Types of collagen synthesised by the lens epithelial cells of human cataracts

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Abstract

Aims/Background—Residual lens epithelial cells (LECs) undergo fibrous proliferation after cataract surgery, resulting in capsular fibrosis. The purpose of this study was to determine the types of collagen produced in cultured LECs derived from human cataract LECs.

Methods—A circular section of the anterior capsule, about 5 mm in diameter, with LECs attached was obtained by anterior capsulotomy during cataract surgery and cultured directly without dispersion of the cells in a well, on the bottom of which a disc-shaped, thin plate of poly(methyl methacrylate) had been placed. At 5 to 6 weeks of culture, the proliferated cells of the culture were stained immunohistochemically with antibodies against human collagens I-VI by the avidin-biotin-peroxidase complex method.

Results—Collagens I, IV, V, and VI were positive in the cultured cells. Types IV and V were strongly present in almost all the cells whereas types I and VI were only observed in a few cells. Collagens II and III were negative.

Conclusions—Since the lens capsule is known to be comprised of collagen IV, collagens I, V, and VI seem to be produced newly in culture. The capsular fibrosis seen after cataract surgery in vivo as a wound healing process of the lens capsule, may contain these types of collagens. The present culture model is useful for studying secondary cataract formation.

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After cataract surgery, residual lens epithelial cells (LECs) proliferate and cause capsular fibrosis or Elschnig's pearls representing a

secondary cataract. A number of studies have demonstrated that capsular fibrosis is the result of collagen synthesis by LECs that undergo so called fibrous metaplasia or pseudometaplasia.¹⁻⁴ Recently, there have been some reports suggesting that an intraocular lens (IOL) can induce fibrous metaplasia. Whenever LECs come in contact with the poly(methyl methacrylate) (PMMA) of an IOL postoperatively, capsular fibrosis occurs with a characteristic appearance on the remaining anterior capsule.⁵ After IOL implantation following continuous circular capsulotomy, there is a ring-shaped opaque anterior capsule in the area facing the posterior chamber IOL, while there is no fibrosis outside of the IOL. When the IOL is not implanted, the ring-shaped fibrosis is seen on the posterior capsule, but thinner, narrower, and more irregular, corresponding to the anterior capsulotomy; when the anterior capsule is retained following the envelope technique, there is a disc-shaped opaque anterior capsule when the posterior chamber IOL is present, but no fibrosis is observed when the IOL is not implanted.

Furthermore, in addition to the surgical trauma itself, LECs disrupt the blood-aqueous barrier in vivo after IOL implantation⁶ when they come in contact with the IOL and begin to undergo fibrous proliferation. We have pointed out that these phenomena reflect the LEC compatibility of an IOL as a biomaterial,⁵⁻⁷ and that this compatibility is thus an important criterion for evaluating the biocompatibility of these lenses.

The degree of fibrous proliferation of LECs is reflected quantitatively and qualitatively by collagen synthesis. Thus, in addition to qualitative analysis, such as determining the types of collagen produced, quantitative measurement of in vitro collagen synthesis may be useful in the assessment of the LEC compatibility of various biomaterials proposed for use as IOLs or as their coating material. The purpose of the present in vitro study was to clarify the types of collagen synthesised by human cataract LECs in culture.

Materials and methods

CULTURE OF HUMAN CATARACT LECs

Human cataract LECs were cultured as previously described.^{7,8} Briefly, a circular section of the anterior capsule with LECs attached was obtained by capsulotomy during cataract surgery and cultured directly without dispersion of the cells. After circular capsulorhexis, the section, about 5 mm in diameter, was

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Figure 1 Schematic representation of the culture of human cataract lens epithelial cells.

Figure 2 Microphotograph of the cultured human cataract lens epithelial cells stained with anti-human collagen I. A few cells are stained (bar, 50 μ m).

touched with an irrigation/aspiration tip and withdrawn from the eye by aspiration. Each section was held with fine forceps and washed thoroughly with irrigating solution, then placed immediately into a well (12 mm in diameter) of a 48 well, multiwell plate containing 0.5 ml of Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS), ascorbic acid (3 μ g/ml), penicillin G (100 U/ml), and streptomycin sulphate (100 μ g/ml). Before the procedure, a disc-shaped, thin plate (11 mm in diameter and 0.5 mm in thickness) composed of PMMA had been placed on the bottom of the well. The specimen was then cultured in 100% humidity at 37°C in 5% carbon dioxide atmosphere (Fig 1). The culture medium was replaced every 7 days. Ascorbic acid was additionally given to the culture medium at the concentration of 50 μ g/ml at each medium change.

Figure 3 Microphotograph of the cultured lens epithelial cells of human cataracts stained with anti-human collagen IV. Almost every cell shows brownish staining (bar, 50 μ m).

DETERMINATION OF THE TYPES OF COLLAGEN PRODUCED USING IMMUNOPEROXIDASE TECHNIQUES

The incubation medium was removed at 5 or 6 weeks of culture, and the culture was washed with calcium and magnesium free phosphate buffered saline (PBS) containing 95% ethanol and 5% acetone for 20 minutes. The culture was then washed twice with PBS, each time for 10 minutes, and immunohistochemically stained by the avidin-biotin-peroxidase complex (ABC) method according to Hsu.⁹

As primary antibodies, mouse anti-human collagens I, II, III (Fuji Pharmaceutical Co, Toyama, Japan), IV (Chemicon International Inc, Temecula, CA, USA), V, and VI (Fuji, Japan), each diluted 50 times to final concentration of 10 μ g/ml, were used. The specificities recognised by the antibody for each type are as follows.

Anti-collagen I: immunogen; human amniotic type I collagen. Specificity; $[\alpha_1(I)]_2 \alpha_2(I)$. No cross reactivity with collagens type II, III, IV, V, and VI.

Anti-collagen II: immunogen; human cartilage (os sternum) cleaved collagen II. Specificity; $\alpha_1(II)$ chain. No cross reactivity with collagens type I, III, and IV.

Anti-collagen III: immunogen; human amniotic type III collagen. Specificity; $\alpha_1(III)$ chain. No cross reactivity with collagens type I, II, and IV.

Anti-collagen IV: immunogen; human amniotic type IV collagen. Specificity; $\alpha_2(IV)$ chain. No cross reactivity with collagens type I, II, III, and V.

Anti-collagen V: immunogen; human amniotic type V collagen. Specificity; N-terminal peptide of $\alpha_1(V)$ chain. No cross reactivity with collagen type IV.

Anti-collagen VI: immunogen; human amniotic type VI collagen. Specificity; $\alpha_1(VI)$ chain.

All cross reactivities were determined by western blot at dilutions of 1:1000. As the secondary antibody, a peroxidase Vectastain ABC mouse IgG kit (Funakoshi Pharmaceutical Co, Tokyo, Japan) was used for staining collagens I, II, III, IV, and VI, and a peroxidase Vectastain ABC mouse IgM kit (Funakoshi Pharmaceutical Co, Tokyo, Japan) for staining collagen V. For the diaminobenzidine-hydrogen peroxide reaction, a DAB substrate kit (Funakoshi Pharmaceutical Co, Tokyo, Japan) was used. Five to eight cultures were stained for each type of collagens.

The controls were subjected to the same procedure except that they were not treated with mouse anti-human collagens.

After collagens II and III were found to be negative by repeated staining, the positive controls were made.

Positive control for collagen II: a section of frozen mouse bronchial cartilage on a slide glass was subjected to the same procedure with anti-collagen II.

Positive control for collagen III: normal human dermal fibroblasts (5000 cells/cm², Fibro Pack, Sanko Junyaku Co, Tokyo) were cultured as described. After the culture became almost

Figure 4 Microphotograph of the cultured lens epithelial cells of human cataracts stained with anti-human collagen V. Brown staining is observed in every cell (bar, 50 μ m).

confluent in 10 to 12 days, it was subjected to the same procedure with anti-collagen III. Three specimens were used as the positive controls for each type.

The outgrowth of LECs beyond the lens capsule on the well bottom was observed under a light microscope and photographed.

Results

CULTURE OF HUMAN CATARACT LECs

Because the anterior capsule usually curled upward and the side to which the LECs were attached faced invariably downward spontaneously, the LECs in the centre of the capsule always came into direct contact with the PMMA plate on the well bottom. Where the capsule touched the plate, LECs began to proliferate within 2 or 3 days. All proliferating cells lost their initial polygonal shape and underwent pleomorphic changes, becoming slightly elongated and fibroblastic. As the attached cells underwent these changes and

Figure 5 Microphotograph of the cultured human cataract lens epithelial cells stained with anti-human collagen VI. Only a few cells show brown staining (bar, 100 μ m).

the areas of attachment expanded, the anterior capsule became fixed to the well, and the curled edge gradually flattened out. With the pleomorphic changes, the cell boundaries and nuclei began to appear indistinct under the phase contrast microscope.

At about 1 week after the start of culture, the specimens began to show cell proliferation to confluence on the capsule as well as growth beyond the capsular margin. After 3 to 4 weeks, the cells were still proliferating and had become almost confluent. Cultures after 5 to 6 weeks were used for staining.

TYPES OF COLLAGEN IDENTIFIED

The cultured cells were positive for collagens I, IV, V, and VI. No cells were stained in the controls. Collagens II and III were not detected, but the positive controls were stained. The staining for collagens I and VI was restricted in a few cells. The specimens with anterior subcapsular fibrosis showed reinforced staining with anti-collagen I compared with those without fibrosis. The staining for collagen IV was intense. The staining for collagen V was even more intense, and distributed diffusely at almost all the cells observed (Figs 2–7). Collagens I, IV, and V were consistently positive by the repeated staining. Collagen VI was positive in five of seven specimens.

Discussion

Our culture method is original in the use of a small sized well, the use of a small amount of medium which is replaced only once each week, and the use of a PMMA plate. There have been some attempts to culture the anterior capsule with the attached human LECs.^{10–12} However, to obtain successful cell growth, tissue adhesive,^{10 11} or higher concentrations of calf serum,¹¹ or plasma clot with some growth factors¹² were used to fix the capsule onto the well or to stimulate cell growth. Like in these reports, successful cultures can only be obtained if the capsular section is prevented from moving or floating in the medium so that it remains in contact with the well, particularly in the initial culture period, until the specimen has adhered to the well. We had initially used larger sized wells (24 mm in diameter) that required much more medium, and had changed the medium three times a week. This promoted moving and floating of the capsule with attached LECs in the well, so that the capsule did not adhere to the well, resulting in no cell growth. Changing to smaller (12 mm diameter) wells in a multiwell plate substantially lowered the volume of medium required (0.5 ml per well) and made only one change of medium per week necessary, at least during the first weeks of culture; under these conditions, the capsule adhered in the manner described and the cells grew, although it did not include the germinative zone. Replacement of the medium once per week seems to be sufficient to maintain the cell culture under these conditions.

Figure 6 Control with IgG for the collagens I, II, III, IV, and VI. No cells are stained (bar, 100 μ m).

About 15 types of collagen, some of which are not found¹³ in the ocular tissues, and about 25 types of α chain have been defined so far.¹⁴ In this study, we attempted to detect whether the tissue culture of human cataract LECs contained collagens I–VI, which are common in ocular tissues, and detected collagens I, IV, V, and VI. In collagen synthesis, the collagen precursors, called pro- α chains are first synthesised. They form a procollagen triple helix after hydroxylation of selected prolines and lysines and glycosylation of selected hydroxylysines within cells, for which the presence of vitamin C is essential. After secretion the propeptides of the fibrillar procollagen molecules are removed by specific proteolytic enzymes outside the cell.¹⁴ This converts the procollagen to collagen molecules in the extracellular space contained within a large folding in the plasma membrane.¹⁴ Because the fixation procedure of the cells used in the present study damage the cell membrane, the stainings may include both intracellular and extracellular procollagen and collagen molecules. Collagens II and III were negative, although

Figure 7 Control with IgM for the collagen V. No cells are stained (bar, 100 μ m).

the possibility cannot be excluded that they could have been produced in longer culture.

The biochemical detection of type IV collagen in the mammalian lens capsule has been widely reported,^{13 15–24} and Barnard *et al*¹⁹ presented evidence suggesting the association of collagen types I, III, and IV in the bovine lens capsule. Long term cultured bovine LECs produce collagens I and III in addition to type IV.²³ There are only a few reports of the types of collagen synthesised by human LECs. In the human lens capsule, collagen IV is commonly found and appears to be the main component.^{16 20 22} Collagen IV forming a lens capsule was detected in a long term culture of human LECs.²³ Immunohistochemical staining of the human lens capsule for collagens I and III was negative.^{20 22} Thus, the structure of the human lens capsule as a basement membrane of LECs appears to consist of a uniform layer of collagen IV, although Marshall *et al*²¹ found collagens I and III in addition to type IV in the aged human lens capsule.

In order to evaluate the usefulness of the present culture model for future studies of postsurgical capsular opacification or of LEC compatibility, it is important to determine the types of collagen synthesised newly in vitro in culture and those in vivo being present in the capsule left in situ after IOL implantation. Collagens I, V, and VI detected in the study may have been newly produced. However, the possibility that they are not newly synthesised but are also components of the human lens capsule cannot be excluded, because collagens other than types I to IV have not been evaluated in previous studies of lens capsular specimens. Regarding collagen types in the human capsule left in situ after IOL implantation, unfortunately there is, to our knowledge, only one report. Shigemitsu *et al*,²⁵ who conducted a postmortem immunohistochemical study using anti-collagens I to V and detected collagens I, III, IV, and V in the fibrous capsular specimens obtained from eyes that had undergone cataract surgery with IOL implantation. Our results are partially consistent with these in vivo findings. The lack of detection of collagen III in our culture may be due to the different conditions in vitro or to the relatively short period of culture.

Our results suggest the necessity for further study of whether the human lens capsule also contains types of collagen other than collagens I, III, and IV and of the types of collagen present in the capsule left in situ after cataract surgery.

It is interesting that the specimens from the anterior subcapsular cataract showed further increase of production of collagen I, as increase has been observed in the subcapsular fibrous matrix in vivo,²² compared with those from the other types of cataract. This finding also suggests the necessity for differentiation of the type and degree of cataract in future studies, because it is possible that fibrosis may develop faster in certain types of cataract and that the type of collagen produced may also vary accordingly. In this regard, culturing infantile

lens epithelial cells without cataract may be of interest and informative.

Although it should be taken into consideration that tissue culture conditions are different from the conditions in vivo, for example, glucose concentration in culture is much higher, the fetal calf serum used in the culture medium may contain a host of growth factors which are not present in aqueous humour in vivo, and the specimens used do not contain germinative zone cells, the tissue culture model of human cataract LECs on a PMMA plate used in this study may be useful in the qualitative assessment of the LEC compatibility of an IOL material and in the study of secondary cataract formation. We intend to report the quantitative assessment elsewhere.

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