Intramembrane changes in retinal pigment epithelial cell junctions of the dystrophic rat retina

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A progressive failure in phagocytosis by the retinal pigment epithelium (RPE) occurs in the Royal College of Surgeons rat with inherited retinal degeneration. Another change that can be attributed to a defect in the RPE is a breakdown in the blood-retinal barrier. RPE cell junctions, which form a part of this barrier, become permeable to extracellular tracer during the dystrophic process. We have used the freeze-fracture technique to study the structure of RPE cell junctions in normal and dystrophic retinas. In normal retinas, tight junctions between RPE cells consisted of 8 to 16 anastomosing strands on the cytoplasmic membrane leaflet (P-face) and a complementary pattern of grooves on the external membrane leaflet (E-face). Gap-junctional aggregates of hexagonally packed P-face particles and complementary E-face pits were enclosed within the tight junctional strands. In dystrophic retinas changes were first seen at postnatal day 21. Subtle breaks in P-face tight-junctional strands became more pronounced with time. Eventually the tight junctions appeared to unravel from the gap junctional aggregates, which became isolated and appeared to break off into patches of particle aggregates. The increased density of background particles in the membranes adjacent to disassembling junctions suggested that junctional elements were being removed by dispersal. Endocytosis of junctional elements was observed in both dystrophic and control retinas but may be accelerated in the dystrophic retina. In the late stages of the dystrophy some RPE cell junctions appeared to have proliferated and occupied extensive areas of the RPE membrane. (INVEST OPHTHALMOL VIS SCI 23:305-318, 1982.)

Key words: retinal pigment epithelium, dystrophic retina, freeze fracture, intercellular junctions, blood-retinal barrier, gap junctions, tight junctions

Abnormal functioning of the blood-retinal barrier occurs with many pathologic conditions and is a major factor in the development of retinal disease.1-3 However, although the pathologic effects of retinal permeability changes are well documented, relatively little is known regarding the mechanisms of such changes.

The blood-retinal barrier is located at two levels in the retina: the retinal pigment epithelium (RPE) and the endothelial cells of the retinal vessels.1 The barrier at the level of the RPE cells is comprised of a junctional complex consisting of gap junctions, adhering junctions, and tight junctions, which forms a belt around each RPE cell.4 At the level of the retinal capillaries the barrier is formed by extensive tight junctions that join endothelial cells.5

In the experimental rat model for retinitis
RPE
g
OS
RPE
t
a
Fig. 1. Thin section of pigment epithelial cells from adult control retina, showing apical location and arrangement of junctional complex. Note course of RPE cell junctional complex, which lies in the horizontal plane parallel to the apical and basal surfaces. Gap junctions (g), tight junctions (t), adhering junctions (a), and outer segment (OS) are shown. (x75,000.)

pigmentosa (the Royal College of Surgeons [RCS] strain of rat) the blood-retinal barrier to the extracellular tracer, horseradish peroxidase, has been reported to break down at the level of the RPE cell (Bok D, unpublished observation in ref. 7) and at the level of the retinal capillaries.8 Tracer that crosses the blood-retinal barrier in such experiments has two possible routes: transport across the cells of the blood-retinal barrier and leakage through altered cell junctions of the blood-retinal barrier. Transcellular transport across capillary endothelial cells is thought to be enhanced in the dystrophic retina,8 and RPE cell junctions are thought to become leaky (Bok D, in ref. 7). We have used the freeze-fracture technique to study these leaky pigment epithelial cell junctions in the dystrophic retina and have found intramembrane changes that correlate with the reported changes in permeability and that may provide the morphologic basis for the blood-retinal barrier breakdown.

Materials and methods
Pink-eyed and black-eyed RCS strains of rats (RCS, RCS-p/+ ) and their genetic controls (RCS-rdy⁺; RCS-rdy⁻p/+ ) were used. The animals were maintained on a 12 hr light/dark cycle with 5 ft-c fluorescent lighting. Dystrophic and control rats between 3 weeks and 1½ years old were anesthetized with chloral hydrate and sacrificed in the first half of the light cycle by perfusion through the heart with a fixative containing 2% paraformaldehyde, 2% glutaraldehyde, and 0.5% acrolein in 0.1M cacodylate buffer, pH 7.3, at room temperature. After 15 min of intracardiac perfusion the anterior chamber of the eye was removed and the posterior eyecup was flushed with fixative for 2 min. The eyecup was then removed and stored in the perfuse fixative for 2 hr. The eyecups were then rinsed and stored in 0.1M cacodylate buffer overnight. The fixed retinas were dissected, cut
Fig. 2. Freeze-fracture appearance of normal junctional complex from adult control retina. Anastomosing P-face (pf) strands (closed curved arrows) and E-face (ef) grooves (open curved arrows) encircle gap-junctional aggregates of hexagonally packed P-face particles (g) and E-face pits (asterisks). Small breaks in the tight-junctional strands are present (arrowheads). Apical region of junctional complex is above, basal below. (×68,750.)

into 1 mm squares, equilibrated in 23% glycerol for 2 hr, mounted retinal-side down on gold disks, then frozen in the liquid phase of Freon 22 and stored in liquid nitrogen. This orientation was found to expose a maximal area of junctional intramembrane structure because of the curvature of RPE cell membrane in the region of the junctional complex (see Fig. 1). The RPE cell junctions exposed by this fracture plane were parallel to the plane of the RPE cell layer. Freeze-fracturing and platinum-carbon shadowing were done at —116°C in a Balzers BAF 301 apparatus fitted with an electron-beam gun for platinum and carbon shadowing and a quartz crystal monitor for standardizing replica thickness. Replicas were digested in Purex and mounted on Formvar-coated grids for examination in the electron microscope.

Thin sections were prepared from eyes fixed as described above. Retinal slices were postfixed with osmium tetroxide, stained en bloc with 2% uranyl acetate, dehydrated in a graded series of ethanol, and embedded for electron microscopy.

Results

Control retinas. Junctions between pigment epithelial cells in the genetic control for the dystrophic rat were similar to those described previously in other species. Each pigment epithelial cell was joined at its apical region to the adjacent cells by a junctional complex consisting of three parts: gap junctions, adhering junctions, and occluding junctions. In thin sections, gap junctions were found just basal to the microvilli and could be identified by the close apposition of membranes that formed a gap 2 to 3 nm wide (Fig. 1). The most basal component of the junctional complex was the adhering junctions. The extracellular space in the area of adhering junctions was slightly more dense than elsewhere, and numerous filaments were found in the adjacent cytoplasm, which was free of other organelles (Fig. 1). Tight junctions
Fig. 3. Junctional complex from 21-day-old dystrophic retina. Breaks in tight-junctional strands (arrowheads) are larger and more frequent than in control retinas. Detached chain of tight-junctional P-face (pf) particles is seen at basal region of junction (straight arrow). Small gap-junctional aggregates (g) at the apical region are not enclosed by tight junctions (small arrows). Apical region of junctional complex is above, basal below. (×68,750.)

were interposed between the region of gap junctions and adhering junctions and were identified in thin sections on the basis of focal membrane fusions (Fig. 1).

In freeze-fracture material, gap junctions appeared as aggregates of uniform, tightly packed particles on the cytoplasmic membrane face (P-face) or as complementary arrays of pits on the external membrane face (F-face) (Fig. 2). Gap junctions ranged in size from small clusters of a few particles to large aggregates containing hundreds of particles. By freeze-fracture no characteristic structure of the adhering junction was identifiable. Rather, the absence of cytoplasmic organelles in the adjacent cross-fractured cytoplasm delineated the extent of the adhering junction (Fig. 2). Tight junctions in the freeze-fracture material formed a continuous network of anastomosing strands around the perimeter of each cell (Fig. 2). On cytoplasmic membrane faces (P-faces) eight to 16 anastomosing tight-junction strands enclosed gap junctions of various sizes. These strands were usually continuous, but occasional breaks were seen. External membrane faces (E-faces) showed complementary patterns of eight to 16 anastomosing grooves. Orientation of junctional complexes in this material could be recognized on the basis of the apical position of gap junctions and the basal position of the region of adhering junctions.

Dystrophic retinas. At postnatal day 21, junctional complexes in the dystrophic retina were similar to those in normal retinas (Fig. 3). Although the arrangement of tight- and gap-junctional elements resembled that in the control retina, there was a suggestion of more
Fig. 4. Junctional complex from 43-day-old dystrophic retina is labeled as in Fig. 2. Many short chains of tight-junctional P-face (pf) particles (straight arrows) are present on the basal aspect of the junctional complex. Tight-junctional strands are fewer in number and more fragmented than in the control retina. Background particle density of membrane adjacent to junction is high. Apical region of junctional complex is above, basal below. (x68,750.)

subtle differences. For example, breaks in the P-face strands were larger and appeared to occur more frequently than in control retinas. In a few replicas there appeared to be an unraveling of the basal strands of tight junctions, which was characterized by the presence of detached tight-junctional P-face strands at the base of the junctional complex. In some areas gap-junctional aggregates were seen apical to the tight junctions (Fig. 3).

At postnatal day 43, the apical region of the junctional complex resembled that at 21 days, but more obvious changes were seen basally in some complexes (Fig. 4). Short chains of tight-junctional particles or grooves terminated without intersection at the base of these junctional complexes (Fig. 4). In addition, tight-junctional strands and grooves were fewer in number, more fragmented, and intersected less frequently than in the control retinas. The background particle density on the P-face membrane adjacent to such areas usually appeared higher (Fig. 4) than at normal junctions (Fig. 5). Particle density increases appeared to involve only the region of abnormal junctional complexes and were not observed in apical or basal RPE cell membrane. In some areas endocytotic vacuoles or invaginations containing gap- and tight-junctional membrane were found (Fig. 6). Such profiles were observed less frequently in normal retinas. At later stages in the dystrophic retina the incidence of areas of increased density of background particles around abnormal junctions and of endocytotic vacuoles containing junctional membrane was similar to this period.

The frequency of abnormal junctions in-
increased with age, and by 72 and 113 days many abnormal junctions were found. In these junctional complexes, tight-junctional strands and grooves were fragmented and few in number, with relatively few intersections (Figs. 7 and 8). Unraveled P-face particle chains or E-face grooves were frequently seen at the base of junctional complexes, and tight junctions, which normally surrounded gap junctions at the apical portion of the junction, were fragmented or absent in some areas (Fig. 7).

In retinas of dystrophic animals 6 months of age and older, many junctional complexes were grossly abnormal (Fig. 9). The tight-junctional strands or grooves were fragmented, few in number, and rarely intersected, giving an unraveled appearance to the junctional complex. In some abnormal complexes the number of gap junctions was reduced. When present, gap junctions were usually not enclosed by tight junctions (Fig. 9), and numerous small clusters of gap-junctional particle aggregates (Fig. 10) were adjacent to some larger gap-junctional arrays, suggesting that the particle aggregates were breaking off and dispersing into the surrounding membrane. Unusual areas of particle clearing were found on the P-faces of some gap junctions during this period, and some of the areas contained small aggregates of particles (Fig. 10).

In some replicas of animals 6 months of age and older the orientation and location of pigment epithelial cell junctions as well as their configuration were disorganized. Instead of being restricted to a narrow belt in the apical region of the RPE cell membrane, these junctions were scattered over vast expanses of the membrane surface, and the tight junctions had a fragmented and an unraveled appearance and did not enclose gap junctions (Fig. 11).
Fig. 6. A, Endocytotic vacuole containing gap-junctional membrane from 43-day-old dystrophic retina. A membrane profile containing E-face gap-junctional pits (asterisk) protrudes from cross-fractured RPE cell cytoplasm (large arrow). B, Endocytotic invagination containing both gap- (g) and tight-junctional (curved arrow) membrane from 43-day-old dystrophic retina. (×75,000.)

An explanation for this apparent proliferation of cell junctions was suggested by the thin-section appearance of the pigment epithelium during this period. In some areas several layers of RPE cells were superimposed, and in some of these, capillaries had invaded the RPE and were surrounded by RPE cells. Junctions were seen between RPE cells in these areas, but the contacts between RPE cells were not always between lateral surfaces. In some areas the RPE cells were stacked with junctions covering much of the area of contact between apical and basal cell surfaces. The proliferated junctions seen in freeze-fracture may correspond to these areas where junctions were not confined to the normal apical position but occupied the entire membrane face between the two RPE cells.

Discussion

The changes observed in the membrane structure of the dystrophic retina suggest a progressive breakdown of the pigment epithelial cell junctions. In comparison to the normal junctional complex from control retinas (Fig. 12), the junctional complex in the dystrophic retina is grossly abnormal. The changes begin at 21 days, with subtle breaks in tight-junctional P-face strands. Over time the tight junctions become increasingly fragmented, beginning basally, and eventually they appear to unravel from the area of gap-junctional aggregates (Fig. 13). At the same time, gap-junctional aggregates become isolated by the unraveling of tight-junctional strands and appear to break off into patches of particle aggregates. As the junctional elements become altered, the background particle density of membranes adjacent to junctions increases and suggests that junctional components are removed by dispersal into the surrounding membrane. Another mechanism for junctional removal may be by endocytotic uptake of gap- and tight-junctional membrane...
Fig. 7. Junctional complex from 72-day-old dystrophic retina. Tight-junctional strands (curved arrow) are fewer in number, more frequently interrupted, and intersect less frequently than in control retinas. Apical tight junctions are fragmented (arrowheads) and do not surround gap junctions (g or asterisk) completely. Chains of P-face (pf) particles are seen at the base of the junctional complex (straight arrow). Apical region of junctional complex is above, basal below. (x68,750.) ef, E-face.

by the RPE cells, which is suggested by the observation of endocytotic vacuoles consisting of junctional membrane. Similar observations of endocytotic vacuoles in normal RPE cells may reflect a normal ongoing autophagic process for membrane removal, which becomes more active in dystrophic RPE cells. As the RPE junctional breakdown continues in the later stages of the dystrophy, in some areas both gap and tight junctions appear to have proliferated and occupy extensive areas of RPE membrane. These zones of proliferating junctions are abnormal in configuration and may represent areas where regenerating RPE cells have become superimposed and where junctions have been reformed subsequent to the initial junctional breakdown.

These changes in freeze-fracture appearance of RPE cell junctions occur at the same time as other changes in the dystrophic retinal pigment epithelial cell layer. Beginning around 15 days, the greatly diminished rate of outer-segment phagocytosis by the RPE cells results in a progressive accumulation of membranous debris in the interphotoreceptor space, accompanied by a gradual degeneration of photoreceptor cells.9 13 Between 40 and 72 days, the RPE cell layer becomes noticeably attenuated in thin-section material, many RPE cells change in shape from columnar to elongated, and both gap and tight junctions disappear or become markedly displaced from their normal location (Caldwell RB, McLaughlin BJ; unpublished observations). Late in the dystrophic process the membranous debris disappears, the RPE
cells regenerate, and the RPE cell layer resumes a more normal appearance. These changes could be expected to affect the function of RPE cell junctions. We have recently demonstrated that tight junctions in the dystrophic retina are abnormally permeable to extracellular tracers at 42 days and later. Although the freeze-fracture appearance of RPE cell junctions was not dramatically altered at this time, permeability changes have been found to occur in other systems without obvious changes in tight junction membrane structure. The function of RPE cell gap junctions in the normal retina is unknown. In view of the current freeze-fracture evidence of gap junction breakdown in the dystrophic retina, however, alterations in gap junction functions would also be expected.

Effects on tight junctional structure similar to those observed in this study have been reported in epithelia exposed to hypertonic solutions and calcium-chelating agents. In these studies, the total number of intact tight-junctional strands is reduced and some strands are broken up into rows of particles. As was true in our study, the most severe changes usually are found basally and the sequence of fragmentation progresses apically.

Two processes have been proposed for the removal of cell junctional elements. Some workers have found evidence suggesting that gap junctions and tight junctions are removed by a process of internalization. In this process the junctions are thought to be internalized by endocytosis and degraded by lysosomal digestion. Others argue that junctions are removed by dispersion of their component particles into the background membrane. In this case, dissolution of
Fig. 9. Junctional complex from 6-month-old dystrophic retina. Tight-junctional strands (curved arrows) are few in number, fragmented, and intersect rarely. Gap-junctional aggregates (g) are not enclosed by tight junctions and a particle-free area is present within a gap-junctional aggregate (arrowhead). Apical and basal orientation of the junctional complex is no longer evident. (x55,000.) pf, P-face.

Junctional components is thought to occur by a reversal of the process of junction formation; that is, the junctions first break up into smaller particle strands or aggregates, which then break up further to single particles that disperse into the surrounding membrane.

Both internalization and dispersal of cell junctions may occur in the dystrophic retina. Internalization probably represents a normal renewal process, since endocytotic vacuoles containing gap and tight junctions were found in both normal and dystrophic animals of all ages. Autophagy of other cellular components is known to occur normally in the pigment epithelium but appears to become more active in dystrophic RPE cells, since endocytotic vacuoles containing junctional membranes were observed more frequently in the dystrophic retinas. Dispersal, on the other hand, may represent the means of removing junctions that are undergoing disassembly. Fragmentation of tight junctions, small clusters of gap-junctional aggregates surrounding larger gap-junctions, and increased density of background particles surrounding abnormal junctions are observed only in the dystrophic retina. These changes are all suggestive of the removal of junctions by dispersal of their component elements into the surrounding membrane.

These dispersed particles may remain in the membrane and participate in the proliferation of abnormal junctions, which takes place late in the dystrophic process when the
RPE cells have regenerated. Proliferation of tight junctions has been observed in other epithelial tissues undergoing regeneration\textsuperscript{33}; in tissue undergoing retinoic acid–induced metaplasia\textsuperscript{34}; in tissue exposed to sublethal gamma irradiation,\textsuperscript{35} phalloidin treatment,\textsuperscript{36} or prolonged treatment with N-nitrosomorpholine\textsuperscript{37}; as well as in various in vitro conditions.\textsuperscript{38–46} In these studies, new tight-junctional strands often spread along the entire length of the epithelial lateral plasma membranes and represent a dramatic increase in tight-junctional strand length,\textsuperscript{35, 37–40} similar to what we have observed in the older dystrophic retina.

We are grateful to Rebecca Krell and Edward Rutkowski for help in manuscript preparation and to Nigel Cooper for helpful suggestions during the course of this investigation.

REFERENCES

Fig. 11. RPE cell membrane from 1½-year-old dystrophic retina. Gap (g) and tight junctions (curved arrows) cover a wide area of the cell membrane. Tight-junctional P-face (pf) strands (closed arrows) and E-face (ef) grooves (open arrow) are more fragmented and intersect less frequently than normal. Gap junctions are not enclosed by tight junction strands. (×9500.)


8. Essner E, Pino RM, and Griewski RA: Permeability of retinal capillaries in rats with inherited retinal

**Fig. 12.** Diagram showing the characteristic appearance of normal junctional complexes in the control retina. A complex network of anastomosing tight junctions encloses gap junctions of various sizes.

**Fig. 13.** Diagram of the dystrophic junctional complex. Over time the tight junctions decrease in number, become increasingly fragmented, and intersect rarely, giving an unraveled appearance. Gap junctions are not enclosed by tight junctions.

33. Yee AG and Revel J-P: Loss and reappearance of gap...


