Physiological function of regenerating endothelium

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A defined area, 4 mm. diameter, cryothermal injury was created on a rabbit cornea. Corneal thickness was measured at four distances from the limbus to the center of the cornea during the swelling phase after endothelial damage, and during the recovery period. Rapid initial swelling was followed by a period of stable maximum thickness over 24 hours. More swelling occurred centrally than peripherally. Eight days after injury the peripheral cornea regained normal thickness, and the central portion was normal thickness after 10 to 12 days. Histologic examination of corneal endothelium showed that early migration of cells into the denuded area occurred 6 hours after injury. By 2 days, most of the denuded area was covered by endothelial cells, although the cells were large and irregular. The number of normal cells increased, and of irregular cells decreased, over the next five days, until two weeks after freezing all cells had a normal appearance. The recovery of physiologic endothelial function lags behind the histologic recovery by about four to five days, indicating that recovery of the normal endothelial permeability is possibly related to the status of the cellular junctions rather than covering of the posterior surface by cells per se.

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The mode of endothelial regeneration following a variety of mechanical, chemical, and thermal injuries has been studied on numerous occasions.1-11 There are, however, very few reports on the functional recuperation of the endothelium during its regenerative process.

In 1873, Leber1 created endothelial injury and corneal swelling by passing a blunt hook across the posterior surface of the cornea; he was the first to point out the importance of endothelium in corneal transparency. A variety of mechanical and chemical techniques have been used since then by Nagano,2 Maurice and Giardini,3 Stacke r,4 Binder and Binder,5 Morton, Ormsby, and Basu,6 McDonald,7 and others. These techniques generally involved opening of
the anterior chamber and, in some instances, rupture of Descemet's membrane which undoubtedly influenced the rate of endothelial regeneration. Furthermore, in none of these techniques could the exact area of endothelial damage be controlled.

Cogan\textsuperscript{4} induced endothelial damage by inserting a magnetic foreign body into the anterior chamber, and caused corneal edema which resolved after a day or two and disappeared in one to two weeks. Using a similar technique, Honegger\textsuperscript{11} followed the course of corneal swelling by measuring corneal thickness. Chi, Teng, and Katzin\textsuperscript{9} induced mechanical injury to one-fourth of the corneal endothelium and noticed complete coverage of the defect in 72 hours and clearing of corneal edema one to two days later.

Maumenee and Kornblueth,\textsuperscript{3} using solidified carbon dioxide, were the first to show epithelial, stromal, and endothelial cell destruction by cold temperature. They used a temperature of \(-78^\circ\) C. and noticed complete cell destruction in all three layers within three to five seconds. Chi and Kelman,\textsuperscript{14} using both a 4 and 8 mm. brass applicator, have shown complete corneal endothelial cell destruction with a temperature of \(-80^\circ\) C. for 20 seconds. A temperature of \(-20^\circ\) C. with an 8 mm. applicator produced partial damage, whereas a temperature of \(-10^\circ\) C. over the same period of time had no effect on the corneal endothelium. In their experiment, a 4 to 4.5 mm. diameter endothelial defect was covered by regenerating endothelium within 3 days.

Faure, Kim, and Graf\textsuperscript{12} showed endothelial damage by a 10 mm. diameter application of liquid nitrogen on the rabbit cornea and the defect was covered in three days. With 6 mm. diameter endothelial damage induced by a freezing device, Capella\textsuperscript{13} reported complete coverage of the area within six days in the rabbit and seven to ten days in the monkey.

Using corneal thickness as an index of endothelial function, we have followed the time course of corneal swelling and de-turgescence after cryothermal injury to the rabbit corneal endothelium. This has been correlated with the morphologic appearance of the endothelium during its regenerative process.

**Methods**

The experiments were carried out on 40 eyes of 20 adult albino New Zealand giant rabbits weighing 3 to 4 kilograms. Lightly proptosed eyes were anesthetized by topical application of proparacaine HCl (Ophthaine, Squibb, New York). A cryoprobe, 2 mm. in diameter, was used to freeze the central region of the cornea, and a temperature of \(-65^\circ\) C. was maintained for 15 seconds. This technique created an ice ball of about 4 mm. in diameter which extended into the anterior chamber.

Corneal thickness was measured at four sections from the limbus to the center of the cornea at about equal distances, using a Haag-Streit Model 360 slit lamp with a pachometer attachment,\textsuperscript{15} prior to freezing, half an hour after freezing, then every two hours for 12 hours, and every 24 hours for 8 days. The zones in which thickness was measured were centrally (or about 7.5 mm. from the limbus) and at 6 mm., 4 mm., and 2 mm. from the limbus. Thickness was measured once on each cornea at each time interval. Obviously, because of the use of eyes for histology, the numbers of eyes became less as time progressed.

For histologic observation the rabbits were killed immediately after freezing, and at 6 hours, 24 hours, 2, 3, 4, 5, 6, and 7 days, 2 weeks, 3 weeks, and 3 months after freezing. The cornea was removed with a rim of sclera attached, and flat preparations of endothelium were made using the technique of Smolin.\textsuperscript{16} The silver-hematoxylin-celestine blue-staining technique was utilized, which resulted in the cell boundary delineated by the silver nitrate and some cellular detail by the hematoxylin-celestine blue stain.

**Results**

**Corneal thickness.** The average corneal thickness in these animals (40 eyes) was $0.375 \pm 0.004$ mm. (S.E.M.) centrally, $0.381 \pm 0.005$ mm. near the limbus, $0.379 \pm 0.003$ mm. and $0.378 \pm 0.003$ mm. approximately 4 and 6 mm. away from the limbus. The initially frozen corneas showed swelling by the time of corneal thickness determination, but the corneas of the second half of the group of rabbits did show some thin-
Fig. 1. Central corneal thickness of cryothermally injured corneas. The corneas were frozen serially with thickness measurements immediately after the completion of freezing in the total group of animals. Only after freezing were all thickness measurements made. It was noticed that the second half of the group of animals all showed a thinning of the cornea (delay from freezing about 10 minutes), whereas the first half of the group showed only a swelling (delay from freezing about 40 minutes). This phenomenon is presumably related to the time after freezing that corneal thickness was measured and it appears that the initial response to freezing is thinning of the cornea by about 25 per cent.

Although the general pattern of corneal thickness fluctuations after freezing and during the healing process was identical in all four regions measured, there was a distinct difference in the amount of swelling in the central and peripheral portions of the cornea (Fig. 3). The swelling was almost always both greater and at a higher rate at the very central portion of the frozen zone and least near the limbus.

**Histologic observations.** Immediately after freezing, all of the endothelial cells were lost over the frozen area, except for those cells which underwent complete disruption and portions of their cellular contents remained adherent to Descemet's membrane. There was a zone of partially damaged endothelial cells around the margin of the central denuded area (Fig. 4), which was approximately 4 mm. in diameter. Six hours after freezing, early signs of migration of endothelial cells from the periphery of the denuded area could be seen. In 24 hours, there was an active migration of endothelial cells from the periphery to the center. The cells were elongated and looked like fibroblasts (Fig. 5). Within 48 hours, all the denuded Descemet's membrane was covered by regenerated endothelial cells, with the exception of the very central portion. The cells were large and irregular with some multinucleated cells, with some other scattered cells showing mitotic activity (Fig. 6). After three days, the previously denuded central area was completely covered, but the cell patterns were identical to the previous specimens. Over the subsequent four days (days 4, 5, 6, and 7) there was a progressive increase in the number of normal cells throughout the destroyed area concurrent with a gradual reduction in the number of irregular cells. Two weeks after freezing, except for a few scattered islands of large irregular cells, all the cells had regained a normal appearance (Fig. 7). Three months after freezing, the endothelial cells had a normal appearance although scattered multinucleated cells were still visible.
Fig. 2. Corneal thickness at various distances from the limbus during the initial 24 hours of freezing. • • •, central corneal thickness; • • •, 6 mm. from the limbus; • • •, 4 mm. from the limbus; • • •, 2 mm. from the limbus. Error bars are ± S.E.M.

Fig. 3. Corneal thickness at various distances from the limbus over eight days after freezing. • • •, central corneal thickness; • • •, 6 mm. from the limbus; • • •, 4 mm. from the limbus; • • •, 2 mm. from the limbus. Error bars are ± S.E.M.

Discussion

We have used a technique which provided a uniform complete endothelial injury, in a well-defined area, without damage to Descemet's membrane. The immediate effect of freezing was a transient thinning of the frozen portion of the cornea. There was no immediate epithelial loss and we attribute this thinning phenomenon to the shrinkage of the corneal stroma as a function of the extreme low temperature. Corneal swelling started within an hour and reached a maximum within 24 hours; at this time the peripheral 1 mm. of defect was covered by regenerating endothelium.

In 48 hours, although most of the de-
Fig. 4. Histologic appearance of the endothelium immediately after freezing. Note completely denuded area of Descemet's membrane, and dead endothelial cells at the margin of the lesion. x37.

Fig. 5. Histologic appearance of corneal endothelium 24 hours after freezing. Note the appearance of elongated, fibroblast-like cells emerging from the edge of the lesion. x37.

Fig. 6. Appearance of corneal endothelium 48 hours after freezing. Note the almost complete covering of the previously denuded area of Descemet's membrane. The appearance of the cells, however, is abnormal with many giant and multinucleate cells. x100.

Fig. 7. Appearance of corneal endothelium two weeks after freezing. Note the almost normal appearance of the endothelium with a few islands of large multinucleate cells. It is impossible to distinguish the previous margin of the lesion. x37.

nuded area was covered by endothelium, there was very little reduction of corneal swelling. On the third day, the corneal thickness was almost twice as much as that of a normal cornea, despite complete coverage of the denuded Descemet's membrane. As the number of endothelial cells in the injured area increased progressively between days three and eight, there was simultaneous reduction of corneal edema. This observation indicates that the functional capacity of regenerated endothelium lags behind the apparent morphologic repair of the area by four to five days. The large flattened endothelial cells apparently are not able to provide the adequate physiologic function needed for normal corneal deturgescence. The clinical counterpart of this phenomenon can be seen in Fuch's endothelial dystrophy: when loss of endothelial cells is compensated for by flattening of adjacent cells; these cells are not able to provide adequate physiologic function and this leads to stromal and epi-
Regenerating endothelium

The rate of deturgescence of isolated normal rabbit cornea is 60 µ per hour\textsuperscript{17, 18} and if complete covering of Descemet's membrane is achieved after freezing with a functionally sound endothelium one would expect the cornea to recover normal thickness in eight to ten hours from the thickness found here. The lag, therefore, between the morphologic and physiologic recovery may be related to the formation of fully operational cellular functions to assure the recovery of an endothelial permeability which is within normal limits; although migrating cells cover the denuded area, obviously a more complete morphologic recovery is necessary for corneal deturgescence. Another possibility is that the regenerating cells lack the capacity to operate fully to produce corneal deturgescence through operation of a pumping mechanism. Since the new, invading cells are not damaged, however, a full physiologic function of the cell from the moment of coverage of Descemet's membrane seems likely and the junctional difference appears more likely as an explanation for the impaired recovery of physiologic function.

In our experiment, using a 2 mm. applicator, we found a complete loss of endothelium over an area 4 mm. in diameter with a temperature of \(-65^\circ\text{C}\) for 15 seconds. The rate of endothelial regeneration in our series was identical to that reported by Chi and Kelman,\textsuperscript{19} but slower than that described by Faure, Kim, and Graf.\textsuperscript{12} The difference is probably due to the presence of intact or partially injured cells at the center of a frozen 10 mm. diameter area, due to the inability to adequately freeze such a large area, which led to complete coverage in three days.

REFERENCES