Morphological Characteristics and Intercellular Connections of Corneal Keratocytes

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Purpose: To investigate the morphological characteristics of keratocytes and the interconnection of keratocytes with adjacent keratocytes using the flat preparation method and scanning electron microscopy with a frontal section of the human corneal stroma.

Methods: The thin, corneal collagen lamellae were carefully dissected from the cornea (n=7), which had been stained by the flat preparation method. The remaining tissue was fixed in 3% glutaraldehyde and observed by transmission electron microscopy following the frontal section.

Results: The flat preparation revealed the corneal fibroblasts between the lamellae of the collagen fibers and showed that the ramifying cellular processes of the keratocytes were in contact with the cytoplasmic processes or cell bodies of neighboring fibroblasts. Two types of discrete subpopulations of keratocytes were identified: a smaller, cellular type of keratocyte with spindle-shaped nucleus with heterochromatin, and a larger, cellular type with a large indented nucleus with relatively scanty cytoplasm. Collagen fibers ran parallel to each other toward the fenestration of the cytoplasmic wall of the keratocyte.

Conclusions: These flat preparation method results showed that the keratocytes within the corneal stroma are interconnected with the adjacent keratocytes, which indicates the presence of a functional communicating network through the keratocyte circuits within the stroma. A smaller, cellular type of keratocyte with spindle-shaped nucleus was morphologically differentiated from a larger, cellular type with a large, indented nucleus by flat preparation and transmission electron microscopy. *Korean Journal of Ophthalmology 19(3): 213-218, 2005*

Key Words: Collagen, Electron microscopy, Flat preparation, Intercellular space, Keratocyte

Keratocyte is a major cell comprising approximately 10% of the corneal stroma, and keratocytes are approximately 2-5% of the total volume of the cornea.[1] The number of keratocytes in adults is 24,000,000 cells.[2]

The regular arrangement of collagen fibers in the corneal stroma[3-6] and the filling of proteoglycan between the arrangement of collagen fibers are important factors to maintain the corneal transparency.[7] Numerous studies have reported that the arrangement of keratocytes within the corneal stroma is an essential factor for the maintenance of the corneal transparency.[1,8] However, there are almost no reports on the intercellular connection network except a few reports by scanning electron microscope (SEM).[9-11]

It has been reported that the removal of cornea epithelial cells reduced the number of corneal keratocytes due to apoptosis.[12,13] This occurrence of the apoptosis of corneal keratocytes after the removal of corneal epithelial cells suggests the formation of a connection network of corneal keratocytes with adjacent corneal keratocytes.

Various methods have been used to examine keratocytes;[14-17] nevertheless, by ordinary cross sectional observation, the arrangement of keratocytes can not be examined in detail, and furthermore, it is especially difficult to examine the intercellular connections.

In our study, to examine the characteristic of corneal keratocytes and their connection with adjacent corneal keratocytes, the corneal stroma was examined by the flat preparation method. In addition, to assess the morphological characteristics of keratocytes and their relationship with collagen fibers, after the serial sectioning of the corneal stroma by the frontal sections, they were examined by transmission electron microscope (TEM).

Materials and Methods

1. Materials

Seven eyes enucleated because of intraocular tumors, such as retinoblastoma, choroidal melanoma or eyeball rupture (age range from 1 to 67 years), were divided into two on the

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equator using a blade and the lens was removed. In the eyeball of the anterior segment containing the cornea, using the limbus as the edge, the cornea was separated using micro-scissors.

2. The flat tissue preparation of the corneal stroma

The separated cornea was divided in half using a blade and underwent electron microscope examination. On the edge area of the incised cornea, the lamellae of the corneal stroma were separated as much as possible into a single layer using micro-scissors. Since the anterior area of the corneal stroma is narrower and arranged more irregularly than the posterior area, during the lamellar corneal transplant, the separation of the corneal lamellae from the posterior area is easier than the anterior area.[18] Therefore, in our study, the anterior area of the corneal stroma was separated thicker than the posterior area because it is more difficult to separate as a single lamella than the posterior area. The sections separated as a single lamella were placed on slides, dried, stained with hematoxylin-eosin, and examined with a light microscope.

3. Electron microscopic examination

The arrangement of cells within the corneal stroma was prepared by the flat preparation method and underwent TEM examination of the serial frontal section, as it was impossible to examine the general sections by TEM.

Each tissue was fixed in 3% glutaraldehyde, postfixed with osmium tetroxide, dehydrated with ethyl alcohol, and embedded in the epoxy. The embedded sections were sectioned by an ultra-microtome into thicknesses of 1 μm, and stained with toluidine blue. The area to be observed was selected by light microscope, ultra-sectioned from 60 to 100 nm in thickness, double stained with uranyl acetate and lead citrate, and examined by TEM (Hitachi, Japan).

Results

1. Findings of the flat preparation method

For the collagen fiber layers that were separated into several layers and stained, the keratocytes were arranged in various directions (Fig. 1). Keratocytes in the collagen fiber layers were separated as 1-2 lamellae were arranged by maintaining a relatively constant distance and shape. In the flat preparation, the main morphology of the keratocytes was a star shape with 4-5 cytoplasmic processes (Fig. 2). The nucleus of the keratocytes was very big and thus occupied the most central area, around which spindle-shaped or very thin, thread-like cytoplasmic processes were observed.

In the collagen fiber lamellae separated as a single layer, keratocytes were arranged in one direction, and in such an arrangement, long and thin-shaped, cytoplasmic processes were characteristically observed (Fig. 3). Similarly, in kerato

Fig. 1. Multi-directional arrangement of the keratocytes is shown in the flat preparation of the thick-layered, lamellar flap. H-E stain ×100.

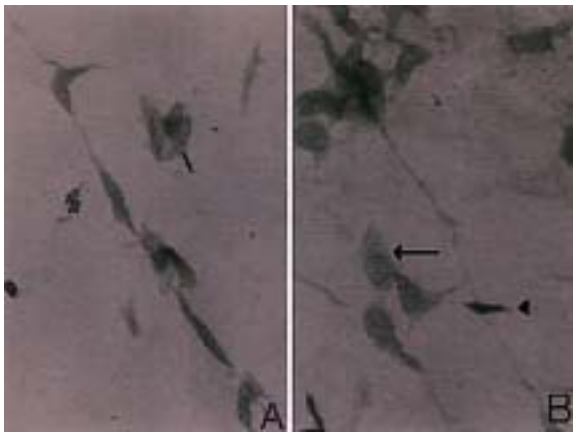

Fig. 2. (A) Star-shaped cytoplasmic processes are evident in the keratocytes of thin-layered, lamellar flap. (B) The nuclei occupy most of the central part of the keratocyte with spindle-shaped cytoplasmic processes. Long, slender cytoplasmic processes are also shown. Flat preparation, H-E stain ×400.

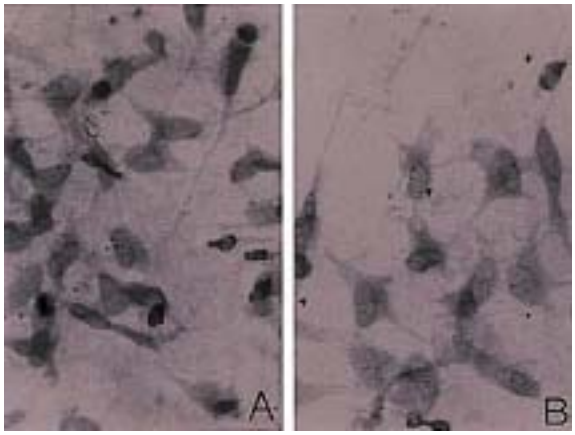

Fig. 3. (A) One-directionally arranged keratocyte showing the typical, long, slender cytoplasmic processes. (B) Two patterns of keratocytes staining are observed: small cell with strong staining (arrowhead) and large cell with weak staining (arrow). Flat preparation, H-E stain ×400.

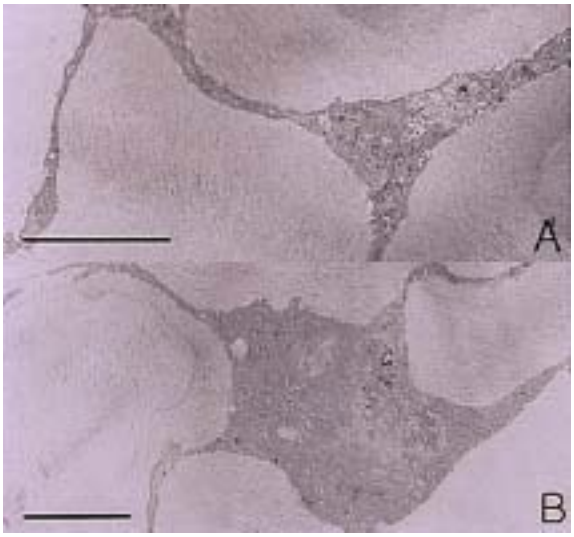

Fig. 4. (A) Syncytial arrangement of the keratocytes showing the thin, slender cytoplasmic processes connected by cellular junctions on one lamellar plane of collagen fibers in the frontal section. (B) Cytoplasmic processes formed by several keratocytes showing a star-shaped arrangement. Bar, 1 μm.

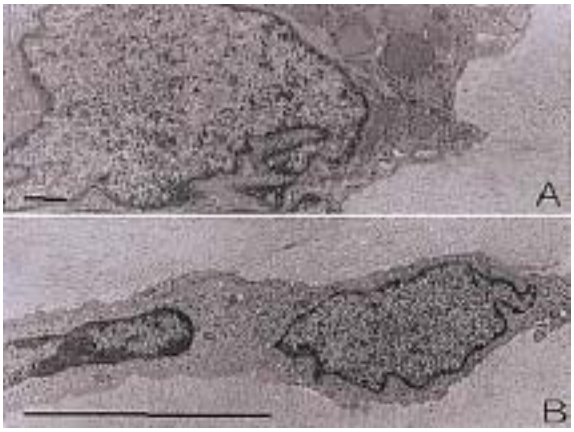

Fig. 5. (A) The indented nucleus occupying the major part of the cell. The cytoplasm surrounds the nucleus and has numerous microorganelles such as many stacks of rough endoplasmic reticulum and Golgi bodies with vesicles. Bar, 0.1 μm. (B) Two discrete subpopulations of keratocytes are connected by intercellular junction. The small, cellular type of keratocyte has a spindle-shaped nucleus with heterochromatin and the large, cellular type of keratocyte has a large, indented nucleus with relatively scanty cytoplasm. Bar, 1 μm.

cytes with long and thin, cytoplasmic processes, the nucleus occupied the most central area, and bilateral, thin and long cytoplasmic processes were presented. According to the shape of the processes, two types were observed: star-shaped cells with asteroid cytoplasmic processes and bipolar cells with thin and long, cytoplasmic processes. In addition, they could be classified into two types depending on cell size and staining condition. The staining condition of cells with a large nucleus and plenty of cytoplasm was slightly bright, while that of cells with a small nucleus and a small amount of cytoplasm appeared to be slightly dark (Fig. 3).

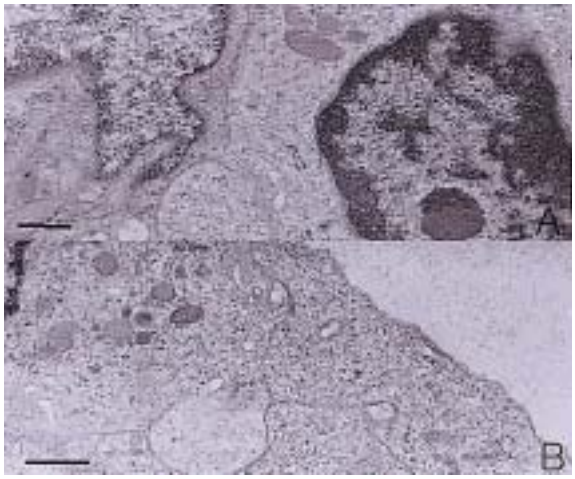

Fig. 6. (A) Small cellular type of keratocyte has a prominent nucleoli with peripheral heterochromatin in addition to numerous microorganelles within the cytoplasm. (B) Three or more keratocytes are connected by intercellular junction. The cells have many stacks of rough endoplasmic reticulum, and Golgi bodies with vesicles within the cytoplasm. Bar, 0.1 μm.

2. The results of electron microscope

In a single lamella of collagen fibers, several keratocytes were inter-connected, cytoplasmic processes became thinner in the periphery and they were connected to the adjacent keratocytes (Fig. 4). The nucleus was partially indented and occupied a large amount of cells, while micro-organelles in the cytoplasm of the vicinity of the nucleus comprised Golgi complex, vesicles, and stacks of rough endoplasmic reticulum (Fig. 5A). The nucleus was big, with a large-sized cell, and heterochromatin was detected in the nucleus. In addition, a cell pattern of small-nucleus cells contacting large-nucleus cells was observed (Fig. 5B). These two cell types were connected by the intercellular connection, and intracellular micro-organelles were more abundant in the small-nucleus cells than in the large-nucleus cells (Fig. 5, 6).

The keratocyte cytoplasm was filled with Golgi complex, vesicles, and stacks of rough endoplasmic reticulum, while the keratocytes were connected to the adjacent cells by intercellular connections. In this manner, the inter-connection within the corneal stroma between the corneal keratocytes was formed by 3 or more keratocytes (Fig. 6).

In keratocytes, numerous fenestrations were observed in the cytoplasmic wall, and collagen fibers traveled toward the direction of fenestration (Fig. 7). Along the frontal-section direction of the tissue keratocytes, the travel of collagen fibers was well detected, and along the fenestration of the cytoplasmic wall of the keratocytes, each collagen fiber traveled in a different direction (Fig. 7, 8).

Fig. 7. The keratocyte has multiple fenestrations on the cytoplasmic wall. The collagen fibers run toward the fenestrations of the cytoplasmic wall of the keratocyte. Bar, 0.1 μm.

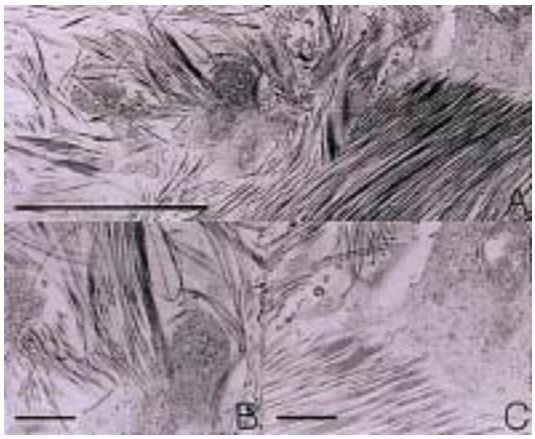

Fig. 8. (A) Many keratocytes are alternated with collagen fibers in this oblique section. Bar, 1 μm. (B) (C) Higher magnification of Fig 8A. Collagen fibers run parallel to each other toward the fenestration of the cytoplasmic wall of the keratocyte. Bar, 0.1 μm.

Discussion

Corneal stroma layers are formed by lamellae,[1,19-21] as keratocytes in the corneal stroma lamellae are connected by long cytoplasmic processes between collagen fiber layers,[21,22] and thus form a connection network of cells over a wide range within the corneal stroma.[8] In our study, collagen fiber lamellae were separated into several layers and stained, and it was found that keratocytes were arranged in several directions. This phenomenon appeared when keratocytes arranged in one direction in a single corneal stroma lamella were separated, thereby resulting in the arrangement of keratocytes in several directions. However, when collagen fiber lamellae were separated thinly, the ratio of keratocytes overlapping among the lamellae was lowered, and thus they were arranged in collagen fiber lamellae while maintaining regular intervals and shapes. In addition, besides the arrangement of keratocytes at regular intervals, an additional characteristic is the appearance of the keratocyte arrangement to specific directions. The above findings imply that keratocytes present in the corneal stroma that formed as lamellae are arranged regularly, and according to the layer of corneal stroma lamellae, that the arrangement and direction of keratocytes are different.

Keratocytes present between collagen fiber layers are thin and long, the nucleus is distinct, and the cytoplasm occupies most of them.[21] However, in our study, in the flat preparation, the most frequent keratocyte pattern was cytoplasmic processes forming star-shaped patterns. In addition, the keratocyte nucleus was big and thus occupied the most central area of the cell body, and the cytoplasmic processes were spindle shapes or very thin, thread-like shapes. Therefore, instead of the cytoplasm occupying the greater part as is generally known, the keratocyte nucleus occupied the greater part of the central area of the cell body. Our study findings were from the flat preparation results, and hence the morphological characteristics of the keratocytes may differ according to the method of observing them.

When the rat cornea was treated with trypsin and observed by SEM, the keratocytes formed numerous, thin cytoplasmic processes with a flat star-shaped pattern, were connected to the cytoplasmic processes of adjacent keratocytes and formed a continuous network parallel to the surface of collagen fiber lamellae.[10] Keratocytes were connected mutually to the collagen fiber lamellae of the corneal stroma in parallel; nevertheless, they may be connected across the lamellae.[1,9,20,22] The inter-connection across the lamellae could not be detected in our study due to the use of the flat preparation separation method according to the corneal lamellae in this study.

In the flat preparation, two keratocyte patterns were observed according to the pattern and size of cytoplasmic processes as well as the staining patterns. They were the star-shaped pattern with star-shape, cytoplasmic processes, and the bipolar shape with thin and long, cytoplasmic processes. Star-shaped cells are the cells forming a relatively regular arrangement with an unclear cell direction. In contrast, the characteristic of bipolar-shaped cells with long cytoplasmic processes is the direction rather than a regular arrangement. It has been reported that two keratocyte types were detected in different areas of the corneal stroma, and hence keratocytes are not functionally identical to each other.[16] In other words, cytoplasmic processes in the anterior corneal stroma are wide and thus it is difficult to distinguish them from the cell body, while the cytoplasmic processes in the posterior area are long, thin and branched occasionally.[16] However, these reported results are different from our two keratocyte types that we obtained in the flat preparation. In addition, the physiological difference of these two cell types has not been elucidated yet. Nevertheless, they were considered to be keratocytes showing a reaction different from each other in response to various physiological and physical reactions in the cornea. Nonetheless, the mechanism of these reactions has not been elucidated yet.

In addition, according to cell size and the staining pattern, keratocytes are divided into two patterns: cells with a large cell body nucleus and cell size and a somewhat weak staining pattern, or a small cell body nucleus and cell size with a somewhat strong staining pattern. These two types of cells are connected by intercellular connections, and micro-organelles in the cytoplasm of cells with a small cell body are more numerous than those in the cytoplasm of cells with a large cell body.

According to recent studies,[23,24] keratocytes are active, not inactive, cells, and micro-organelles in the cytoplasm are quantitatively few. The micro-organelles' Golgi complex, various vesicle shapes, and numerous stacks of rough endoplasmic reticulum, imply that keratocytes are very active cells involved in the synthesis and storage of protein, and in the absorption and release of substances from extracellular matrix or to the matrix. 8 Keratocytes are classified into bright cells and dark cells according to the high or low electron density, and it is thought that this variation in electron density is due to the difference of cell activity.

Most collagen fibers run along the surface of keratocytes,[21] and collagen fibers show the pattern that runs toward the keratocyte surface or originates from it.[9] In our study, in the cytoplasmic keratocyte wall, numerous fenestrations were observed, and collagen fibers ran toward the direction of such fenestration. Along the fenestration of the cytoplasmic keratocyte wall, collagen fibers ran to various directions. In the SEM examination of human cornea treated with chemicals, the collagen fiber layers were basically arranged in parallel on the corneal surface, and occasionally were connected to a portion of adjacent collagen fibers.[17] The fenestration of such keratocytes accelerates the diffusion of substances, and also involves the attachment of collagen fibers.[8]

In our study, there were 3 or more junctions among keratocytes: between the cell body and keratocyte cell body, between the keratocyte cell body and the keratocyte cytoplasmic processes, and between the cytoplasmic processes and the keratocyte cytoplasmic processes. In this manner, the keratocyte cytoplasmic processes protruded from one layer of collagen to another layer, which implies the mutual and continuous connection of the corneal stroma in the front and back. Keratocytes are thought to form a connected circuit though the gap junction between cells, and it has been reported that through such a network, they respond together to the injury of the cornea or inflammatory reaction and thus chemical signals or electric signals are transmitted.[25]

In addition, between corneal stroma lamellae, the inter-cellular contact of various keratocytes was observed, and the various directions of cytoplasmic processes and the intercellular interaction through the keratocyte network suggest a direct communication ability for the maintenance and recovery process of the stroma.[26]

In conclusion, from the results of using the flat preparation method, it was suggested that keratocytes within the corneal stroma are mutually connected to adjacent keratocytes and form a circuit among the keratocytes within the corneal stroma, and that they thus form a functional junctional relation. Using the flat preparation method and TEM, keratocytes with a pattern of a spindle-shaped nucleus and small cell size were distinguishable morphologically from those with a pattern of big cells with an indented nucleus and relatively small cytoplasm.

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