Maturation of the Corneal Endothelial Tight Junction

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Apical tight junctional formation of the rabbit corneal endothelium was examined by freeze-fracture analysis and measurement of paracellular permeability to 5(6)-carboxyfluorescein. Freeze-fracture analysis indicated that apical tight junction formation of the rabbit corneal endothelium is a dynamic process. At birth, there are few tight junctional strands present and a minimal barrier for paracellular diffusion. As the rabbit matures, a more complex network of anastomosing tight junctional strands begins to encircle the cell perimeter under the apical folds. However, even in the mature animal (3 months), there are discontinuities and free ends in the network, thus suggesting that the barrier is not complete even at this stage. Paracellular permeability measurements using 5(6)-carboxyfluorescein as a tracer corroborate these anatomic findings. Endothelial paracellular flux measurements steadily decrease as the rabbit matures from birth to young adult. This indicates that the tight junctional network is increasing in complexity and progressively limiting the flow of substances through the intercellular space. Invest Ophthalmol Vis Sci 32:2757–2765, 1991

The cornea is an avascular tissue and receives most of its nutrients through leaking of aqueous humor across the endothelium in an aqueous-to-stroma direction. In the adult of most species, the endothelium functions as a "leaky" semipermeable membrane as a result of the presence of low-resistance junctions between cells, thus allowing the cornea to imbibe aqueous humor at a slow continuous rate. The discontinuous occluding junction apparently has a role in the paracellular diffusion of substrates necessary for corneal nutrition. Conical thickness and transparency are controlled by the active removal of fluid that has leaked into the stroma. A pump-leak hypothesis describes the control of stromal hydration. It states that the passive flow of fluid into the stroma across the leaky endothelial junctions is balanced by active fluid removal by the endothelial pump. According to this theory, the role of the apical endothelial tight junctions is of paramount importance in the maintenance of transparency. Should the junctions be perturbed, the stromal hydration increases causing loss of stromal proteoglycans, disarray of the collagen fibrils, and loss of transparency.

Previous transmission electron micrographic studies in the rabbit showed the presence of apical electron-dense regions between adjacent endothelial cells as early as 21 days' gestation. They are considered to be well developed within a few days after birth. These areas were interpreted as being tight junctions. However, no attempt was made to compare the completeness and/or paracellular permeability of the junctions at the various developmental stages of the rabbit. Others used freeze-fracture techniques to examine the morphology of the tight junctions in the adult rabbit corneal endothelium. These studies concluded that the junctions were an incomplete barrier between adjacent cells in the adult. Another study examined corneal endothelial wound repair in the cat. As the wound healed, the endothelial tight junctions developed first as isolated randomly arranged strands. Gradually, the complex junctional structure of the adult, similar to eyes without wounds, was formed.

The tight junction is the ultrastructural basis for limiting paracellular movement between cells. Anatomic studies describe junctional strand formations but do not reveal the physiologic function of the complex. Therefore, the permeability of the endothelium to 5(6)-carboxyfluorescein (CF; Eastman Kodak, Rochester, NY) was measured in conjunction with freeze-fracture analysis of the junctional complex. This was done to follow the maturation of the endothelial apical tight junction formation both anatomically and physiologically and to obtain a more complete analysis of its development.

Materials and Methods

Animals

All experimental animal procedures adhered to the ARVO Resolution on the Use of Animals in Re-
search. New Zealand white rabbits of either sex were studied in all experiments. The animals were anes-thetized for all procedures intramuscularly with ketamine HCl (10–15 mg/kg) and xylazine (6 mg/kg). They were killed by intracardiac injection of sodium pentobarbital (80 mg/kg). The animals were examined at the following ages: newborn, 7 days (sodium-potassium adenosine triphosphatase pump sites first observed on lateral membranes of corneal endothelial cells3-10), 13 days (palpebral fissure opens), 20 days (onset of transparency6), and 3 months (young adult, the age most frequently used in corneal studies). Their eyelids were opened surgically if the palpebral fissure was still closed at that age. Young rabbits, newborn through 20 days, were housed with their mothers until the time of the experimental procedure.

Transmission Electron Microscopy

Three rabbits of each age were killed. Their corneas were excised and immersion fixed in a trialddehyde fixative consisting of 1% paraformaldehyde, 1% glutaraldehyde, and 0.5% acrolein, pH 7.3–7.5 in 0.1 M sodium cacodylate buffer. The corneas were rinsed in cacodylate buffer, and the central cornea was cut into squares and osmicated in 2% osmium tetroxide in buffer for 1 hr. The tissue was rinsed in buffer, en bloc stained with uranyl acetate for 1 hr, dehydrated through a series of ethanols, embedded in Spurr’s resin, and polymerized at 60°C for 3 days. The blocks were sectioned, stained with uranyl acetate and lead citrate, and viewed in a Hitachi H600 electron microscope (Conroe, TX).

Freeze-Fracture Analysis

Three rabbits of each age were killed. Their corneas were removed with a scleral rim and immersion fixed in the same trialddehyde fixative used for transmission electron microscopy. The tissues were then rinsed in 0.1 M cacodylate buffer, cut into squares, and infiltrated with 23% (v/v) glycerol for 2 hr. The tissue squares were mounted endothelial-side up on gold specimen carriers, frozen in Isotron (Pennwalt Corp., Philadelphia, PA) cooled by liquid nitrogen, and stored in liquid nitrogen. The corneas then were fractured on a Balzer BAF-400T freeze-etch apparatus (Hudson, NH) at −114°C, and after the proper fracture plane was obtained, they were coated with platinum and carbon. The tissue was digested with 5% hypochlorite, the replica was rinsed with distilled water, placed on a copper grid, and examined with a Zeiss CEM-902 transmission electron microscope (Thornwood, NY).

Measurement of Paracellular Permeability With CF

Five to six rabbits of each age were killed. Their eyes were removed with the lids still attached as described previously.11 The epithelium was removed with a Gill knife. The excised corneas with attached conjunctiva were mounted in modified Ussing chambers for the permeability studies.12-14 The corneas were mounted as pairs; both epithelium and endothelium removed from one cornea and epithelium only removed from the other. Glutathione bicarbonate Ringer’s solution (GBR, containing NaCl 6.519 g/l, KCl 0.359 g/l, CaCl2·2H2O 0.153 g/l, MgCl2·6H2O 0.159 g/l, NaH2PO4 0.103 g/l, NaHCO3 2.453 g/l, glutathione 0.092 g/l, and glucose 0.903 g/l) was added to the reservoir bathing the epithelial side, and GBR containing 10−6 M CF was added to the endothelial reservoir. Constant mixing and pH 7.4 were maintained by an airlift siphon using 95% air and 5% CO2, and a constant temperature of 36.5°C was maintained by a circulating water bath. After a 1-hr equilibration period, the epithelial chamber was evacuated and the GBR, discarded. The reservoir was refilled and emptied into a tared test tube. This reading was used as a background or residual count. Subsequent samples from the epithelial chamber were collected at 30-min intervals for a 2-hr period. After the final collection of the fluid from the epithelial chamber, the endothelial chamber was collected and rinsed. The pH of all samples was adjusted to the range of 7.5–7.6. Fluorescence was measured with a Gilford Flore IV Spectrofluorometer (Ciba Corning, Oberlin, OH). The relationship between dye concentration and fluorescence emission was linear in the concentration ranges used in this study. The permeability coefficient, Ktrans, was calculated according the method of Maffly and co-workers,15 using the formula:

$$K_{trans} = \frac{\text{#CF molecules on the de-epithelialized side}}{\text{conc of CF on endo side \times area of cornea \times time}}$$

The Ktrans value has the units of centimeters per second.

Statistics

Data obtained from paracellular permeability to CF experiments were analyzed by one-way analysis of variance followed by planned comparisons in which the t values were based on the pooled mean-square error from the analysis of variance table.

Results

Freeze-Fracture Analysis

Apical surface: Transmission electron microscopy and freeze-fracture analysis of the apical surfaces showed that the tortuosity of both the lateral and apical cell borders was minimal in the newborn (Figs. 1A–B). Gradually, with age, the lateral membranes

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Fig. 1. Micrographs shown in Figures 1–5 are displayed in a similar format and are arranged as follows: (a) transmission electron micrograph showing the interdigitiation of the lateral membrane and the presence of the apical tight junction; (b) freeze-fracture micrograph illustrating the overlap of the apical membranes between adjacent cells (fracture plane is between the bilayers of the apical membrane); (c) freeze-fracture micrograph showing the tight junctional formation (fracture plane is between the bilayers of the apicolateral region of the cell); and (d) freeze-fracture micrograph of an apicolateral gap junction (fracture plane is between the bilayers of the apicolateral region of the cell).

Transmission electron and freeze-fracture micrographs of the corneal endothelium of the newborn rabbit. The apical junction as seen in the transmission electron micrograph [arrowhead in (a)] is small in comparison to the apical junction in the endothelium of older rabbits (see Figs. 4a and 5a). The tight junctional complex as viewed with freeze-fracture microscopy consists of a few isolated particle strands in the newborn [arrows in (c)] and cell overlap in the apical plane is minimal (b). Large and prominent gap junctions are present on the apicolateral membranes [arrows in (d)] as well as within the strands of the junctional complex [arrowhead in (c)]. Bar = 1 μm in the transmission electron micrograph and 0.2 μm in the freeze-fracture micrographs.

became more interdigitated (Figs. 2A, 3A, 4A, 5A). This same pattern of increased tortuosity of the apical cell borders was evident in the freeze-fracture micrographs (Figs. 2B, 3B, 4B, 5B) where finger-like projections overlapping onto the juxtaposed cell were seen.

The intramembranous particle density was low in the apicolateral membrane of the young rabbits and gradually increased with age (Figs. 1B, 2B, 3B, 4B, 5B).

Junctional complex: In the newborn corneal endothelium, most areas of the apical perimeter did not
have junctional strand formation. In isolated areas, however, a single isolated strand such as that shown in Figure 1C was found. With age, more strands are added to the developing junctional complex (Figs. 2C, 3C). Gradually the junctional strands interconnected and began to form a complex network of anastomosing intramembranous particle ridges that involved a larger area of the cell perimeter (Fig. 4C). As seen in Figure 5C, the junctional complex of the young adult rabbit was nearly complete with many interconnecting junctional strands. However, even in the 3-month-old rabbit, interruptions can be seen between areas of complexity.

**Gap junctions:** The gap junctions of the newborn rabbit corneal endothelium were abundant and prominent (Fig. 1D). With maturity the size, abundance,
Fig. 3. Transmission electron and freeze-fracture micrographs of the corneal endothelium of the 13-day-old rabbit. The size of the tight junctional complex is increased over that of the 7-day animal (Fig. 2c), as seen in the electron micrograph [arrowhead in (a)] and the freeze-fracture micrograph (c). The number of anastomosing tight junctional strands is elevated over that of the 7-day rabbit (arrows in (c)). The overlapping projections at the cell apical surface are becoming more prominent and abundant (arrows in (b)). The gap junctional aggregates are still apparent among the tight junctional strands (arrowhead in (c)), but are becoming smaller on the apicolateral membrane (arrows in (d)). Bar = 1 μm in the transmission electron micrograph and 0.2 μm in the freeze-fracture micrographs.

and prominence of the gap junctions decreased (Figs. 2D, 3D, 4D) until (Fig. 5D) the gap junction was decreased in size and distinction in the young adult corneal endothelium. At all ages, smaller gap junctions could be seen among the apical junctional complex (Figs. 1C, 2C, 3C, 4C, 5C).

Paracellular Permeability to CF

Endothelial permeability to CF is shown in Figure 6. The endothelial permeability to the fluorescent marker steadily decreased from $19.3 \times 10^{-6}$ cm/sec in the newborn to $3.3 \times 10^{-6}$ cm/sec in the young adult. Even at 20 days of age when the cornea was optically transparent, the endothelial paracellular flux was elevated above that of the adult ($P < 0.05$). Maximum permeability was determined by denuding the cornea of both epithelium and endothelium. The maximum permeability of the newborn cornea to CF was increased significantly ($P < 0.05$) over that of the corneas of the 3-month-old rabbits. There was, however, no difference between the maximum permeabilities of 7-day-old, 13-day-old, 20-day-old, and 3-month-old rabbits.
Fig. 4. Transmission electron and freeze-fracture micrographs of the corneal endothelium of the 20-day-old rabbit. The size of the tight junctional complex [arrowhead in (a)] has increased in size over those of the younger rabbits. The freeze-fracture micrograph of the junctional complex indicates that the particle strands that comprise the junction are increasing in number and interconnections [arrows in (c)]. The overlapping projections at the cell apical surface are increasing in number and size [arrows in (b)]. The gap junctional aggregates are present among the tight junctional strands [arrowheads in (c) and (d)], but are becoming less prominent. Bar = 1 μm in the transmission electron micrograph and 0.2 μm in the freeze-fracture micrographs.

Discussion

The freeze-fracture replicas in this study showed that there are many structural changes occurring in the membranes of corneal endothelial cells during development from birth to adulthood. The changes, however, did not stop when transparency was achieved at 20 days. The junctional strands of the newborn endothelium began as single linear arrays of P-face ridges. Over time, more particle strands formed in these localized regions, making the junctions deeper in the apical-to-basal direction. Gradually, with age, more junctional strands developed, and a more complicated junctional network was formed.
Fig. 5. Transmission electron and freeze-fracture micrographs of the corneal endothelium of the 3-month-old rabbit. The tight junction is evident on the transmission electron micrograph (arrowhead in (a)), and is a complex network of particle strands, as indicated in the freeze-fracture micrograph (arrows in (c)). The bases of finger-like projections are visible on the apical surface of the endothelial cells (arrows in (b)). Gap junctional aggregates are evident among the strands of the tight junction (arrowheads in (c)), and are also found on the lateral membranes (arrows in (d)). Bar = 1 μm in the transmission electron micrograph and 0.2 μm in the freeze-fracture micrographs.

However, the junctional complex was not a complete belt-like structure, circumscribing the entire perimeter even at 3 months of age. A similar observation was reported earlier. Analyses after tight junction formation in other tissues showed similar developmental stages. The first indication of the formation of junctional strands in culture, embryos, or fetuses is the appearance of a raised area of particle-free plasma membrane. A few small clusters of particles then emerge. These form short strands that eventually fuse to form an anastomosing junctional network.

Correlation of freeze-fracture analyses and CF permeability measurements defined both anatomic and physiologic apical junctional structure and permeability. The solute permeability of the corneal endothelium is an important factor in the regulation of corneal hydration and therefore transparency. Because of this, knowledge of the permeability coefficient is
important to the understanding of the mechanisms involved in the pump–leak control of stromal water content. The completeness or “tightness” of the apical junctional belt greatly influences the paracellular permeability. The structure of the junctional complex is not the only limiting factor determining the passive transepithelial permeability, but it appears to be the dominant factor. With maturity, the length of the intercellular space increased with the elevated tortuosity of the lateral membranes. This increased path length also may retard the movement of CF, thus contributing to the decreased paracellular permeability found during the aging process. A physiologic measure of the corneal endothelial permeability was assessed using CF as an in vitro paracellular permeability marker. This dye is hydrophobic in nature; therefore, it will cross the endothelial cell layer through the paracellular shunt rather than through the cell membranes and give an accurate measurement of intercellular permeability. The permeability coefficient (K\text{trans}) was defined as the amount of a given substance that crossed a 1-cm² area per time under a given driving force.

Our results showed that endothelial permeability to CF steadily decreased with age as the tight junction became more complex. These data suggest that the paracellular permeability of the endothelial cell layer was elevated in the neonates even up to and including 20 days, the time when transparency and adult hydration levels were present. Although not analyzed quantitatively, by freeze-fracture analysis, it appeared that the strand number and area of the perimeter occupied by the strands was lower in the newborn through 13 days compared with the adult rabbit. At 20 days, however, the junctional complex appeared morphologically similar to the adult although paracellular flux measurements showed that the complex allowed more CF to pass through the junctions at this age. Therefore, anatomic findings alone cannot be used to estimate the tightness of the junction unless a more quantitative study is undertaken.

Corneal endothelial permeability was measured in the normal adult rabbit, in the cornea denuded of endothelium, after inflammation, and after wounding. The experimental measurement of the permeability to CF we obtained fell close to the predicted values of others who tested in vitro adult rabbit corneal endothelial permeability to seven substances of varying molecular weights, ranging from 35,000–90,000 daltons.

Freeze-fracture micrographs reveal additional ultrastructural information regarding the corneal endothelium. Concomitant with the development of the junctional complex, there is a gradual appearance of more intramembranous particles on the apicolateral P-face. The intramembranous particles appeared as the apical surface area of the endothelial cell was increasing, suggesting that additional protein synthesis and insertion of the molecules into the membrane were occurring. It has been proposed that some of the intramembranous particles on the lateral membrane are the protein of the sodium–potassium adenosine triphosphatase, an integral component of the endothelial pump mechanism. The overlap of adjacent endothelial cells in the apical plane gradually increased until the crossed finger-like expansions described previously were abundant at 3 months of age. This overlap may act along with the tight junction to limit paracellular flux.

Small gap junctions were present among the junctional strands at all ages, and large, prominent gap junctions were found on the apicolateral membrane of the neonatal corneal endothelium. The prominence, size, and abundance of the lateral gap junctions diminished with age. In the developing system, it is believed that gap junctions are necessary for the coordination of developmental processes. The presence of the communicating junctions were correlated with growth regulation, the differentiation of cells, and the synchronization of cell activity. Gap junctions are permeable to small ions and intercellular messengers. During the time that the corneal endothelial layer is organizing into a hexagonal pattern and forming the apical junctions, there may be a need for increased intercellular communication. Thus this
may be the reason for the increased size and abundance of the gap junctions. Similar results were observed during corneal repair where it was determined that, early in wound healing, gap junctions were approximately 25% larger than normal.9

Key words: corneal endothelium, development, tight junction, freeze-fracture, permeability

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References