Corneal fine structure in experimental scorbutus

D. F. Sulkin, N. M. Sulkin, and H. Nushan

Cornea of scorbutic but otherwise normal guinea pigs were studied with the electron microscope. The corneal epithelium of the guinea pig on an ascorbic acid-deficient diet showed variable degrees of changes depending on the duration of the nutritional insult. The fine structural alterations observed included marked epithelial edema with enlarged intercellular spaces, but with intact desmosome attachments, dilated endoplasmic reticulum, and swollen mitochondria. Also observed were degenerating cells in the middle layer and extensive changes in the basal cell profiles with dense cytoplasm, loss of hemidesmosomes, interrupted basement lamina, and protruding cytoplasm into the stroma with concurrent changes in the stroma. The collagen pattern was disrupted, some of the modified keratoocytes showed great activity. Vascular invasion was noted in two of the corneas studied. The alterations observed here are similar to those described in a variety of pathologic conditions of the cornea. It is suggested that ascorbic acid has a direct influence on maintaining the integrity of the cornea.

Key words: ascorbic acid-deficiency, cornea, corneal epithelium, basement lamina, anterior stroma, collagen, vascularization, electron microscopy, corneal edema, hemidesmosomes.

Ascorbic acid is widely distributed throughout the tissue of the animal body and a high concentration of the vitamin occurs in the eye, especially in the corneal epithelium. Very little progress has been made in determining the function of ascorbic acid in the metabolism of the eye. It has been well established that ascorbic acid is necessary for the maintenance of mesenchymal cells and the fibrous protein, collagen, which they produce. Collagen is the main constituent of the corneal stroma.

A comprehensive study by McTigue on the fine structure of the normal human cornea and certain corneal dystrophies has resulted in a re-examination of the layers of the cornea. This study has emphasized the presence of a basal lamina separating the epithelium from the Bowman's layer as a separate anatomic entity. Its adhesive role to the corneal epithelium is still not well understood. Recent observations deal with epithelium-stroma interaction and indicate that the epithelium may play an important role in stromal metabolism.
In our laboratory, studies have shown that a vitamin C-deficient diet in the guinea pig will result in severe cellular changes in the autonomic ganglion cells and their satellite cells. Increase in agranular vesicles, breakdown, and distention of the rough endoplasmic reticulum and loss of ribosomes suggest faulty protein synthesis. These alterations were reversed after the administration of ascorbic acid. The reappearance of collagen in the endoneural spaces in recovery stages, notably absent during the nutritional insult, also suggest that the satellite cells may be involved in collagen synthesis. A further study on the spinal ganglia during ascorbic acid deficiency supports these findings and adds to the growing body of evidence that cell types other than the fibroblast are involved in the synthesis of collagen.

The purpose of this present investigation is to study the effect of vitamin C-deficiency on the corneal epithelium and the influence the epithelium and basement membrane have on the anterior portion of the stroma.

Material and methods
The guinea pig was used in this study since it is one of the only known nonprimates which cannot synthesize its own vitamin C. A total of 20 young female guinea pigs weighing 200 to 250 grams were placed on a scorbutigenic diet (Nutritional Biochemical, Cleveland Ohio). These animals were maintained on the diet as long as 32 days. Four additional animals were fed ad libitum with a supplement of fresh leafy vegetables. These were used as controls. The animals were closely observed. They gained weight on the scorbutogenic diet for the first ten days. Their weight leveled off and then started to decrease. By the twenty-fifth day all animals were visibly affected. A previous experiment in which a group of normal healthy animals were pair-fed with a group on a scorbutogenic diet indicated that inanition was not involved in the experimental changes.

Animals undergoing vitamin C deficiency were anesthetized at appropriate intervals by an intraperitoneal injection of 0.35 mg per gram chloral hydrate. The central corneal tissues were removed by a number of techniques, followed by several types of fixation. Best results were obtained in removing the whole eye, placing it in 4 per cent buffered glutaraldehyde for several hours, dissecting out the central portion of the cornea, and placing it back into the fixative. This was followed by post-fixation in 2 per cent osmium tetroxide. Blocks of tissues were dehydrated rapidly through a graded series of cold ethanol and embedded in Epon 812. Flat capsules were used to facilitate orientation of the layers of the cornea. Thick sections 0.5 to 1 μ were cut with glass knives on a Porter Blum ultramicrotome stained with 0.1 per cent toluidine blue in 1 per cent borax and studied with the light microscope for identification purposes. Thin sections, light gold to silver, were then cut with a diamond knife, mounted on 300 mesh uncoated grids and double stained with uranyl acetate and lead citrate. The sections were examined with a RCA EMU-3F electron microscope.
Results

The ultrastructural profile of the normal cornea of the guinea pig showed the well organized characteristic layers. Minute villi were observed projecting from the surface cell of the epithelium (Fig. 1). A tear film covered this irregular surface explaining the smooth appearance usually seen in the light microscope. The cell membranes of the epithelium were seen closely opposed and interdigitated and connected by numerous desmosomes (Figs. 1 and 2). The cytoplasm of the basal epithelial cell was granular and highly filamentous, giving it an electron dense appearance. Hemidesmosomes were distinct and formed sites of attachment of the basal cells to the basal lamina. The basal lamina was continuous. The Bowman's layer was composed of irregularly arranged collagen (Fig. 2).

The rest of the stroma was characterized by an extremely orderly arrangement of collagen fibrils and keratocytes (Fig. 3).

The corneal epithelium of the guinea pigs on an ascorbic acid-deficient diet showed variable degrees of changes depending on the duration of the nutritional insult. Extensive degenerative changes were seen in animals which had been maintained on a vitamin C-free diet for at least 25 days. A majority of the surface cells appeared edematous with intercellular spaces markedly enlarged and presumably filled with fluid (Figs. 4 and 5). The mitochondria appeared swollen and deteriorated (Fig. 5). Desmosomes were still intact although the spaces were distended (Fig. 4). The middle layer also showed varying stages of edema, degenerate mitochondria, and dilated endoplasmic reticulum (Figs.
Fig. 3. Stroma of cornea taken from control guinea pig is composed of collagen fibrils particularly well oriented. Keratocyte in the middle field appears to have an elongated nucleus with a minimal amount of cytoplasm. (x24,000.)

6 and 7). Some of the cells seemed to be in a disintegrating state (Fig. 7).

The cytoplasm of the basal cells appeared dense and the filaments less prominent due to the general flattening of the cells. Prominent vacuoles and sequestration of dense bodies were sometimes observed (Fig. 8). Cytoplasmic projections were prominent contributing to the irregular infoldings of the epithelial base with basement membrane thickening in the areas of these projections (Fig. 9). The basement lamina was interrupted frequently, the hemidesmosomes absent or incomplete (Fig. 8).

When extensive alterations were observed in the epithelium, changes were also found in the stroma. The Bowman's layer was indistinct or absent in some areas, and replaced by disorganized collagen fibrils (Fig. 8). The collagen pattern was disrupted and modified in the anterior stroma (Figs. 8 and 10). The keratocytes showed great activity, vacuoles were prominent, and large filamentous bodies with free ribosomes were noted (Fig. 10). The regular spaces between keratocytes were no longer maintained and it was not uncommon to see an aggregate of two or three fibroblasts (Fig. 10). In two of the animals studied, vascular invasion of the central cornea was observed (Fig. 11). These vessels were found in the anterior portion of the stroma. In both instances
Fig. 4. Corneal epithelium taken from animal on vitamin C-deficient diet for 26 days. Edema present as indicated by markedly enlarged intercellular spaces. (×34,000.)

the animals had been exposed to the nutritional insult for the maximum time.

Discussion

The fundamental role played by ascorbic acid in metabolic processes is not well understood. Heath described the distribution and possible functions of ascorbic acid in the eye. Although scurvy is now a rare disease, he reviewed a previous work by Hamilton who described four cases of intraocular hemorrhage which was attributed to dietary ascorbic acid deficiency and which responded well to ascorbic acid therapy.

Previous studies have shown experimentally the importance of vitamin C in metabolism of the cornea. Campbell and Ferguson reported an experiment in which they produced heat injuries to the cornea of control and scorbutic guinea pigs. They found that vascular invasion occurred with significantly greater frequency in the scorbutic group than in the controls. Thomas in his review concluded that ascorbic acid is believed to have an influence in the healing of corneal ulcers and a deficiency of this vitamin may result in a slow repair of such a pathologic process; that repair of collagenous tissue is dependent upon ascorbic acid which is known to occur in high concentration in the cornea.

Recently, electron microscopic studies of
Fig. 5. Superficial corneal epithelial cells taken from guinea pig on a vitamin C-deficient diet 28 days. Edema present, intercellular spaces were enlarged with intact desmosomes. Note swollen mitochondria. (x35,000.)

The studies of various types of corneal dystrophy have indicated severe alterations in the epithelium and stroma. Changes in the corneal epithelium are enlargement of intercellular spaces, swollen mitochondria, some separation of cells, absence of or interruption of thickened basement membrane, and stromal alteration.

In recent publications, emphasis has been placed on metabolic interaction of stroma and epithelium. These investigations have dealt mainly with stromal wound healing. In experiments with corneal incisions in rats, Dunnington and Weimar have shown that removal of epithelium delays transformation of kerocytes into fibroblasts. Gnadinger and co-workers, in an autoradiographic study, demonstrated that the epithelium participated actively in the biosynthesis of stromal collagen. Tritiated proline was administered to eyes with perforating corneal incision 5 to 9 days after the incision, to
**Fig. 6.** Intermediate layer of cells of corneal epithelium of guinea pig on scorbutic diet for 26 days. Edema and degenerating mitochondria. (×34,000.)

**Fig. 7.** Intermediate layer of cells of corneal epithelium of guinea pig on vitamin C-deficient diet for 25 days. Effect of diet shows distended endoplasmic reticulum and degenerative forms of mitochondria. In upper cornea a degenerating cell is present. (×24,000.)
evaluate the biosynthesis of collagen under different experimental conditions. There was significantly less proline uptake in wounded cornea in which the epithelium was scraped as compared to wounds with the epithelium left undisturbed. The fact that the epithelium was heavily labeled with the isotope strengthens the hypothesis of the epithelium being directly involved in the production of collagen or its precursors. Dohlman in a recent review emphasized the importance of interaction of epithelium and stroma both in noninjured stroma and stromal healing. He also postulated that stromal ulcerations may be caused by enzymes that are released by...
cells which become abnormal by the disease process. This process causes changes in the epithelium which in turn responds by releasing proteolytic enzymes which can float out to the tear film and also digest some of the stroma.

The data presented in this study indicate that the alterations observed in the cornea of the scorbutic but otherwise normal intact animals are very similar to those described in a variety of pathologic conditions of the cornea. These alterations included marked edema with enlarged intercellular spaces, dilated endoplasmic reticulum and swollen mitochondria in surface cells, degenerating cells in the middle layer and extensive changes in basal cell profiles with dense cytoplasm, loss of hemidesmosomes, accumulation of dense bodies, interrupted basement membrane and protruding cytoplasm with concurrent changes in the stroma and, in some instances invasion of blood vessels into the central cornea. Since vascularization was not a constant alteration observed in the scorbutic cornea, more experimental data will be necessary to establish its relationship to avitaminosis C.

The nutritional insult may very well initiate changes in the corneal cells similar to those observed in injury caused by chemical, bacterial, genetic, or viral factors. These changes are reflected both in the epithelium and the anterior stroma, and suggest that ascorbic acid has a direct influence, not yet understood, on maintain-
Fig. 11. Corneal stroma from guinea pig on vitamin C-deficient diet for 30 days. Vascular invasion and disorganized stromal lamellae. (x7,800.)

ing the integrity of the corneal epithelium, the basement membrane and, consequently, the stroma.

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REFERENCES

17. Kayes, J., and Holmberg, A.: The fine struc-