Intercellular Junctions Between Fibroblasts in Connective Tissues of the Eye of Macaque Monkeys

A Thin Section and Freeze Fracture Analysis

Giuseppina Raviola, Mary Jo Sagaties, and Celeste Miller

Thin-section electron microscopy and freeze fracture were used in the analysis of the intercellular junctions between fibroblasts in connective tissues of the eye of Macaca mulatta, M. fascicularis, and M. arctoides. Fibroblasts located in the subconjunctival loose connective tissue, anterior sclera, scleral spur, ciliary body stroma, and posterior choroid were joined by three kinds of junctions. Gap junctions were of different sizes and frequently composed of a small number of connexons organized in polygonal aggregates or linear arrays. Tight junctions were represented by isolated strands and never composed a continuous belt around the cells. Intermediate junctions were seen in thin sections but did not have any representation in the interior of the plasma membrane. It remains to be established whether, as is the case in other tissues, pathologic conditions of the eye are accompanied by some changes in the morphology and distribution of intercellular junctions between fibroblasts. Invest Ophthalmol Vis Sci 28:834–841, 1987

Cells in direct physical contact may be joined at specialized regions of the plasma membrane known as intercellular junctions. Two types of intercellular junctions have been described between fibroblasts in connective tissue of many different organs. Gap junctions between connective tissue cells have been reported in the embryo,1,2 between fibroblasts of the developing cornea3 and in tissue culture.4 In the adult, gap junctions have been observed between fibroblasts of interstitial loose connective tissue and between other cells of mesenchymal origin (eg, fat cells, osteocytes, and myofibroblasts). Specifically, gap junctions have been described between brown fat cells,4 bone cells,5 and periodontal fibroblasts,6-7 between fibroblasts of chorion laeve of human fetal membranes,8 and between fibroblasts in full-term human placentas.9 The presence of small pieces of incomplete tight junctions has been described between mesenchymal cells of fetal dermis10 and focal tight junctions have been observed between corneal fibroblasts.3

The aim of this study was to identify the types of intercellular junctions found between fibroblasts in a variety of ocular tissues in normal eyes. This study represents a necessary prelude for the analysis of pathologic conditions, which include changes in the functional activity and morphology of ocular fibroblasts.

Materials and Methods

Animals

The eyes of five Macaca mulatta, three M. fascicularis, and one M. arctoides were examined in this study. The animals were young adults or adults of either sex, and were treated according to the ARVO Resolution on the Use of Animals in Research.

Preparation of Tissues

Thin-section electron microscopy: With the animal sedated by an intramuscular injection of Ketamine HCl (10 mg/kg) and subsequently anesthetized with sodium pentobarbital (30 mg/kg), the eye globes were enucleated and opened with an equatorial incision. The tissues were fixed by immersion in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH, 7.4) containing 0.2% Ca Cl2. After dissection, the tissues were subsequently postfixed in a mixture of equal volumes of 2% osmium tetroxide and 3% potassium ferrocyanide in distilled water and stained en bloc with uranyl acetate. The specimens were embedded in a
mixture of Epon-Araldite, thin-sectioned, stained with uranyl acetate and lead citrate, and examined with a JEOL 100 CX electron microscope at 60KV (JEOL USA, Electron Optics Division, Peabody, MA).

Freeze fracture: For freeze fracturing, sections 200 µm in thickness were obtained from immersion-fixed tissue blocks with a Smith-Farquhar tissue chopper (Sorvall, Inc., Newtown, CN). The sections were equilibrated with 20% glycerol in cacodylate buffer (pH, 7.4). After mounting on gold specimen carriers, the tissues were rapidly frozen in the liquid phase of partially solidified Freon 22 (monochlorodifluoromethane) and stored in liquid nitrogen. The specimens were fractured in a Balzers 301 apparatus (Balzers High Vacuum Corp.; Santa Ana, CA) at a stage temperature of —115°C and shadowed with platinum and carbon. Replicas were cleaned in methanol and then sodium hypochlorite and examined under a JEOL 100 CX electron microscope (JEOL USA) at 60 kV.

**Results**

The following regions of the eye were examined in thin sections and freeze fracture replicas: subconjunctival loose connective tissue, anterior sclera, scleral spur, iris stroma, ciliary body stroma, and posterior choroid. In these tissues, fibroblasts could be recognized easily in thin section by their characteristic morphology. In freeze fracture replicas they could be distinguished from the other elements of the connective tissue by their elongated shape, relationship with bundles of collagen fibers, and openings of clusters of plasmalemmal vesicles at their surface. Where fibroblasts could be confused with elongated melanocytes, the latter were identified by the presence of melanosomes in their cytoplasm. In the ciliary body stroma, at the boundary with the ciliary muscle, the distinction between fibroblasts and elongated smooth muscle cells was facilitated by the presence of bundles of actin and myosin filaments in the cytoplasm of the smooth muscle cells. In addition, we knew that the smooth muscle cells of the ciliary muscle are richly innervated but are not joined to one another by gap junctions or other intercellular junctions (Personal observations: G. Raviola).

Three kinds of intercellular junctions were identified between the fibroblasts of the eye: gap junctions, tight junctions and intermediate (or adherens type) junctions. Each of these junctional types was found in all six of the ocular connective tissues examined in this study. In addition, the same morphologic characteristics of each junctional type were consistently observed in these ocular tissues.

**Gap Junctions**

Gap junctions were the most common intercellular junctions found between fibroblasts in all the ocular regions examined. In thin sections, gap junctions could easily be seen as regions of the cell surface at which adjacent plasma membranes run parallel course and the external leaflet of the adjacent plasma membranes were separated by a cleft of 2 nm in thickness (Figs. 1, 2). In replicas of fixed materials, the large gap junctions seen in thin sections appeared as aggregates of 8–9-nm intramembranous particles on the inner leaflet of the
plasmalemma (P-face) (Fig. 3). In addition to large gap junctions, the freeze-fracturing technique revealed the presence of minute gap junctions represented by either linear arrays of gap junctional particles (Figs. 4, 6) or irregular aggregations of small number of particles (Fig. 5).

Tight Junctions

In addition to gap junctions, the fibroblasts of the ocular tissues were linked by tight junctions. These tight junctions did not form a belt around the cells but were incomplete as to suggest fragments of tight junction strands scattered on the plasma membrane. They were difficult to detect with certainty in thin sections and could be better observed and analyzed in freeze fracture replicas. Some of these tight junctions closely resembled normal zonulae occludentes, except that they stopped abruptly without forming a complete band (Fig. 6). In most cases, however, the authors saw scattered strands either found singly or interconnected to a few other elements. That these strands represented points of fusion between plasma membranes was seen clearly when the plane of cleavage fortuitously left the plane of one membrane and crossed the intercellular space before penetrating the adjacent plasma membrane (Fig. 6, inset); on the P-face they appeared as high ridges decorated with a small number of particles, and on the E-face, as deep grooves filled with irregular particles. These short tight junctions could be distinguished easily from linear gap junctions because the strands of tight junctions tended to fracture with the E-face of the plasma membrane although the gap junction particles remained attached to the P-face (Fig. 6). Furthermore, gap junctional particles were usually larger and sepa-
rated from one another, and particles of the tight junctions tended to fuse to one another and form continuous cords or strands. Gap and segments of tight junctions could be found next to one another on the same cell, such as in Figure 7 in which a small gap junction appears surrounded by strands of tight junction.

**Intermediate Junctions**

In thin sections, intermediate junctions were found between fibroblasts and were characterized by a mat of fine filaments on the cytoplasmic surface of the junctional membranes and by an irregular intercellular cleft, 12-15 nm wide (Fig. 8, inset). In freeze fracture replicas, intermediate junctions could not be identified in the interior of the plasma membrane.

**Discussion**

The authors have observed that the fibroblasts of the ocular tissues are connected by three kinds of junctions: gap junctions, tight junctions and intermediate junctions. Such a finding has been possible through the application of two different techniques with complementary results. Linear and small gap junctions as well as isolated strands of tight junctions could be identified with certainty in freeze fracture materials but not in thin sections. On the other hand, intermediate junctions were evident in thin sections; however, as is the case with other tissues, they did not show any specialization in the interior of the plasma membrane.

The authors have observed that the gap junctions between fibroblasts in the connective tissues of the eye were of different sizes and possessed a pleomorphism that ranged from the planar hexagonal lattice of typical

---

**Fig. 4.** *Macaca mulata,* ciliary body stroma. A simple linear gap junction can be identified between fibroblasts. The arrowheads indicate the opening of caveolae as seen on the P-face (P) of the plasma membrane (×325,000).

**Fig. 5.** *Macaca mulata,* ciliary body stroma. The interior of the plasma membrane of a fibroblast shows clusters of gap junction particles (arrows). E = E-face and P = P-face (×235,900).
Fig. 6. Macaca mulatta, subconjunctival loose connective tissue. The P-face (P) of a fibroblast can be identified in the upper part of the figure. A small gap junction (GJ) made of two linear aggregates of particles is present. In the lower-right part of the figure, a tight junction (TJ) made of several furrows packed with continuous particles can be seen on the E-face (E) of the plasma membrane. The openings of caveolae that appear as shallow volcanoes are indicated by arrowheads. The inset shows a series of discontinuous tight junctions, and the arrowhead indicates the point at which the furrow of a tight junction on the E-face (E) of the plasma membrane continues across the intercellular space onto the P-face (P) of the adjacent cell. On this face, the junction appears as a ridge on the plasma membrane with a discontinuous row of intramembranous particles (x91,200, inset x55,800).
gap junctions to a simple linear aggregate. Similar diminutive junctions have been found in other tissues to be the only elements connecting electrotonically coupled cells.\textsuperscript{12-13}

The authors do not know whether the gap junctions present between fibroblasts in all the connective tissue of the eye allow communication between these cells throughout the entire organ. The changes in transmembrane potential of cells at various distances from a cell in which a current is injected has been recorded in different tissues. For instance, it has been observed that osteoblasts respond to the stimulus introduced into a cell at a distance of 44.2–320.2 \( \mu m \), salivary gland at 750–1100 \( \mu m \), and toad bladder at 18 \( \mu m \).\textsuperscript{5} These results may reflect the different geometry of the tissues and the electrical properties of the cells as well as the limitations of the techniques presently available; thus, the authors cannot exclude the possibility that fibroblasts can communicate for long distances.

It has been widely documented that gap junctions may be involved in developmental differentiation processes, contact inhibition of movement, and control of secretion of extracellular materials.\textsuperscript{14} Several investigators have postulated that gap junctions may play a role in pathologic conditions involving communication and transport mechanisms. It has been demonstrated in vivo that a decrease in the number of gap junctions resulted in a decrease of metabolic coupling between affected cells.\textsuperscript{15} Similarly, it has been shown that cultured cells demonstrating defective metabolic cooperation did not form gap junctions.\textsuperscript{16} A decreased area of gap junctions by 50% and a decreased number of gap junctions by 40% has been found in rats in which squamous cell carcinoma was present.\textsuperscript{17}

Tight junctions are of common occurrence in vertebrate epithelia as membrane specializations between adjacent cells that physically separate the interstitial and luminal spaces. Most commonly in fixed material,
strands of tight junctions separate with the P-face of the membrane and corresponding grooves are found in the E-face. We found this distribution reversed in the discontinuous tight junctions of ocular fibroblasts, a feature common to unixed material, and for reasons unknown to other cell types such as the endothelial cells of blood vessels and of the canal of Schlemm.

Freeze fracture of ocular fibroblasts demonstrated groups or short segments of strands, but in no case did these strands form a continuous or almost continuous barrier as observed in the fibroblasts of the lamina fusca in the hamster eye. The presence of small pieces of incomplete tight junctions has been described between mesenchymal cells of fetal dermis and in early chick embryos in cells that have left the epiblast to form mesenchymal structures (Hensen's node, juxtanodal mesenchyme, primitive streak mesenchyme). Because well-developed zonulae occludentes are found in the epiblast, the idea has been advanced that the focal tight junctions of the mesenchymal cells are remnants of the junctions between neighboring cells in the epiblast that become "diluted" either through cell division and/or cell movement. Similarly, focal tight junctions have been observed between corneal fibroblasts and interpreted as remnants from the epithelial precursors of the mesenchymal cells. The significance of these segmental tight junctions remains obscure. At present, the authors do not have precise information on the chemical composition of tight junctional strands, and whether tight junctions perform some other function in addition to composing epithelial and endothelial barriers. Therefore, the authors cannot exclude that discontinuous tight junctions might represent morphologic structures responsible for some unknown form of communication between adjacent cells.

The intermediate or adherens type junctions seem to provide attachment and mechanical stability to the network of cell processes of the fibroblasts. In these cell contacts, actin is characteristically associated with the cytoplasmic aspect of the plasma membrane; more recently, it has been shown that two additional proteins, vinculin and a 135-kilodalton protein, are found at these intercellular contacts. During development, it has been observed that intermediate junctions are consistently present in regions of the plasma membrane, which later contain gap or tight junctions. Such a temporal and spatial pattern of junctional development suggests that intermediate junctions are necessary for the establishment of gap and tight junctions. Although the life span and turnover of gap and tight junctions between fibroblasts is unknown, the authors cannot exclude the possibility that the presence of intermediate junctions represents, also in the adult animal, a necessary step in the establishment of other types of junctions.

At present, there is some important evidence that fibroblasts are involved in pathologic conditions of the eye. It has been reported recently, in human glaucomatous eyes, that an increase of extracellular materials is found not only in the inner and outer walls of the Schlemm canal but also in the anterior portion of the ciliary muscles where fibroblasts showed an increase of cytoplasmic organelles. On this basis it has been postulated that in human glaucoma a common factor stimulates both the cells of the trabecular meshwork and the fibroblasts of the ciliary body to produce specific substances that are then deposited into the sheaths of the elastic-like fibers or the basal lamina. Because these studies were performed on specimens originating from trabeculectomies, it was not possible to establish whether this "activation" of the fibroblast is a phenomenon limited to the region of the Schlemm canal and anterior portion of the ciliary body or whether it extends to the connective tissue of the whole eye. In addition, a more detailed analysis of the "activated" fibroblasts could be useful in detecting possible changes in the intercellular junctions between these cells.

Another interesting observation related to fibroblasts in glaucomatous eyes is that although the aqueous humor from cataract patients consistently inhibited the growth of their own subconjunctival fibroblasts, the aqueous humor of some glaucoma patients did not. Of important practical significance was the observation that a correlation exists between the success of a filtering operation and the ability of the patient's aqueous humor to inhibit the growth of fibroblasts in tissue cultures. A detailed morphologic study of the fibroblasts in these different groups of patients was not performed. It would be of great importance to establish whether fibroblasts belonging to glaucomatous eyes display morphologic characteristics, including intercellular junctions that are different from those of normal eyes.

Key words: fibroblasts, electron microscopy, eye, freeze fracturing, intercellular junctions

References

6. Shore RC, Berkovitz BKB, and Moxham BJ: Intercellular con-