The overall morphology of corneal fibroblasts in the rat was examined by scanning electron microscopy after removal of stromal connective tissue elements by trypsin digestion and HCl hydrolysis. Fibroblasts thus exposed were flattened and stellate cells with a diameter of about 10 μm and had a number of ramified cytoplasmic processes. The processes of neighboring fibroblasts contacted with each other to form an extensive and continuous network structure parallel to the plane of collagenous lamellae. The cellular network appears to constitute an integrated system which may be involved in the synchronized regulation of the metabolic and physiologic homeostasis for the maintenance of corneal transparency. Invest Ophthalmol Vis Sci 29:1887–1890, 1988

The corneal stroma occupies about 90 to 95% of the total thickness of the cornea. The corneal stroma is responsible not only for the maintenance of the physical strength and the curvature, but also for the maintenance of transparency of the cornea. The major cellular components of the corneal stroma are the corneal fibroblasts (keratocytes). Although these cells are located dispersedly throughout the entire corneal stroma and they occupy only 2 to 3% of the total volume of the corneal stroma, the corneal fibroblasts play an important role in the active synthesis and secretion of collagen, proteoglycans and other extracellular proteins. Thus, the corneal fibroblasts serve for the maintenance of metabolic and physiologic homeostasis of the cornea.2,3

A considerable number of studies by transmission electron microscopy (TEM) have elucidated structural characteristics of the corneal fibroblasts. Hogan et al illustrated the three-dimensional morphology of the cells diagrammatically based on the observation of ultrathin sections.4 Histological studies under a light microscope after silver or gold staining showed the possible network structure of the corneal fibroblasts and suggested the apparent continuity between the processes of adjacent corneal fibroblasts.5-8 Although there were many ideas suggested about the network structure of the corneal fibroblasts, there was no direct observation on the intercellular connections between the fibroblasts in the corneal stroma. Recently, we reported the presence of gap junctions between the corneal fibroblasts of rabbits by transmission electron microscopy and by a freeze-fracture technique.9 The junctional complexes between the corneal fibroblasts previously reported were hemidesmosomes6,10 or macula occludens.4 However among these junctional complexes, small molecules and ions can be transmitted between the neighboring cells only through the gap junctions.11-13 Thus each connecting cell can exchange information through gap junctions. The presence of gap junctions and posited network structure of the corneal fibroblasts suggested to us that the cells coordinated and synchronized to main-

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tain the metabolic homeostasis of the corneal stroma. However, because of the limitation of a light microscope, the resolution of those previously reported figures was not satisfactory. The current study is an attempt to disclose the overall shape, arrangement and interrelationship of the corneal fibroblasts by scanning electron microscopy (SEM), by applying the denudation method which selectively removes extracellular connective tissue components. This sort of information provides a structural basis for our better understanding of the corneal function as a whole.

Materials and Methods. Young adult albino rats, Wistar-KY strain, were used. This study adhered to the ARVO Resolution on the Use of Animals in Research. Immediately after the animals were sacrificed by an overdose of chloroform, the whole eyeballs were dissected out and washed in phosphate-buffered saline (PBS, pH 7.2). The specimen preparation for SEM was basically the same as reported previously. In brief, the eyeballs were incubated in PBS buffered trypsin solution (Sigma type II, 2.5 mg/ml, St. Louis, MO) at 37°C for 30 min, followed by glutaraldehyde fixation. The corneas were excised and were post-fixed by OsO four. They were separated into two layers near the Descemet's membrane by fine forceps. The samples of the stromal layers were then treated with 8 N HCl at 60°C for 15 min. The specimens were pro-
cessed for SEM through conventional procedures and were observed with a JEOL JEM 840 scanning electron microscope (Tokyo, Japan).

**Results.** In mechanically teased preparation of the cornea without enzymatic digestion and acid hydrolysis, corneal fibroblasts were totally concealed within dense collagenous lamellae. After removal of collagen and extracellular materials, the fibroblasts were readily viewed by SEM (Fig. 1). Here the cells were more densely distributed than we had expected from the published TEM pictures. They were stellate and about 10 μm in diameter and were flattened in the direction parallel to the plane of collagenous lamellae. They possessed short and often branching processes ranging in diameter from 1 μm to 5 μm.

The processes of the neighboring cells overlapped and attached to each other, forming a continuous network which spread out in the plane parallel to the collagenous lamellae (Fig. 2). In some areas, the intercellular connections between the cells located in the different planes could be observed (Fig. 3). Here, very fine filaments less than 10 nm in diameter were present, which crisscrossed and overlapped each other to form an intricate network intermingled with the cellular network described earlier. As judged from their diameter and arrangement, these filaments appeared to represent individual collagenous fibrils distributed between the collagenous lamellae.

What appeared to be nerve fibers or fiber bundles were occasionally encountered in the cellular network (Fig. 4). They tended to take a straight course with a diameter of 2 to 3 μm along their length. They could be followed for a distance of more than 100 μm.

**Discussion.** In the current study, we demonstrated by SEM, for the first time, the overall morphology of corneal fibroblasts by removing stromal connective tissue components. In such tissues as skeletal muscle, which contain relatively small amount of collagen, the stromal cell surface is readily exposed for SEM by HCl hydrolysis alone. By contrast, the cornea contains a great amount of collagen fibrils densely aggregated in compact lamellae, while cellular components, mostly fibroblasts, occupy only 2 to 3% of the total volume of the cornea. We have tested various denudation methods, including HCl hydrolysis and collagenase digestion, but these resulted in insufficient removal of collagen fibrils and often caused serious tissue shrinkage and disruption. So far, tryptic digestion followed by HCl hydrolysis gives the most consistent and best results without obvious artificial products. There was little cell shrinkage; the size of fibroblasts in our materials almost coincides with that measured in published TEM pictures. The shape of the cells thus observed is quite similar to those observed in vivo by a tandem scanning reflected light microscope, as reported by Lemp et al.

Fibroblast-like cell networks have been demonstrated in the previous SEM studies in several tissues and organs and have been interpreted in many ways despite their basic morphological resemblance in different systems. Those found in the lamina propria of the rat intestine may act as a contractile cytoskeleton. Those in the cod liver have been suggested to form an organocytoskeletal system. In the rabbit...
colon, interstitial cells of Cajal were also shown to form a similar network and may play a mechanical role, namely, to safeguard intramural ganglia and nerve bundles from the vigorous movement of intestinal muscle contraction.\(^1\)

The fibroblasts in the cornea are generally accepted to be involved in the synthesis of collagen and other extracellular substances,\(^2,3\) but their continuous network formation has not been well-depicted previously. Although the intercellular junctional sites could not be detected by the current procedure, we have demonstrated a frequent occurrence of gap junctions between the rabbit corneal fibroblasts in ultrathin sections and freeze-fracture preparations,\(^9\) suggesting that the fibroblasts might function synchronously through the gap junctions and might be responsible for metabolic activities essential for the maintenance of corneal transparency.

In the present preparation, the fibroblasts were seen to form essentially a two-dimensional network spreading between the collagenous lamellae. However, some cells extended the processes to the different planes (Fig. 3). They may form a three-dimensional network throughout the whole thickness of the corneal stroma. Yukioka,\(^20\) in fact, reported the perpendicular connections between the cells in different layers in the edematous cornea.

Key words: network structure, intercellular connection, corneal fibroblasts, rat, scanning electron microscopy

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