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Collagen production by Cultured Human Retinal Pigment Epithelial Cells

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We investigated extracellular matrix produced by human retinal pigment epithelial cells (RPE) in vitro using electron microscopy and enzyme linked immunosorbent assay (ELISA). The thickness of the matrix under the cell layer was about 30 μm after 360 days of culture. It consisted mainly of fibrous and granular components. Type IV and V collagen were detected but type I and III were not detected by ELISA. It appeared that RPE can secrete type IV and V collagen and form a thick membrane which may cause proliferative vitreoretinopathy (PVR) by contraction. Control of RPE proliferation and secretion of extracellular matrix is indispensable for prevention of PVR.

(Key words: collagen, human, proliferative vitreoretinopathy, retinal pigment epithelium)

INTRODUCTION

The retinal pigment epithelium (RPE) is a single layer of cells around the outer circumference of the retina. The RPE cells play a vital role in providing anatomic, mechanical, and metabolic support for the photoreceptors and outer retina. Mitoses are extremely rare in adult cells, but they multiply and produce excessive amounts of extracellular matrix in certain pathological conditions. Proliferative vitreoretinopathy (PVR) is the most common cause of failure of rhegmatogenous retinal detachment surgery. PVR involves the uncontrolled proliferation of non-neoplastic cells capable of forming membranes, which may occur on either surface of the retina or along the detached surface of the vitreous gel. Contraction of these membranes creates tractional forces which can distort or detach the retina. Five major cellular constituents have been identified in vitreous and epiretinal membranes removed during vitrectomy : RPE cells, macrophages, fibroblast-like cells, fibrous astrocytes, and myofibroblast-like cells (3). The origin of these cells is controversial, but numerous reports attribute the major role in PVR formation to RPE cells (2, 5, 7, 9, 15). A recent report (4) using immunohistochemical techniques stated that the membrane stroma was composed primarily of type I, II and III collagen and in discrete regions within the stroma, laminin, both heparan sulfate proteoglycans and type IV and V collagen were also present. The source of type I and III collagen was assumed to be fibroblast-like cells, glial cells, and RPE cells. Therefore, the role of extracellular matrix production by RPE cells is important in understanding the pathobiology of PVR. In this study we examined the extracellular matrix secreted by long-term cultured human RPE cells using electron microscopy and enzyme linked immunosorbent assay (ELISA) to clarify what kind of collagen was produced by the cells.

MATERIALS AND METHODS

Cell culture

An eyeball was obtained from a 26-year-old male patient who underwent orbital excenteration secondary to a large facial hemangioma which had involved the right

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orbit, resulting in blindness. The methods of RPE cell culture was described elsewhere (6). The harvested RPE cells were placed in 60-mm plastic petri dishes (Corning ,NY, USA) containing 3 ml of Ham's F12 medium supplemented with 16% fetal calf serum (GIBCO, Grand Island, NY, USA), 0.75% $NaHCO_3$, penicillin (100 U/ml) and streptomycin (100 μg/ml) (GIBCO). The medium was changed twice a week. Primary culture cells were incubated 360 days without passaging.

Morphologic examination

After 360 days of culture, the cells in the culture dish were used for morphologic examination. The cell layer with extracellular matrix was separated from the dish with forceps. The membrane was cut into two pieces with scissors. The first piece was fixed in buffered 10% formalin and embedded in paraffin for light microscopy. Hematoxylin-Eosin, Mallory, PAS, Elastica and Alcian-blue staining were performed. The second piece was fixed in 2% glutaraldehyde, post-fixed in 1% osmium tetraoxide, dehydrated with graded ethanol and embedded in Epon for transmission electron microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 100B transmission electron microscope (JEOL, Tokyo).

Enzyme linked immunosorbent assay (ELISA)

Another culture dish was used for preparation of collagens (11) and ELISA (14) as follows. The peeled membrane composed of the cell layer and the extracellular matrix was washed in cold 0.05M Tris-HCl buffer, pH 7.4, containing protease inhibitors (4 mM EDTA, 2 mM phenylmethylsulfonyl fluoride and 10 mM N-ethylmaleimide) and then homogenized. The homogenate was suspended in 0.5 M acetic acid and pepsin (Boehringer Mannheim, Manheim, Germany) was then added in an enzyme/substrate weight ratio of 1:40. The reaction mixture was stirred at 4 ℃ for 24 hours. The undigested residue was removed by centrifugation at 10,000 g for 1 hour and the supernatant was adjusted to 1.8 M NaCl by addition of crystalline NaCl to precipitate collagen. The solution was stirred for 24 hours at 4℃ and centrifuged for 1 hr at 100,000 g. The precipitate was dissolved in 0.15 M NaCl/0.05M Tris-HCl buffer, pH7.6, and 0.2 ml of a 20 μg/ml solution was dispensed into microtiter wells (Nunc-Immuno Plate II-96 U, Roskilde, Denmark) and incubated overnight at 4 ℃. After washing with the same buffer, 0.2 ml of 1% BSA was added to each well and incubated for 1 hour at 37 ℃ for after coating. After washing with PBS-Tween, 0.2 ml of antibody solution (anti-human type I, III, IV and V collagen) (10) diluted at 1:10, 1:100, or 1:1000 with PBS-Tween-BSA was added to each well and incubated overnight at 4 ℃. After washing with PBS-Tween, 0.2 ml of second antibody solution (alkaline-phosphatase labelled anti-rat IgG produced in rabbits) (DAKO, Copenhagen, Denmark) diluted to 1:500 with PBS-Tween was added and incubated for 1 hour at 37 ℃. After washing with PBS-Tween, 0.15 ml of paranitrophenyl phosphate solution (5 mg/ 5 ml of 10% diethanol-amine buffer) (SIGMA, St. Louis, USA) was added to each well and incubated for 30 minutes at room temperature. The reaction was terminated by adding 0.1 ml of 3N NaOH, and the absorbance of the products of the enzyme reaction was measured at 405 nm. This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association.

RESULTS

Light microscopic findings

Primary culture cells retained epithelioid morphology even after 360 days of culture (Fig. 1). The membrane including the cell layer and extracellular matrix was separated from the dish easily with forceps (Fig. 2). The perpendicular sectional view of the membrane showed a monolayer of the cells and fibous materials under it. The fibrous extracellular matrix was stained positively with PAS and Alcian blue stain, and stained blue with Mallory stain, but was negative for Elastica stain.

Electron microscopic findings

The cultured RPE cells were flatter than those in vivo. Microvilli were seen on the free surface. Pigment granules, mitochondria and lysosomes were observed in the cytoplasm. The thickness of the extracellular matrix under the cell layer was about 30 μm. It consisted of a fibrous component, granu-

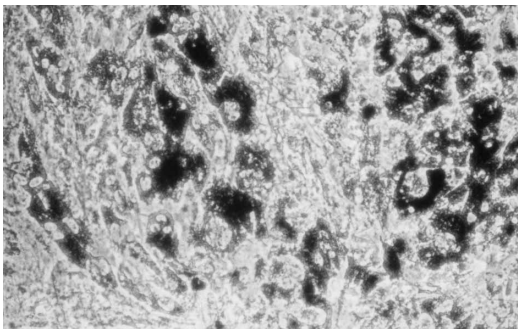

Fig. 1 A phase contrast micrograph of long-term cultured RPE cells. There are large cells abundant in pigment granules and relatively small cells with few pigment granules.(original magnification ×50)

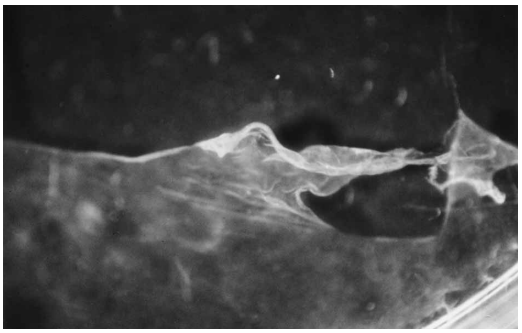

Fig. 2 The membrane including cell layer and extracellular matrix is separated from the dish easily with forceps.

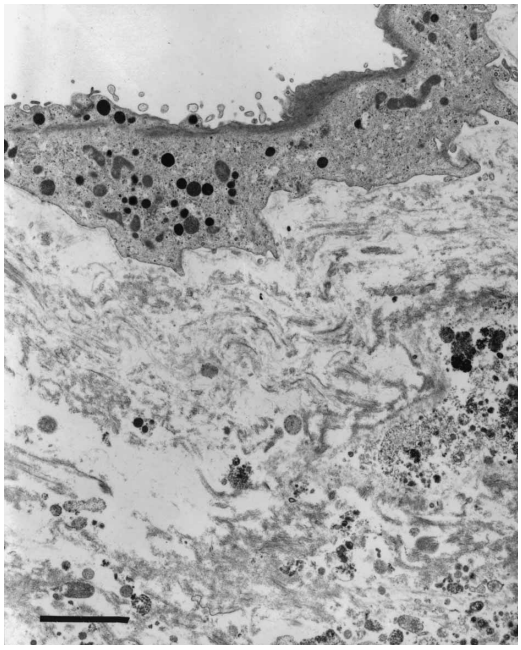

Fig. 3 (A) Electron micrographs of long-term cultured RPE cells and extracellular matrix.

Cultured cells have microvilli on the free surface. Pigment granules, mitochondria and lysosomes are seen in the cytoplasm. The thickness of the extracellular matrix under the cell layer is about 30 μm. It consists of fibrous components, granular material and fragments of the cells.(bar = 5 μm)

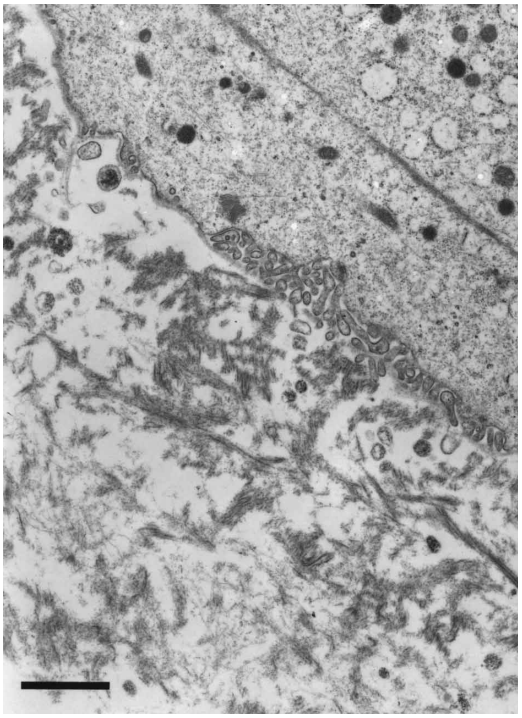

Fig. 3 (B) Electron micrographs of long-term cultured RPE cells and extracellular matrix.

Basal infolding of the cells and basal lamina are observed.(bar = 1 μm)

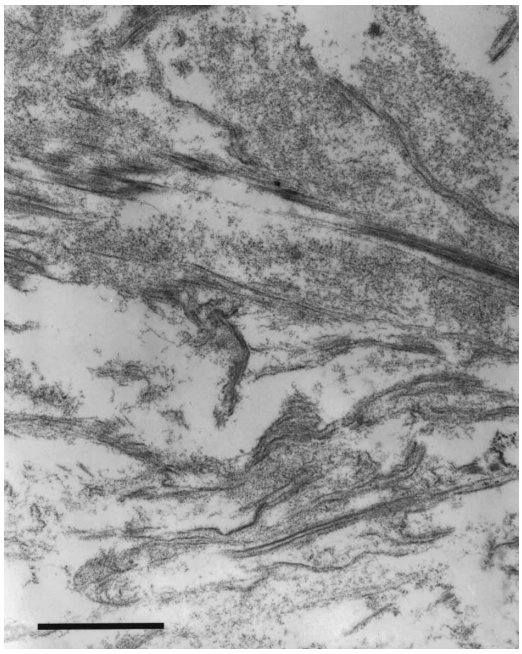

Fig. 3 (C) Electron micrographs of long-term cultured RPE cells and extracellular matrix.
The maximum diameter of the fibrils in the extracellular matrix is 30 nm and they have an indistinct banded pattern. They form bundles and pass through the granular matrix at random.(bar = 1 μm)

lar material and fragments of the cells (Fig. 3-A). Basal infolding and basal lamina were recognized (Fig. 3-B). The maximum diameter of the fibrils in the extracellular matrix was about 30 nm and they had an indistinct banded pattern. They formed bundles and passed through the granular matrix at random (Fig. 3-C).

ELISA

As show in Table 1, dilution-dependent positive reactions were observed with type IV and V collagen. With type I and III collagen, the reactions were very low in background levels (0.02～0.09). Therefore the major types of collagen produced by cultured human RPE in this study were type IV and V, and type I and III were very minor components or not secreted.

Table 1 ELISA Results

dilution antibody	1:10	1:100	1:1000
anti-type I	0.078	0.033	0.035
anti-type III	0.072	0.056	0.043
anti-type IV	0.340	0.279	0.238
anti-type V	0.627	0.508	0.397

(Absorbance : 405nm)

DISCUSSION

There are several reports on collagen production by cultured RPE. Newsome and Kenyon (12) reported that cultured RPE of the chick embryo produced basement-membrane material and striated collagen fibrils as extracellular deposits beneath the basal surface of the cells. The fibrils were oriented randomly and had a diameter of 15-45 nm. Although longitudinal macroperiodicity was not clearly discernible in fibrils of small diameter, a definite macroperiod ranging from 55 to 60 nm could be found in larger fibers. These extracellular deposits were not examined biochemically in the report. Li et al. (8) noted that feline RPE produced an extracellular matrix in vitro which was located between the basal surface of the RPE and the culture plate. The matrix had three morphological components: bundles, granules and fibrils. Basal membrane formation was not evident. The bundles exhibited a macroperiodicity of 118 ± 23 nm. Some of the fibrils showed a periodicity but a periodic band pattern of about 67 nm which is usually seen in interstitial collagen fibrils was not observed. Type IV collagen was detected biochemically but type I collagen was not detected. Campochiaro et al. (1) reported that human cultured RPE was found to produce collagen types I, II, III, and IV, but not type V using immunohistochemical staining techniques. Newsome et al. (13) reported using the same technique that cultured RPE of humans and monkeys produced collagen types I, III, IV and V, but not type II. In our study, a basal lamina, a fibrous component and a granular component were produced by lomg-term cultured human RPE. The maximum diameter of the fibrils was about 30 nm and they had an indistinct banded pattern. With ELISA, type IV and V collagen were detected but type I and III were not apparent. There are some discrepancies among these results in morphological and biochemical findings, probably due to differences in culture conditions.

RPE cells behave as an epithelial cell line under normal conditions, but under abnor-

mal conditions they may be transformed into mesenchymal cell-like cells. Many pathological reports on PVR note that fibrils are assumed to be synthesized by metaplastic RPE cells, but it is not clear what kind of cell synthesizes each extracellular matrix component in PVR membranes. In this study, it was found that cultured human RPE cells formed a thick membrane including a large number of fibrils and consisting of type IV and/or V collagen. This membrane may cause PVR by contraction. It is indispensable for prevention of PVR to control RPE proliferation and secretion of extracellular matrix.

REFERENCES

1) Campochiaro PA, Jerdan JA, Glaser BA: The extracellular matrix of human retinal pigment epithelial cells in vivo and its synthesis in vitro. Invest Ophthalmol Vis Sci 27: 1615-1621, 1986
2) Clarkson JG, Green WR, Massof D: A histopathological review of 168 cases of preretinal membrane. Am J Ophthalmol 84: 1-17, 1977
3) Glaser MG: Pathology of PVR. In Freeman HM, Tolentino FI (eds.): Proliferative Vitreoretinopathy (PVR). New York, Springer-Verlag, 1988, 12-21 pp.
4) Jerdan JA, Pepose JS, Glaser BM, et al: Proliferative vitreoretinopathy membranes. An immunohistochemical study. Ophthal-mology 96: 801-810, 1989
5) Kampik A, Kenyon kR, Michels RG, et al: Epiretinal and vitreous membranes: Comparative study of 56 cases. Arch Ophthalmol 99: 1445-1454, 1981
6) Kigasawa K, Soushi S, Tanaka Y, Obazawa H: Morphologic and chromosomal study of a human retinal pigment epithelial cell line. Jap J Ophthalmol 38: 10-15, 1994
7) Laqua H, Machemer R: Clinical-pathological correlation in massive periretinal proliferation. Am J Ophthalmol 80 : 913-929, 1975
8) Li W, Stram LE, Agurre GD, Rocky JH: Extracellular matrix production by cat retinal pigment epithelium in vitro: Characterization of type IV collagen synthesis. Exp Eye Res 38: 291-304, 1984
9) Machemer R and Laqua H: Pigment epithelium proliferation in retinal detachment (massive periretinal proliferation). Am J Ophthalmol 80: 1-23, 1975
10) Minamoto T, Ooi A, Okada Y et al: Desmoplastic reaction of gastric carcinoma: A light- and electron-microscopic immunohistochemical analysis using collagen type-specific antibodies. Human Pathology 19: 815-821, 1988
11) Miller EJ, Rhodes RK: Preparation and characterization of collagens. Methods in Enzymol 82: 33-64, 1982
12) Newsome DA, Kenyon KR: Collagen production in vitro by the retinal pigment epithelium of the chick embryo. Dev Viol 32: 387-400, 1973
13) Newsome DA, Pfeffer BA, Hassel JR, et al: Detection of extracellular matrix molecules synthesized in vitro by monkey and human retinal pigment epithelium: Infuluence of donor age and multiple passages. Exp Eye Res 46: 305-321, 1988
14) Rennard SI, Berg R, Martin GR et al: Enzyme-linked immunoassays (ELISA) for connective tissue components. Anal Biochem 104: 205-214, 1980
15) Smith RS, van Heuven WAJ, Streeten B: Vitreous membranes: A light and electron microscopical study. Arch Ophthalmol 95: 1556-1560, 1976
16) Wallow IHL, Miller SA: Preretinal membrane by retinal pigment epithelium. Arch Ophthalmol 96: 1643-1646, 1978