Retinal Pigment Epithelial Cells From Dystrophic Rats Form Normal Tight Junctions In Vitro

Chih-Wei Chang,* Dennis M. Defoe,† and Ruth B. Caldwell*

Purpose. In the genetically defective Royal College of Surgeons (RCS) rat model for retinal degeneration, a breakdown occurs in the retinal pigment epithelial (RPE) cell tight junctions just as the photoreceptors begin to degenerate. These experiments sought to determine the impact of the RPE genetic defect on this alteration in the RPE cell tight junctions.

Methods. Retinal pigment epithelial cell cultures prepared from RCS and control rats were treated with hormonally defined medium (HDM), base medium conditioned by RCS or control retinas, or unconditioned base medium. The tight junctions formed by these cultures were assayed functionally by measuring transepithelial electrical resistance and permeability. Junction structure was evaluated by immunolocalization of the tight junction protein zonula occludens 1 and of the junction-associated actin microfilaments.

Results. Retinal pigment epithelial cultures from dystrophic rats formed structurally and functionally normal tight junctions when maintained in hormonally defined medium. The junctions remained stable when the medium bathing the apical surface was switched to base medium preconditioned by normal retinas. In contrast, cultures treated with medium preconditioned by degenerating dystrophic retinas or with unconditioned medium exhibited a breakdown in their tight junctions.

Conclusions. Retinal pigment epithelial cells isolated from dystrophic RCS rats can form tight junctions normally in vitro. Normal, but not dystrophic, retinas release factors that support RPE tight junctions. Therefore, the junctional abnormality seen in dystrophic rat RPE cells in vivo is probably caused by the loss of trophic factors normally provided by the healthy neural retina rather than by a direct effect of the genetic defect on the tight junctions. Invest Ophthalmol Vis Sci. 1997;38:188-195.
i.e., the RPE tight junctions are intrinsically defective. The second is that the RPE tight junctions are normal but become altered because of changes that occur in the surrounding environment secondary to the dystrophic process.

In this study, we demonstrate that RPE cells from RCS rats form tight junctions in vitro that are structurally and functionally indistinguishable from those formed by normal RPE cells. We also show that medium conditioned by the healthy neural retinas of normal rats contains factors that maintain RPE cell tight junctions, whereas unconditioned medium or medium conditioned by degenerating dystrophic retinas fails to support RPE tight junctions. These observations support the hypothesis that the RPE cell tight junction alterations in RCS rats occur secondarily to the photoreceptor degeneration and are not caused by direct effects of the mutation on the tight junctions.

MATERIALS AND METHODS

Cell Culture

Retinal pigment epithelial cell cultures were prepared from 6- to 8-day-old rat retinas as described previously. The rats were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Retinas were obtained from breeding colonies of pink-eyed and black-eyed dystrophic rats, congenic controls for RCS and Long Evans rats. Retinal pigment epithelial cells were dissociated into single cells and small clusters and were plated into 24-well Transwells (0.4 μM pore size; COSTAR, Cambridge, MA) that had been coated with matrigel (1:1 ratio in NCTC-135 medium; Collaborative Research, Bedford, MA). They were seeded at the density of 2 × 10⁵ cells/cm² and were maintained in hormonally defined medium (HDM) prepared using NCTC-135 base medium supplemented with growth factors, proteinase inhibitors, and antibiotics as described.

In some experiments, cells were plated initially in plastic dishes, amplified by culture for 3 to 5 days in DMEM with 10% fetal bovine serum, and harvested for the preparation of Transwells as described.

Conditioned Medium Preparation

Conditioned medium was prepared using the method described by Rizzolo and Li. Briefly, neural retinas from 1-month-old dystrophic or control rats were placed in NCTC base medium that contained 50 mM vitamin C, 1 mM glutathione, 1% bovine serum albumin, 20 mM Hepes, and 2% penicillin–streptomycin (two retinas per milliliter). After incubation for 6 hours at 37°C under 95% O₂ and 5% CO₂, retinas were discarded and the conditioned media were collected, filtered, and stored at −70°C. For treatment of cultures, conditioned medium was added to the apical compartment of the Transwell, and hormonally defined medium was added to the basal compartment. The volume of medium in the apical (200 μl) and basal compartments (800 μl) was adjusted to avoid hydrostatic pressure differences. Medium in the apical compartment was changed every other day; that in the basal compartment was changed every day.

Lactate Dehydrogenase Activity

Lactate dehydrogenase (LDH) was assayed using a kinetic ultraviolet light method. Samples (100 μl) were mixed with 2.5 ml of phosphate buffer containing 16.2 mM pyruvate and 0.194 mM nicotinamide–adenine dinucleotide at 25°C. Generation of NAD⁺ was read at 340 nm after 30 seconds and was monitored at 1-minute intervals for 3 minutes after the initial reading. One unit of LDH activity is defined as the amount of enzyme that catalyzes the formation of 1 mM/1 of NAD⁺/minute.

Transepithelial Electrical Resistance

An Epithelial Voltohmeter (World Precision Instruments, Sarasota, FL) with STXII electrodes was used to measure RPE cell transepithelial electrical resistance (TER). Values for TER for the filters alone, without cells, were measured as background and subtracted from values for RPE cultures; TER was expressed as ohm · cm².

Permeability Assay

Sodium fluorescein (376 Da) and horseradish peroxidase (40 kDa) were used as permeability tracers. Tracers (25 μg SF/ml and 50 μg HRP/ml) were constituted in HDM, added to the apical compartments alone or in combination, and incubated with the cultures at 37°C with 5% CO₂ for consecutive intervals of 2 to 60 minutes. The amount of tracer that crossed the monolayer to reach the basal compartments was determined for each time point. Sodium fluorescein was read directly in a CytoFluor II Microplate Fluorescence Reader (Millipore, Marlborough, MA). Horseradish peroxidase concentration was determined by the reaction of 20-μl samples with 150 μl of freshly made substrate [o-phenylene-diamine 400 μg/ml in 0.05 M citric acid, 0.1 M phosphate, and 0.012% H₂O₂, pH 5]. The peroxidase reaction was terminated by the addition of 50 μl 0.25 M H₂SO₄, and the product was read in a 7520 Microplate reader (Cambridge Technology, Watertown, MA). The tracer concentration for each sample was calculated by comparison with standard curves prepared using tracer samples from time 0.

Immunocytochemistry

Retinal pigment epithelial cultures were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 10 minutes at room temperature. For ZO-1 labeling,
Tight Junctions of Retinal Pigment Epithelial Cells From Dystrophic Retinas Form Normally In Vitro

To test whether the increased tight junction permeability observed in dystrophic rat RPE cells in vivo could be a direct effect of the genetic RPE cell defect, cultures of normal and dystrophic RPE cells were analyzed in parallel. Tight junction formation was examined by measuring TER and by permeability assay. One day after cell plating, TER was measured, and this continued for 6 days. After the last TER reading, permeability was assayed. Data showed that TER levels comparable to those in normal RPE cells developed in RPE cells from the mutant retina (Fig. 1A). Permeability assay showed that mutant RPE cells, like normal RPE cells, could restrict the passage of sodium fluorescein (Fig. 1B) or horseradish peroxidase (Fig. 1C). We used first-passage cultures for this analysis so the cultures could be prepared simultaneously using the same batches of media, tracers, and other reagents. This was done because our initial studies showed that cultures prepared from freshly isolated RPE cells exhibited slight differences in maximum TER levels reached because of slight variations in cell attachment and survival after trypsinization. In other experiments using different batches of fresh RPE isolates, absolute TER levels, ranging between 200 and 300 ohm·cm², were higher than those shown in Figure 1. In fresh RPE isolates from RCS retinas, TER levels were sometimes a little higher, sometimes a little lower, than in controls. No significant differences were detected between the two strains.

Dystrophic RPE cells also were normal morphologically as indicated by the localization of junction-associated proteins. Phalloidin labeling showed actin concentrated apicod laterally in a circumferential microfilament bundle that highlighted the junctional complex zone (Fig. 2). No differences between the dystrophic and control rat strains were evident in terms of their junctional morphology, cell shape, or cell size, nor did immunolabeling for ZO-1 show any differences between dystrophic and control RPE cells (data not shown).

Medium Conditioned by Normal Retinas Supports the Maintenance of Retinal Pigment Epithelial Tight Junctions In Vitro

Next we tested whether the RPE tight junctions in the dystrophic retina become altered because of changes in the cellular milieu that occur secondarily to retinal degeneration. We reasoned that the tight junctions might be affected adversely, either by negative factors released by the degenerating retina or by the loss of positive factors normally released by the healthy retina. To test these possibilities, media preconditioned by normal or dystro-
Retinal Pigment Epithelial Tight Junctions of RCS Rat

FIGURE 2. Cellular morphology of dystrophic retinal pigment epithelial (RPE) cells appears normal in vitro. Cultures prepared using RPE cells isolated from normal (A) or dystrophic (B) retinas were maintained in hormonally defined medium for 6 days and were stained with phalloidin. Actin microfilaments are distributed peripherally at the region of the cell-cell junctional complex. Magnification, x275.

The pattern of the RPE cultures' responses to the different media treatments was highly consistent between different batches of primary RPE cultures. Results showed that when HDM in the apical compartments was replaced by medium preconditioned by normal retinas, the cultures prepared from either normal (Fig. 3A) or dystrophic (Fig. 3B) rats could maintain their TER levels for up to 5 days (analysis of variance [ANOVA] showed no significant decline in TER measures between successive days of treatment). In contrast, when the apical compartment HDM was replaced with medium preconditioned by dystrophic retinas, TER declined steadily, dropping 60% during the 5-day test period (Fig. 3C; \( P < 0.001 \), ANOVA). Retinal pigment epithelial cultures maintained in unconditioned base medium without additional supplements showed similar TER decreases (Fig. 3D; \( P < 0.001 \), ANOVA). Results of permeability assays using the low-molecular-weight tracer sodium fluorescein confirmed the RPE cell tight junction instability shown by these TER assays. As TER decreased in the cultures treated with unconditioned base medium, their permeability increased, as indicated by increased flux of sodium fluorescein across the monolayer (data not shown).

Immunolabeling analysis of cellular morphology in the same cultures used in these experiments showed that cells were organized as a continuous monolayer of tightly packed hexagonal cells as described. ZO-1 was located at the cell-cell contact zone, outlining the cells' polygonal shape (Fig. 4). No differences in the morphology or viability of the RPE cells were detected under the different medium treatments, nor were any obvious differences in junction morphology detected. Actin labeling also showed similar morphology in all groups (data not shown). Apparently, the functional assays of tight junction formation are much more sensitive to tight junction alterations than these immunolocalization approaches.

The actin-labeled cultures were analyzed using morphometry to determine whether cell density was affected by the conditioned medium treatments. Cell counts in multiple, randomly selected microscope fields showed that cell density was slightly lower in the cultures treated with medium conditioned by normal retinas (mean ± SD = 1.8 ± 0.1 \( \times 10^5 \) cells/cm\(^2\)) than in the cultures treated with medium conditioned by the dystrophic retinas (2.4 ± 0.2 \( \times 10^5 \) cells/cm\(^2\)).

Results of the above experiments with conditioned medium suggest that the healthy retina releases soluble factors needed to support stable tight junctions. These factors may be altered or missing in the media conditioned by the degenerate dystrophic retina. Alternatively, dying cells in the degenerating retina may release factors that are damaging to the RPE cells and their tight junctions. To evaluate this possibility further, the extent of any cell lysis that might have occurred during the preparation of conditioned medium was examined by assaying LDH activity. This analysis showed that LDH released into the conditioned medium during the 6-hour incubation period at 37°C was significantly lower in the dystrophic retinas than in the normal control retinas (132 ± 44 U/l versus 302 ± 115 U/l, respectively). Thus, it is unlikely that lytic factors released by the dystrophic retina during preparation of the conditioned medium are responsible for the tight junction changes observed in our experiments. Furthermore, when the total amount of LDH in the retinas before culture was assayed in samples subjected to three cycles of freezing...
and thawing, the amount in the healthy retina was found to be more than twice that in the dystrophic retina (2249 U/l versus 1004 U/l). The reduction in the total amount of LDH activity in the dystrophic samples, compared with the control samples, probably results from the fact that cell density is reduced substantially in the 1-month-old dystrophic retina because many of the photoreceptors have degenerated. Because the percentage of LDH activity in the retina-conditioned medium relative to the total retinal LDH activity was the same in both strains (13%), it can be presumed that equivalent amounts of cell lysis occurred in both media preparations. Thus, it is unlikely that lytic factors released by the degenerate retina are responsible for the tight junction instability shown in these experiments.

**DISCUSSION**

Increased permeability of the blood–retinal barrier is an early complication in most retinal diseases and is thought to contribute to the development of proliferative retinopathy. The dystrophic RCS rat provides an excellent animal model for analysis of this disease progression because its RPE cell tight junctions become leaky just as the photoreceptors begin to degenerate. As the RPE cell breakdown progresses, the retinal capillaries also become permeable and proliferate within the subretinal space and in vitreoretinal membranes. Numerous pathologic changes that may contribute to this disease progression have been described within RPE and glial cells of the dystrophic rat retina, but the primary stimulus for the RPE...
FIGURE 4. Cellular morphology of retinal pigment epithelial (RPE) cultures treated with medium preconditioned by normal or dystrophic retinas appears normal in vitro. Normal RPE cell cultures treated with normal (A) or dystrophic (B) retina-conditioned medium, as described in Figure 3, were immunoreacted with an antibody against ZO-1. In both treatments, ZO-1 was distributed peripherally at the zone of the cell–cell junctions. Magnification, ×275.

cell permeability breakdown has not been investigated. The experiments reported here were designed to address this issue by testing whether the RPE cell permeability increase in RCS rats results from the inability of the mutant cells to form stable tight junctions or whether they occur secondarily to retinal degeneration.

Our analyses of TER, permeability, and junction morphology in RPE cultures prepared from 6- to 8-day-old normal and dystrophic rat retinas showed that the dystrophic RPE cells can form tight junctions that are functionally and structurally indistinguishable from those formed by normal RPE cells. In interpreting these data, it should be kept in mind that the RPE cell tight junction breakdown first becomes evident in vivo during the third postnatal week. Thus, the results obtained here might represent an intermediate phase of development in which the normal RPE junctions are not yet fully mature and in which a genetic defect in tight junction stability has not yet manifested itself. Technical difficulties in obtaining sufficient quantities of healthy, intact RPE cells from older animals precluded testing this premise specifically, but comparison of our TER data with results obtained in previous studies of mature bovine retinas suggests that the tight junctions of rat RPE cells are already functionally mature at 6 to 8 days. The TER values we obtained ranged from 100 to 300 ohm cm⁻², whereas TER values for freshly isolated RPE–choroid preparations from adult bovine retinas ranged from 110 to 220 ohm cm⁻².²⁷ Retinal pigment epithelial cells from 6- to 8-day-old rat retinas are also functionally mature in terms of their phagocytic activity. The genetic defect in phagocytosis is clearly evident in culture, even though phagocytosis does not begin in vivo until the photoreceptors mature during the third postnatal week.²⁴,⁵ Thus, it seems likely that the genetic defect in RCS rats does not directly impair the assembly or stability of the RPE tight junctions.

After demonstrating that tight junctions of dystrophic RPE cells could form normally in vitro, we tested whether the tight junction alterations seen in vivo could be caused by changes in the cellular milieu of the RPE monolayer that occur secondary to the photoreceptor degeneration. Our data showed that medium conditioned by normal retinas supported the high TER levels developed by normal and dystrophic RPE cultures in HDM. In contrast, neither unconditioned base medium nor medium preconditioned by dystrophic retinas was able to maintain the stability of the tight junctions. Moreover, cell density determinations showed that the decrease in TER in these cultures was not caused by a loss of RPE cells. In fact, cell density was slightly higher in the cultures treated with medium conditioned by dystrophic retinas than in those treated with medium conditioned by normal retinas. The cause of this cell density increase is unknown. One possibility is that the medium conditioned by the dystrophic retinas induced cell proliferation, leading to increased numbers of smaller sized cells. Another possibility is that it resulted from greater shrinkage of the cultures during fixation secondary to weakening of their junctions. Whatever the reason for the cell density difference, the beneficial effects of the medium conditioned by the normal retina indicate that this medium contains trophic factors that support tight junction stability. This finding is consistent with previous analyses using RPE cells isolated from the developing chick retina showing that soluble retina-derived factors promote the development of RPE tight junctions in vitro.¹⁴

The negative effects of the medium preconditioned by dystrophic retinas on RPE tight junction stability probably reflect the fact that trophic factors
released by the healthy cells in normal retinas are decreased or lost as the photoreceptors degenerate in the dystrophic retina. A possible alternative explanation is that lytic factors that impair tight junction stability are released by dying cells in the degenerating dystrophic retina. We think we can rule out the latter explanation based on the results of the LDH assay. First, the amount of LDH in the medium preconditioned by the dystrophic retinas was substantially lower than that in the medium conditioned by healthy retinas. This indicates that the amount of cell lysis in the 1-month-old dystrophic retina was even less than that in the healthy, age-matched control preparation, which had a beneficial effect on tight junction stability. Second, the total amount of LDH in the freeze-thawed dystrophic retinas was only half that found in the normal retinas, indicating a 50% decrease in living cells probably because of photoreceptor degeneration in dystrophic rats, which is extensive at 1 month (for review, see reference 2). Because the dystrophic retina at 1 month has fewer total living cells than the normal retina, it probably contains reduced levels of trophic factors normally released by healthy cells. This could explain why the dystrophic retina-conditioned medium failed to support the stability of the tight junctions developed by normal RPE cultures. This idea has been supported by recent experiments testing the effects of conditioned medium supplemented with the hormones and growth factors normally included in HDM (Chang and Caldwell, unpublished data, 1995). The data showed similar effects of the normal and dystrophic retina-conditioned media preparations on tight junction stability, suggesting that the negative effect of the dystrophic retina-conditioned medium reflects the absence of a positive factor rather than the presence of a negative factor.

The LDH analysis also suggests that cell lysis did not contribute to the beneficial effects of the medium preconditioned by normal retinas. Absolute levels of LDH activity in the conditioned medium were very low (<1 U/ml), indicating that very little cell lysis occurred during medium preparation in either rat strain. Release of cytoplasmic material from retinal cells damaged during the preparation of the conditioned medium was unlikely to contribute to the positive effects of the normal retina-conditioned medium on RPE cells. Presumably, the putative trophic factor(s) was released by intact cells in the normal retina.

It should be noted that the gradual decline in tight junction stability observed in the RPE cultures treated with unconditioned medium or medium conditioned by dystrophic retinas was not associated with any obvious alterations in tight junction structure, as indicated by immunolocalization of ZO-1 or actin. Further analysis with more sensitive morphologic methods, such as electron microscopy, will be necessary to determine the structural basis for the alterations revealed by our functional assays. Biochemical analyses using Western blot or Northern blot procedures may help to reveal quantitative differences in expression of junctional proteins.

In conclusion, these investigations are the first to show that genetically defective RPE cells from dystrophic rats have the potential to form tight junctions normally in vitro and that the stability of these junctions can be supported by soluble factors from normal retinas, but not by those from dystrophic retinas. Further work is necessary to identify factors from normal or dystrophic retinas that affect tight junction stability and to rule out conclusively the presence of negative factors in medium conditioned by the dystrophic retina. Retinal cells are known to produce a number of factors that modulate RPE cell growth, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor, transforming growth factor-beta, and others.28—32 These factors have been found to interact with each other in modulating the rate of RPE cell growth, and they also may affect other RPE cell responses, including tight junction formation and function. Interestingly, a deficiency in bFGF receptors has been reported in RPE cells of dystrophic rats.33 The specific effects of this deficiency on RPE cell functions have not been examined. Analyses using conditioned medium approaches, together with bFGF and other growth factors and their neutralizing antibodies, will help to identify the factors involved in stabilizing or destabilizing RPE cell tight junctions. Our data show that RPE cells from normal and dystrophic rats develop comparable tight junctions when maintained in a defined medium containing 0.1 ng/ml bFGF. We have not determined whether the presence of bFGF or other media components is required for tight junction formation or stability in vitro. However, preliminary studies with neutralizing antibodies against bFGF suggest that it may have a modest role in stabilizing tight junctions of normal RPE cells. Additional experiments examining the potential interaction of bFGF with other growth factors should help to clarify the role of this factor in tight junction formation and to show whether a bFGF receptor deficiency in dystrophic rats has any effect on tight junction stability.

Key Words
blood–retinal barrier, cell adhesion, retinal cell culture, retinal degeneration, retinal pigment epithelium, tight junction

References
2. LaVail MM. The retinal pigment epithelium in mice...
Retinal Pigment Epithelial Tight Junctions of RCS Rat


