, our results may not be the direct effect of IL-1β but of factors activated during the many intervening steps, such as an upregulation of other cytokines.

Occludin and claudins are two integral membrane components that form tight junctions especially in monolayers of epithelium-derived tissues.[38,39] We showed that these molecules were expressed in the RPE cell lines studied. As reported,[3,4,40,41] junctional staining for occludin and claudin-1 was also found in these cells, although the expression level and pattern may be different in the medium with or without IL-1β. These different molecules may participate in the generation of the RPE-specific tight junctions by rearrangements into monophilic or heterophilic strands with the other strands in the tight junction.[42]

Occludin has been shown to be exclusively localized in tight junction strands in various types of epithelial cells[43] and to be involved not only in the barrier but also in the fence functions of tight junctions.[44] Our results showed that the expression of occludin was lower in the medium supplemented with IL-1β than in standard medium by real-time PCR, immunohistochemistry, and Western blot analysis. The occludins between adjacent cells have been reported to interact with each other in a monophilic manner[44] and not to interact with caludin-1 in a heterophilic manner.[42] The paired organization of ARPE-19 in tight junctions therefore may be influenced by the downregulation of occludin. This may be one of the reasons why the downregulation of occludin affected the ARPE-19 cellular tight junctions.

In addition, Chen et al.[40] reported that inhibition of mitogen-activated protein kinase (MAPK) stabilizes occludin by increasing the half-life and tyrosine phosphorylation of occludin, but not claudin-1 in Ras-transformed Madin-Darby canine kidney cells. In contrast, several investigators have reported that IL-1β stimulates MAPK expression in several types of cells[45,46] including RPE cells.[47] From these reports, we can speculate that treatment of ARPE-19 cells by IL-1β may induce MAPK expression and thus influence the stability of occludin, which may then slowly alter the tight junction function of ARPE-19 cells. However, other factors still to be determined are probably activated that influence the expression of these tight junction molecules.

The claudins are part of a multigene family,[48] and more than two distinct claudins are coexpressed in single cells.[49] The expression of claudin-1 seemed to be stimulated by IL-1β, although the expression of claudin-11 and -12 genes was almost at the same level or slightly lower than that in the standard medium. These results appear to be contradictory to the decreased TER and increased permeability in the medium with IL-1β. The TER of ARPE-19 cells in the IL-1β–supplemented medium may be affected by the balance of the function between occludin and claudin-1. The decrease of TER by the downregulation of occludin may be greater than that of the upregulation of claudin-1. The upset in the balance may also generate the gradual decrease of barrier function in ARPE-19 cells.

However, the increased expression of the claudin-1 gene may not always be associated with increased TER in tight junctions. It is generally accepted that claudins in the tight junctions span laterally to associate with other strands in the tight junctions from apposing membranes to form paired strands that can be subclassified into four models. Thus, the homopolymer in one strand was associated laterally in a homophilic or heterophilic manner to the other strand.[30] Unlike occludin, most claudins in the tight junction have been reported to be composed of heteropolymers in one strand associated laterally, in both a homophilic and heterophilic manner, with the other strand.[42] The organization is suspected to be one of the reasons why permeability and ion selectivity of each tight junction varies from the others, depending on the type of

epithelial cell. As shown, the tight junction in ARPE-19 cells was thought to include at least several claudins. Therefore, these claudins may be arranged heterogeneously in one strand and may show monophilic or heterophilic association in the tight junctions. In spite of almost the same expression level of claudin-11 and -12, the upregulation of claudin-1 in IL-1β–supplemented medium (Fig. 5) may change not only the distribution in one strand, but also the paired organization of ARPE-19 cells and lead to changes of the ARPE-19–specific tight junction function.

In summary, the tight junctions of ARPE-19 cells are altered by IL-1β supplementation, although the intervening steps are probably mediated by other factors. The aberrant expression of the tight junction molecules may have participated in the dysfunction of the RPE tight junctions in these in vitro experiments.

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