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Abstract

To understand the development of the trabecular meshwork of the eye, floating cellular aggregates (multicellular spheroids) were formed from human trabecular cells in a non-adherent environment of culture and incubated for up to one month. Dissociated trabecular cells formed multicellular spheroids within one day in the non-adherent environment, and apoptosis continued to occur in the spheroids which had been initially filled with cells. The final structure after one month appeared as a meshwork of cells with large extracellular spaces. Epidermal and basic fibroblast growth factor (EGF and bFGF) protected trabecular cells in the spheroids from apoptosis and, as a result, kept the spheroids filled with cells even after one month. In the absence of excess EGF or bFGF, the multicellular spheroids grown in vitro from human trabecular cells mimicked the mesh-like structure of normal trabecular tissue. In constrast, under an excess of these growth factors, spheroids of high cellularity, resembling the abnormal trabecular tissues of patients with congenital glaucoma, were formed.

KEYWORDS: human trabecular cells, multicellular spheroids, basic fibroblast growth factor, epidermal growth factor, histology

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Reconstruction of Trabecular Tissue from Human Trabecular Cells as a Multicellular Spheroid

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To understand the development of the trabecular meshwork of the eye, floating cellular aggregates (multicellular spheroids) were formed from human trabecular cells in a non-adherent environment of culture and incubated for up to one month. Dissociated trabecular cells formed multicellular spheroids within one day in the non-adherent environment, and apoptosis continued to occur in the spheroids which had been initially filled with cells. The final structure after one month appeared as a meshwork of cells with large extracellular spaces. Epidermal and basic fibroblast growth factor (EGF and bFGF) protected trabecular cells in the spheroids from apoptosis and, as a result, kept the spheroids filled with cells even after one month. In the absence of excess EGF or bFGF, the multicellular spheroids grown *in vitro* from human trabecular cells mimicked the mesh-like structure of normal trabecular tissue. In constrast, under an excess of these growth factors, spheroids of high cellularity, resembling the abnormal trabecular tissues of patients with congenital glaucoma, were formed.

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Reconstruction of organs or tissues *in vitro* from their constituent cells provides a model for studying their developmental processes and also provides clues as to the regulatory factors in their homeostasis (1, 2). Cellular aggregates, called multicellular spheroids, which are formed in a non-adherent culture environment, can be used as one method to reconstruct a tissue from cells. Cell-cell interactions take place in the three-dimensional architecture of multicellular spheroids, in contrast with the usual adherent conditions of two-dimensional cultures (1-6). Cells in multicellular spheroids, therefore, show a greater tendency toward differentiation than to proliferation (6). Floating culture in a non-adherent environment has also been shown to induce transdifferentiation of retinal pigment epithelial cells to the neural retina (7) or to the lens (8) in the presence of basic fibroblast growth factor.

The trabecular tissue of the eye, located at the angle between the cornea and iris, forms a mesh-like structure of cells and plays a crucial role in regulation of the intraocular pressure. There is still controversy as to how the mesh-like structure, called the trabecular meshwork, develops during embryogenesis (9, 10). We previously demonstrated that multicellular spheroids formed by bovine and porcine trabecular cells produced a mesh-like structure after one month of culture in a non-adherent environment (11). However, large differences in the structure of trabecular tissues of the eye among different species pose a limit to application of the bovine and porcine models to human situations (9, 10). In this study, we used human trabecular cells to form multicellular spheroids and histologically examined their structures.

Materials and Methods

Trabecular tissues excised during trabeculectomy in 3 patients with primary open-angle glaucoma were used for culturing trabecular cells. Informed consent was obtained from each patient, and the procedure was in accordance with the Declaration of Helsinki. Human trabecular cells usually grew out of an explant of trabecular tissue after 2 weeks (12-16), which was placed in a well of a 24-well multidish (Corning Coster Japan, Tokyo, Japan) and

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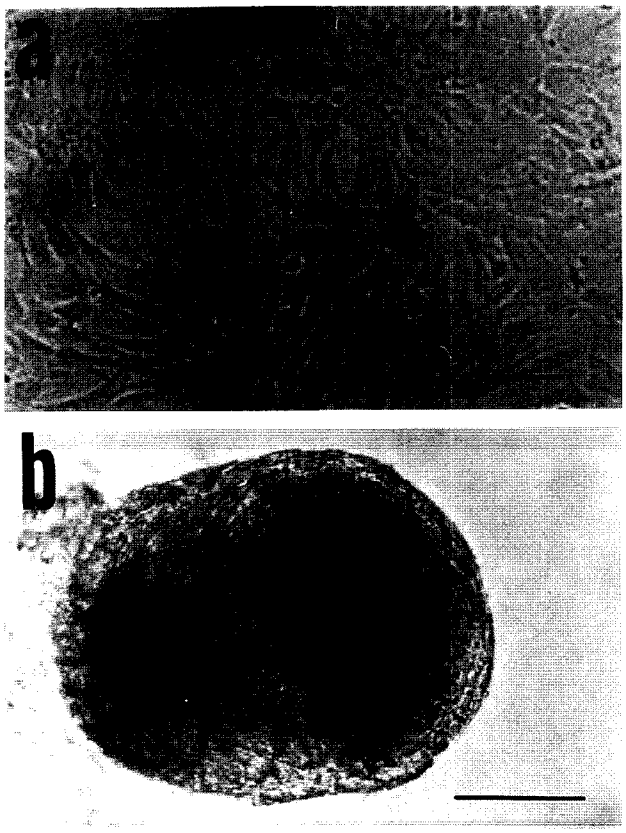


Fig. 1 Phase-contrast micrographs. (**a**) Human trabecular cells in a regular adherent dish. (**b**) A multicellular spheroid formed from human trabecular cells after one month in a non-adherent dish. Bar = 100 μm.

incubated in Dulbecco's modified Eagle's medium (DMEM: Nissui, Tokyo, Japan) supplemented with 10 % fetal calf serum (FCS), 100 mg/L streptomycin, and 100 mg/L ampicillin (Fig. 1a). Cells were then amplified in wells of a 6-well multidish (Corning Coster Japan) for 1–2 weeks. Cells were transferred to wells of a polystyrene 24-well multidish for suspension culture (Sumilon, Osaka, Jakan) after treatment with 0.25 % trypsin and 1 mM EDTA in Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution (Gibco BRL, Grand Island, NY, USA) for 5 min, and incubated in DMEM with 10 % FCS for one month under a humidified atmosphere of 5 % carbon dioxide and 95 % air.

Multicellular spheroids were observed by phase-contrast microscopy as well as by light and electron microscopy after their fixation with 3.7 % formaldehyde and 2.5 % glutaraldehyde, respectively, in 0.1 M phosphate buffer (pH 7.4). For scanning electron microscopy, glutaraldehyde-fixed spheroids were cracked bluntly with a razor blade and coated with gold. Epidermal growth factor (Human EGF, R&D Systems, Minneapolis, MN, USA) or basic fibroblast growth factor [Human FGF basic (157 aa), R&S Systems] was added to the medium at a final concentration of 100 ng/mL, and half a volume of the medium was changed twice a week. Cell lines derived from individual patients were used for the duration of the experiment without mixing or replacement.

Results

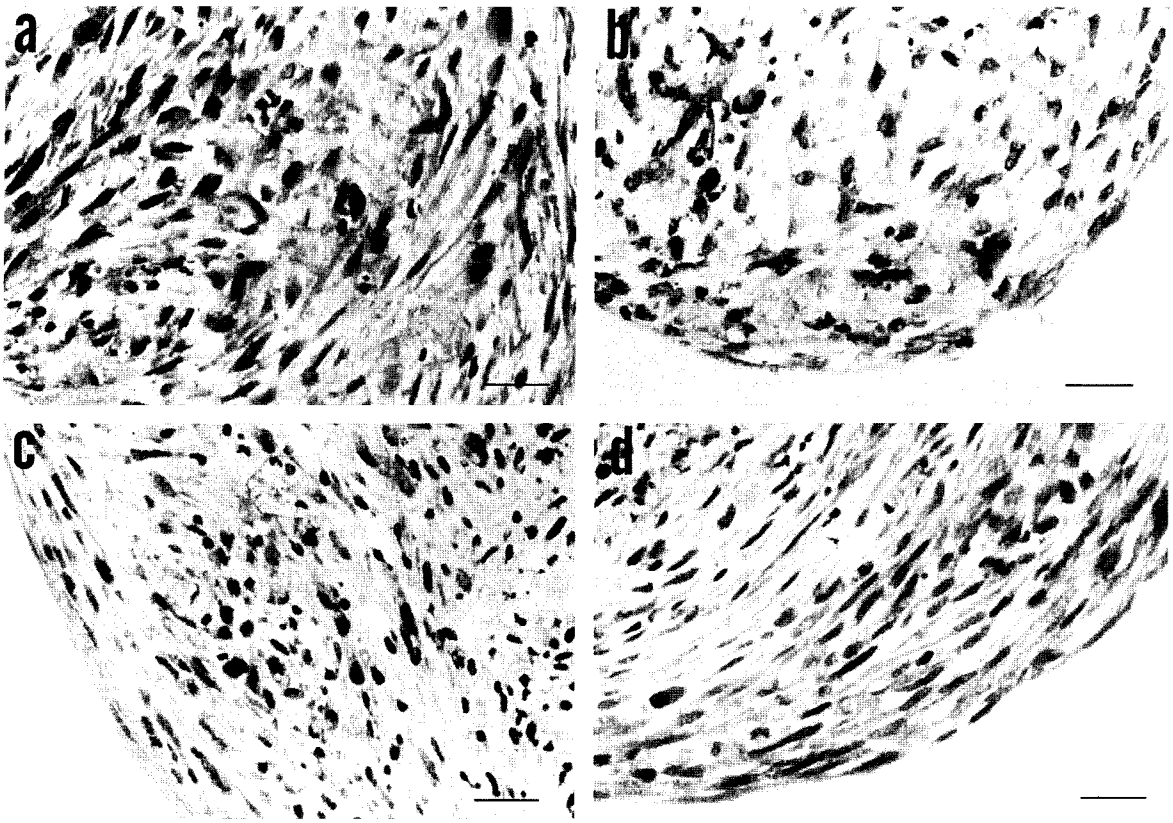
Human trabecular cells formed muticellular spheroids within a day after their dissociation by trypsin and transfer to the non-adherent dishes. The multicellular spheroids grew in size during the first week of culture but remained the same size during the month of observation which followed (Fig. 1b). Light microscopy showed that 3-day-old multicellular spheroids were filled with trabecular cells (Fig. 2a). After one month of culture, cells in the spheroids became sparse and formed a mesh-like structure (Fig. 2b and Fig. 3). Electron microscopy revealed that large extracellular spaces among viable trabecular cells, spreading like a meshwork, contained cell debris, apoptotic cells, and collagen fibers in one-month-old spheroids (Fig. 4, top). Cells showed such characteristics of trabecular cells (13–16) as abundant villous projections and coated vesicles along the cellular surface, osmiophilic cytoplasms with various organelles, and nuclei with a dark spiked band of peripheral chromatin (Fig. 4, bottom).

The presence of basic fibroblast growth factor (Fig. 2c and Fig. 5, top) or epidermal growth factor (Fig. 2d and Fig. 5, bottom) kept multicellular spheroids filled with trabecular cells for one month by reducing the number of cells going through apoptosis. Epidermal growth factor induced a change in the shape of trabecular cells to more elongated configurations.

Cells derived from each of the 3 patients showed the same morphological features of multicellular spheroids in the presence or absence of growth factors as described above.

Discussion

Cells grown from explants of trabecular tissue have been known to contain a small number of scleral fibro-



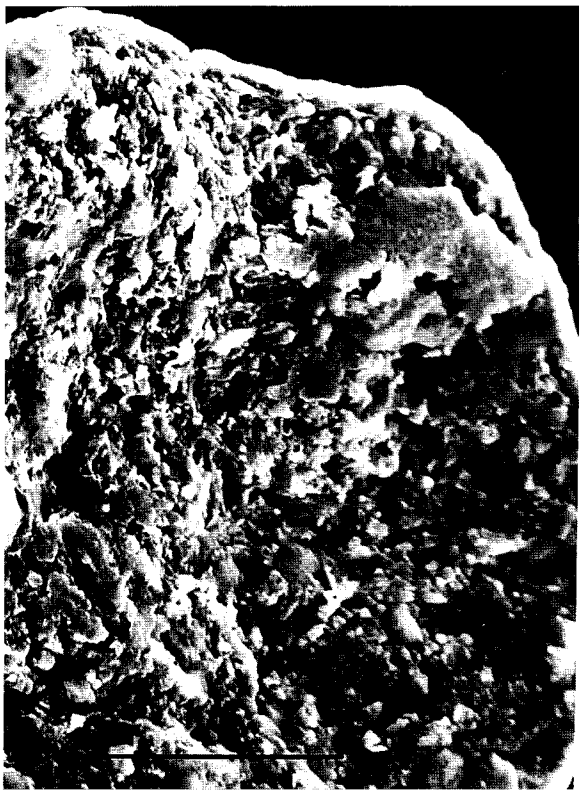


Fig. 2 Light micrographs of multicellular spheroids formed by human trabecular cells. A spheroid is filled with cells 3 days after its formation (**a**). Cells in a spheroid become sparse and spread like a mesh after one month (**b**). Basic fibroblast growth factor (**c**) and epidermal growth factor (**d**) keep spheroids full of cells even after one month. Hematoxylin-eosin stain. Bar = 20 μm.

Fig. 3 (Left) Scanning electron micrograph of the cracked surface of a multicellular spheroid formed by human trabecular cells after one month. Cells spread like a mesh inside the spheroid. Bar = 50 μm.

blasts in addition to the dominant population of trabecular cells (13–16). In this study, the morphological features of cells at confluency were consistent with those described previously for trabecular cells (12, 14, 15), and cells as early as the first and second passage were used for the formation of multicellular spheroids to avoid the growth of these fibroblasts. Electron microscopy confirmed that most cells in the multicellular spheroids had features of trabecular cells.

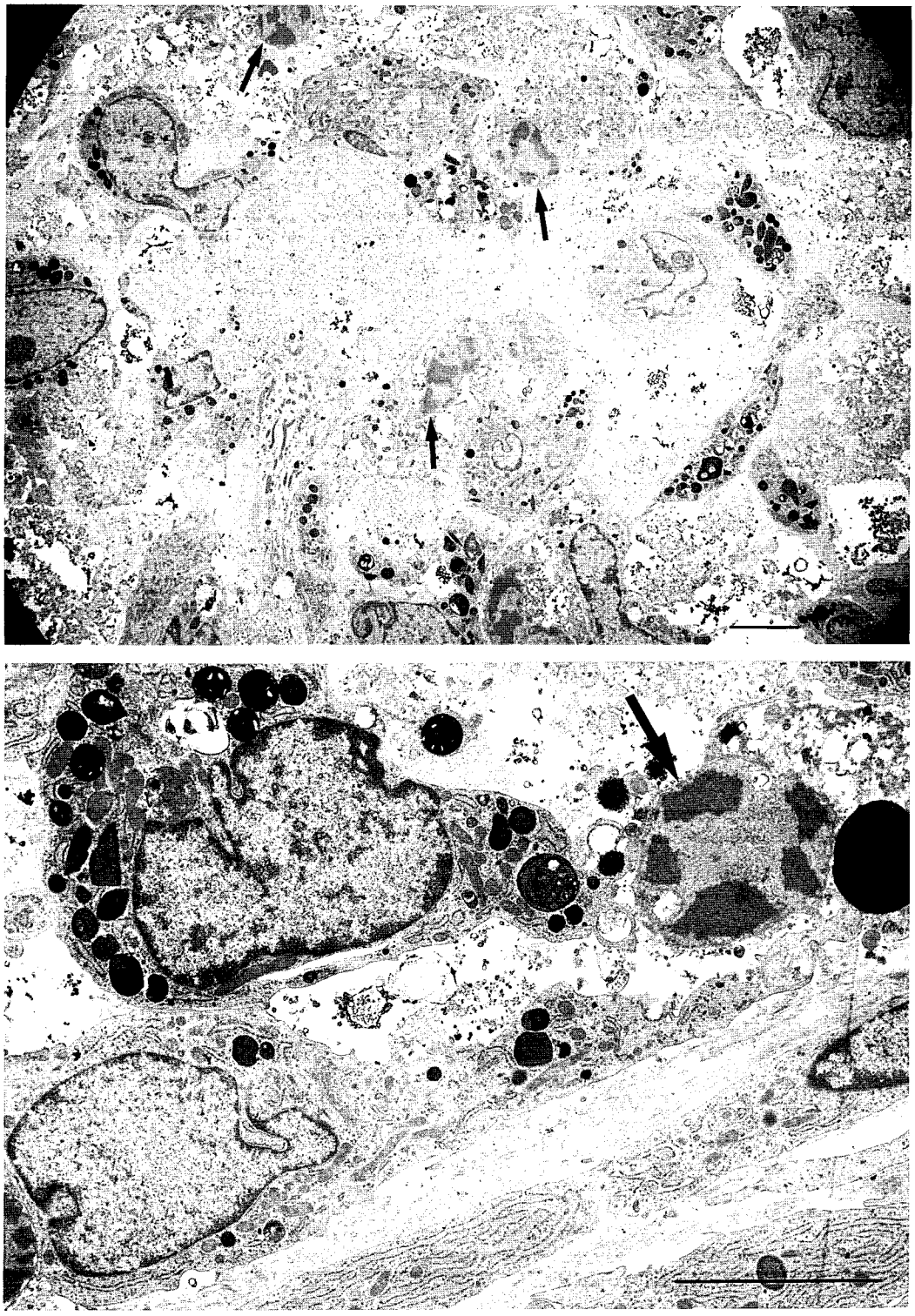


Fig. 4 Transmission electron micrographs of a multicellular spheroid formed by human trabecular cells after one month. Cells spread like a mesh, with large extracellular spaces filled with cell debris, apoptotic cells (arrows), and collagen fibers (top). Cells have villous projections, osmiophilic cytoplasms with various organelles, and nuclei with the dark spiked band of peripheral chromatin, characteristic to trabecular cells (bottom). Note an apoptotic cell (arrow). Uranyl acetate and lead citrate stain. Bar = 5 μm.

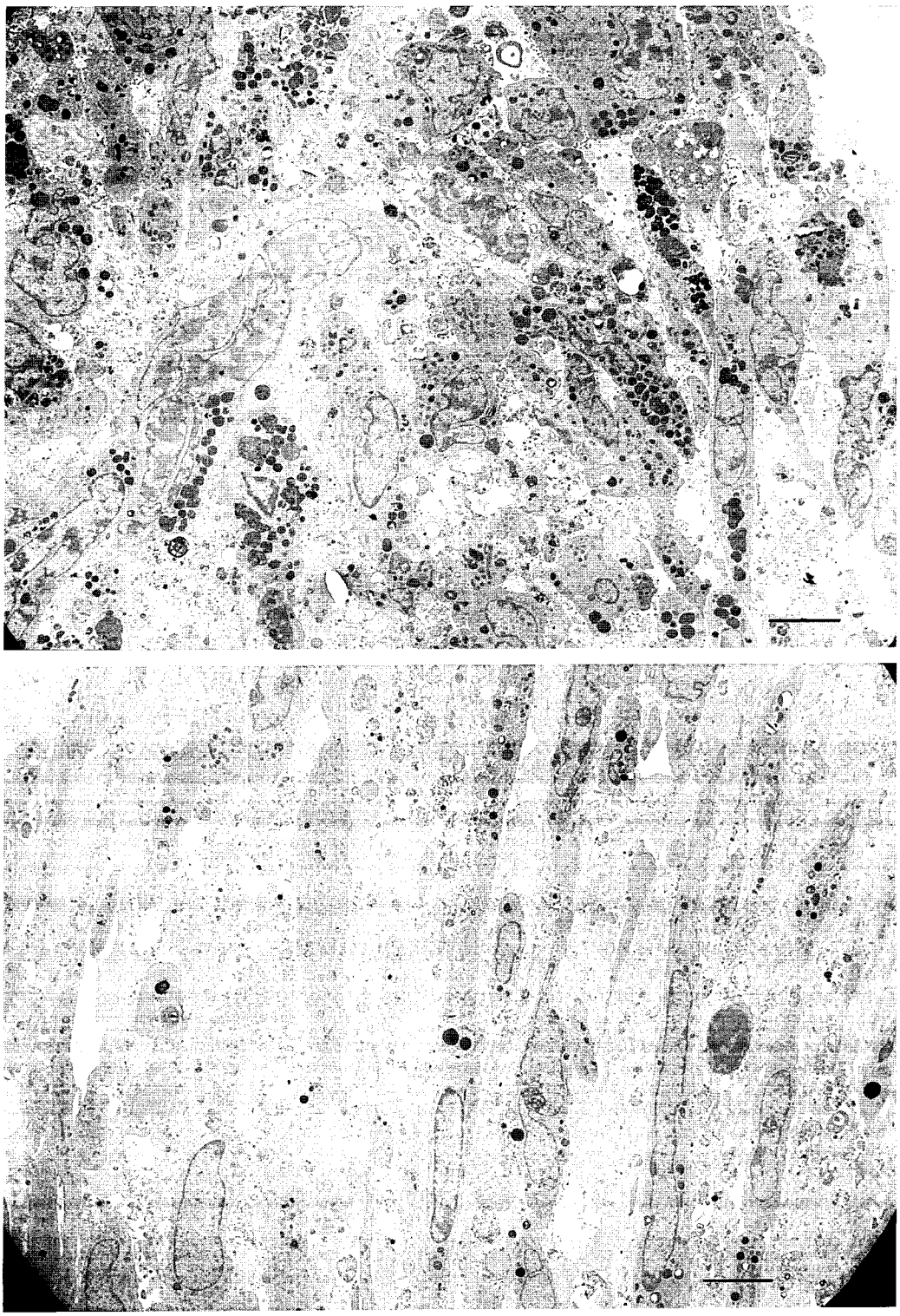


Fig. 5 Transmission electron micrographs. Multicellular spheroids formed by human trabecular cells in the presence of basic fibroblast growth factor (top) or epidermal growth factor (bottom) after one month. Spheroids are filled with cells even after one month. Uranyl acetate and lead citrate stain. Bar = 5 μm.

In the present study, we have shown that human trabecular cells form a mesh-like structure in multicellular spheroids after one month of culture as a result of their continuing apoptosis. The apoptosis of trabecular cells cannot be a result of simple nutritional problems since nutrients and oxygen can penetrate to the center of spheroids of such small size (5). Rather, a selective mechanism induced by loss of interaction with neighboring cells and extracellular matrices might underlie this apoptosis. The structure of multicellular spheroids formed by dissociated human trabecular cells resembles the normal trabecular tissue of the eye *in vivo*, and therefore, shows the possibility of reconstructing this tissue *in vitro*.

The presence of basic fibroblast growth factor or epidermal growth factor apparently prevents trabecular cells from going through apoptosis, and leads to the formation of multicellular spheroids with a larger number of cells. This high cellularity in the spheroids might correspond to such an *in vivo* situation as thickened layers of the juxtacanalicular tissue of the trabecular meshwork of the eye, which is seen in patients with congenital and developmental glaucoma (17, 18). Thus, the present results suggest that epidermal and basic fibroblast growth factors play a role in the development of these goniodysgenetic glaucomas.

In conclusion, multicellular spheroids can be grown from human trabecular cells just as they can be from bovine and porcine trabecular cells (11). The structure of these trabecular spheroids, as well as their response to epidermal and basic fibroblast growth factor, is similar among these species. This suggests that the basic nature of human, bovine and porcine trabecular cells is essentially the same.

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