A Study of Human Lens Cell Growth In Vitro
A Model for Posterior Capsule Opacification

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Purpose. After intraocular lens (IOL) implant surgery for cataract, cell growth on the posterior capsule is responsible for renewed visual impairment in approximately 30% of patients. The authors have, therefore, developed a human lens capsule system to study this growth in vitro.

Methods. Sham cataract surgery, including anterior capsulorhexis, nucleus hydroexpression, and aspiration of lens fibers, was performed on donor eyes. In some cases, a polymethylmethacrylate IOL implant was placed in the capsular bag. The capsular bag was dissected free, pinned flat on a plastic culture dish, covered with Eagle's minimum essential medium supplemented with 10% fetal calf serum and observed by phase-contrast and dark-field microscopy for as long as 100 days. At the end-point, capsules were examined by fluorescence microscopy for actin, vimentin, and chromatin.

Results. Within 24 hours, there was evidence of cell growth in the equatorial region. After 2 to 3 days, cells were normally observed growing from the rhexis onto the posterior capsule and across the anterior surface of the IOL, if present. Growth proceeded rapidly so that the posterior capsule, for example, was totally covered by a confluent monolayer of cells at 5.8 ± 0.6 days and 7.2 ± 0.7 days for capsules aged <40 years and >60 years, respectively. Total cover of the anterior IOL surface generally followed 4 to 5 days behind that of the capsule. Capsular wrinkles became increasingly apparent as time progressed, causing a marked rise in light scatter. An increase in capsular tension also occurred, and the actin filaments became more polarized near the wrinkles.

Conclusions. The model presented here for posterior capsule opacification shows many of the changes seen in vivo, including rapid lens cell growth, wrinkling, tensioning, and light scatter in the posterior capsule. It will be possible to develop strategies for inhibiting cell growth with this system.

In modern extracapsular cataract surgery, to restore the best possible vision, a small artificial lens is implanted into the remaining lens capsular bag from which most of the fiber mass has been removed. This approach is not without subsequent complications, however, and within 2 years as many as 35% of patients experience a significant loss of visual acuity. The degradation of the visual image appears to arise from colonization of the posterior capsule by lens epithelial cells left behind at surgery. The resultant cellular accumulation and changes to capsular structure give rise to an increase in light scatter, and decentralization of the intraocular lens (IOL) also can occur.

The most common and indeed successful method of treatment is to photodisrupt a section of the posterior capsule by a high-energy Nd:YAG laser to create a clear region around the visual axis. This treatment is expensive and not without medical complication because it can give rise to an increase in intraocular pressure, retinal detachment, and, in extreme cases, pitting of the surface and fracture of the IOL adjacent to the cleared region (see refs. 2 and 3 for review). As a consequence of these multiple complications, there have been strenuous efforts to understand more fully the underlying mechanism of posterior capsular opacification, and such fundamental investigations either have been carried out in vivo in clinical studies or in vitro.
animal model systems\textsuperscript{4,5} or have used tissue-cultured cells largely of nonhuman origin.\textsuperscript{6-8} Both approaches have defects because the details of growth are difficult to follow in vivo,\textsuperscript{9} and the tissue culture approach is complicated by the fact that the dynamics of cell growth are both species and substrate dependent (compare data in refs. 6, 7, 8, 10).

We have, therefore, developed a human lens capsular bag culture system that not only permits day-to-day, high-resolution imaging of cell growth on the natural substratum but allows a study of the rate of progression of capsular wrinkling and tensioning, both of which appear to play an important role in the postoperative loss of vision in a large number of patients who have undergone cataract surgery.\textsuperscript{1-3}

\section*{MATERIALS AND METHODS}

After the removal of corneo-scleral discs for transplantation purposes, human donor eyes obtained from East Anglian Eye Bank were used to perform sham cataract surgery, including continuous circular capsulorhexis (5 or 8 mm), hydroexpression of lens fiber mass, and aspiration of residual lens fibers. An IOL could be implanted into the bag if required. The capsular bag was then dissected free from the zonules and secured on a sterile polymethylmethacrylate petri dish. Six to eight entomological pins (D1; Watkins and Doncaster, Kent, UK) were inserted through the edge of the capsular bag to retain its circular shape (Fig. 1). Cultures were maintained in 1.5 ml of Eagle’s minimum essential medium (Sigma, Poole, UK) supplemented with 10% fetal calf serum and 50 mg/l gentamycin, and incubation was at 35°C in a 5% CO\textsubscript{2} atmosphere. The maintenance medium was replaced every 3 to 4 days. Ongoing observations were performed using phase-contrast and dark-field microscopy. The first group of experiments was conducted without an IOL because this facilitated observation of the earliest stages of cell growth in the capsular bag.

Cytoskeletal proteins were visualized by immunocytochemistry and epifluorescence microscopy. All reagents were from Sigma (Poole) unless otherwise stated. Washes were for 3 \times 15 minutes in phosphate-buffered saline (PBS)/bovine serum albumin (BSA)–Nonidet (0.02% and 0.05%, respectively). The pinned capsules were fixed for 30 minutes in 4% formaldehyde in PBS and permeabilized in PBS containing 0.5% Triton-X100, also for 30 minutes. Nonspecific sites were blocked with appropriate serum (1:50 in 1% PBS/BSA). Anti-vimentin (Clone V9) was diluted 1:100 and applied for 60 minutes at 35°C, followed by washing. Vimentin was visualized with fluorescein isothiocyanate-conjugated anti-mouse serum, used at 1:64 for 60 minutes at 35°C. After extensive washing, the actin cytoskeleton was stained with Phalloidin-tetramethylrhodamine isothiocyanate (TRITC) (2 \mu M) for 30 minutes, and cell nuclei with 4,6-Diamidino-2-phenylindole (DAPI) at 1 \mu g/ml for 10 minutes.
FIGURE 2. Low-power epifluorescence micrographs of capsular bag preparations at various stages of growth in the absence of an intraocular lens, (a) immediately after pinning out, (b to d) after 3 days, and (e to h) after 8 days. Red micrographs show F-actin stained with TRITC-phalloidin, green micrographs show vimentin visualized with fluorescein isothiocyanate, and blue micrographs show DAPI staining of cell nuclei. In all cases, micrographs represent a field of view of 568 X 390 μm. (a). In the freshly prepared capsular bag, large patches of cells are present on the anterior capsule, where they remained throughout surgery. However, in some areas (large asterisks) cells were removed; in particular, most cells were removed in the area (small asterisks) immediately adjacent to the rhexis (arrow). Cells are totally absent from the posterior capsule (PC) beyond the capsulorhexis. (b, c) Staining of the dense cytoplasmic network of vimentin filaments in cells after 3 days in culture. (b) Cells growing on the posterior capsule are in focus, whereas the rhexis (between the arrows) is bright but out of focus. With the rhexis in focus (c), it can be seen that cells also grew up onto the outside of the anterior capsule, and out toward the equator. (d) F-actin staining of the same cells growing onto the posterior capsule (the plane of focus is the same as in b). Cells near the rhexis appear radially polarized with parallel stress fibers (see Fig. 4a), whereas cells at the leading edge of the wave of growth have ruffled membranes and actin microspikes (see Fig. 4b). (e). Vimentin staining of original lens cells (in focus) on the anterior capsule, overlaid by new growth (out of focus) on the outside of the anterior capsule. Penetration of the antibody to the interior cells is poor, and they appear lightly stained. Note that cells on the interior of the anterior capsule are small, whereas those growing across the outside surface are much larger. (f to g) Confluent cells on the posterior capsule with one wrinkle, stained to show cell nuclei (f) and corresponding actin (g) and vimentin (h) stain. Note the accumulation of nuclei in the wrinkle (f) and a cell (arrow) undergoing mitosis alongside the wrinkle.

both at room temperature. The stained preparations were again washed extensively, floated onto microscope slides, and mounted in Vectashield mounting medium (Vector Laboratories, Peterborough, UK). Images were viewed with a Zeiss Standard R microscope (Zeiss, Oberkochen, Germany) and recorded on Ektachrome 400 or Tmax 400 pro film (Kodak UK, Hemel Hempstead, UK) with an Olympus (London, UK) OM2 camera. Forty-two capsules were used for all the studies.

RESULTS

The two types of capsular bag preparations used in this study are shown in Figures 1a and 1b. Generally, eight pins were used to preserve the natural circular outline of the bag. The extent of the capsulorhexis can be seen clearly in both cases, and when an IOL was inserted, it slightly distorted the outline of the bag as it would be in vivo. These preparations could be observed by optical microscopy methods for up to 100 days. Immunohistochemical staining for F-actin, performed immediately after pinning out, showed that although the posterior capsule was totally free of cells, large areas of viable cells remained on the anterior capsule (Fig. 2a).

Growth Studies Without Intraocular Lens

To carry out long-term growth studies, the capsule was observed on a day-to-day basis by phase-contrast microscopy. During the first 1 or 2 days after pinning out, there was evidence of cell growth in the equatorial region, but it was difficult to discern whether the growing cells were on the anterior or posterior capsular areas because of the disruption in optical images from adhering fibers and the overlaying of the two areas. However, once the cells progressed beyond the rhexis and were growing toward the center, good optical images were available, and growth into this region usually occurred within 2 to 3 days after pinning out the capsule (Fig. 3).
FIGURE 4. High-power epifluorescence micrographs showing F-actin in cells growing on the posterior capsule, 3 days after pinning out and in the absence of an intraocular lens. In elongated cells (a) growing off the rhexis, which is out of focus (between the arrows), the actin microfilaments are highly polarized along the length of the cell, parallel to the direction of growth (right to left). Cells at the leading edge of the growth wave (b) have ruffled membranes and show actin microspikes at right angles to the plasma membrane, both at the leading edge and in regions of cell-cell contact. Some cells also show polygonal arrays of actin filaments. Micrographs represent a field of view of $105 \times 68 \mu m$.

Vimentin staining clearly showed these cells growing toward the center of the posterior capsule (Fig. 2b), and in another plane of focus, it showed that cells also grew upward from the rhexis and back across the exterior surface of the anterior capsule (Fig. 2c). Staining for fibrous actin (Fig. 2d) of the cells growing on the posterior capsule (see Figs. 2b, 3) showed that they had a well-developed actin cytoskeleton. In cells at the rhexis edge, this appeared to be polarized with the major filaments lying parallel to the direction of growth (Fig. 4a). Cells at the leading edge had fan-shaped, ruffled membranes containing microspikes (Fig. 4b). Small spikes appeared to lie in register with those in neighboring cells. Although original epithelial cells stained brightly with TRITC-phalloidin (see Fig. 2a), the vimentin antibody did not penetrate well into the region under the anterior capsule. Lightly stained original cells can be seen in Fig. 2e, overlaid by bright, but out of focus, new growth on the outside of the anterior capsule.

Within 6 to 10 days the cells became confluent on the posterior capsule and acquired a regular, cobblestone appearance (Fig. 5). Wrinkles began to form by this stage, and they increased in number and magnitude with increasing time in culture (Fig. 6). The wrinkles were regions of light scatter, and this could be seen clearly in the dark-field micrograph (Fig. 6b). Early wrinkles seemed to be initiated beneath individual cells (Fig. 5), but in major wrinkles (Fig. 6), the situation was more complex. DAPI staining of early wrinkles within 2 days of confluency (Fig. 2f) demonstrated that there was already a concentration of nuclei along the wrinkle, with a small area almost devoid of nuclei along each side. The closely packed cells within the wrinkle appeared to have their major actin filaments running in parallel along the length of the wrinkle (Figs. 2g, 7). In regions to each side of the wrinkle, where cell density was comparatively low (Figs. 2f, 2h), major actin filaments were aligned mainly at right angles to the wrinkle (Fig. 7). The net result of the progressive capsular folding was increased tensioning of the capsule, which can clearly be seen as the inwardly bowed region (arrow) between the pins (Fig. 8).

With the capsular bag systems, it was possible to study the effect of donor age and extent of capsulorhexis on the rate of growth on the posterior capsule. This was assessed in terms of time for complete cell cover on the posterior capsule. There was a small but significant effect of donor age on the growth characteristics. The capsule was totally covered by a confluent

FIGURE 5. Formation of a confluent monolayer of cells on the posterior capsule after 9 days in the absence of an intraocular lens. The phase-contrast micrograph of newly confluent cells at the center of the posterior capsule shows their regular cobblestone appearance and reveals that fine wrinkles are already beginning to form. Because the posterior capsule is not totally flat, only part of the field of view is in focus. The micrograph represents a field of view of $0.89 \times 0.58 \, mm$. 
cell monolayer at 5.8 ± 0.6 days and 7.2 ± 0.7 days for capsules aged <40 years and >60 years, respectively. The Student’s t-test applied to these data showed that the means were significantly different at \( P \leq 0.01 \). Increasing the diameter of the capsulorhexis from 5 mm to 8 mm had little effect on the rate of coverage of the posterior capsule (Fig. 9).

Studies With Intraocular Lens Present
Inserting an IOL into the bag again had little effect on the growth rate on the posterior capsule, though in addition there was cell coverage of the anterior surface of the IOL. The pattern and progression of cell growth on the IOL and on the posterior capsule is shown in Figure 10. The two capsular preparations from the same donor (42 years of age) were found to grow at much the same rate. Growth on the posterior capsule was complete after 8 days, and growth on the IOL was complete after 12 or 13 days.

Cell growth onto the IOL proceeded at first in a manner similar to that on the posterior capsule in that the rhexis soon fused onto the IOL by cells growing from the anterior capsule. Once growth was established, the confluent cells had a normal cobblestone appearance (Fig. 11), but cells at the growing edge had a much larger and more irregular appearance than the corresponding cells growing on the posterior capsule (see Fig. 3). When the cells on the IOL became confluent, they increased in density, and most of them had an appearance similar to that of confluent cells on the posterior capsule. During the time course of our experiments (100 days), there was no evidence of cell multilayering or wrinkle formation on the anterior IOL.

DISCUSSION
This study (Fig. 1) confirmed data from a number of earlier reports where it has been shown that a high proportion of viable lens epithelial cells are left behind after all the manipulations and irrigation that take place in conventional extracapsular cataract surgery.\(^1\) The exact proportion of the anterior capsule covered by viable cells was variable from preparation to preparation and ranged from 30% to 80%. This estimate generally was obtained by observing the anterior capsule by phase optics at low magnification where the non-viable cells were apparent by their increased light scatter and prominent nuclei. The cell loss was always greatest nearest the rhexis, but, in spite of the difference in cell mortality, all capsules showed a rapid growth of cells across the posterior surface. It is a measure of the resilience of human lens cell growth that, without exception, total and confluent cell cover of the posterior capsule was obtained. It is the resilient growth of the epithelial cells left after surgery that gives rise to the postoperative opacification of the capsular bag. In vivo cell growth appears to continue for many months and even years, and light scattering cell–protein aggregates (Elschnig’s pearls) are formed in dense array.\(^12\) In the current model, there was little evidence of such pearl formation, at least in capsules cultured for as long as 100 days.

The first evidence of cell growth was observed in the bow region, where cells were seen moving onto the posterior capsule. This is in accord with a recent study\(^13\) of cell growth in the capsular bag, which showed, using the BrdU technique, that mitotic cells are seen first in the bow region. After a few days, cells
move beyond the rhexis and advance rapidly across the posterior capsule. Our findings agree with the movement data of Nagamoto and Miyajima\(^1\), obtained from a human capsular bag culture system immobilized by a ring-supporting system.

As expected from a number of tissue culture experiments,\(^14,15\) cells on capsules from younger donors did grow at a faster rate than those from older individuals, and this agrees with the fact that younger patients show a higher rate of posterior capsule opacification than their older counterparts.\(^1\) The fact that increasing rhexis size has only a minor delaying effect on cell coverage of the posterior capsule indicates that bow region epithelial cells play a major role in driving cell growth. These cells are the least accessible to mechanical disrupting techniques. With our system, the presence of an implant also does not greatly perturb growth on the posterior surface. There is, however, evidence to suggest that satisfactory growth can be obtained from capsules,

serum proteins are present at much higher concentrations than those found in normal aqueous.\(^8,9,11\) It should also be noted that the majority of in vitro models uses lens epithelial cells obtained from noncataractous lenses. There is, however, evidence to suggest that satisfactory growth can be obtained from capsules.

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**FIGURE 7.** High-power epifluorescence micrograph of the actin fibers of cells in the region of a wrinkle (see Fig. 2g). In the numerous cells involved in the wrinkle (see Fig. 2f), major actin filaments appear to be aligned along the length of the fold (between the arrows). In the sparse cells immediately alongside the wrinkle (see Fig. 2h), the major actin filaments are aligned mainly at right angles to the fold. This micrograph represents a field of view of 68 x 68 \(\mu m\).

**FIGURE 8.** Dark-field photograph of capsular bag after 46 days in culture. The increase in tension within the capsule is seen as an inward bowing of the capsular edge between the pins (arrow). Light scatter from the wrinkles is present, but not very apparent, at this magnification (see Fig. 6b). Because of the long working distance of the objective lens used at this magnification, imperfections of the surface of the plastic culture dish are visible. This micrograph represents a field of view of 1.1 x 1.15 cm.

**FIGURE 9.** Rate of cell coverage of the posterior capsule beyond the rhexis with either 5-mm or 8-mm diameter capsularhexis; 100% represents confluency. All donor capsules were >60 years of age, and data were obtained in the absence of an intraocular lens.
and cells removed from lenses that are both aged and cataractous.\textsuperscript{14-16}

After 2 to 3 days in culture, there was evidence of cell growth in the anterior capsule, and cells began to move across the anterior surface of an implanted IOL just as they do in vivo.\textsuperscript{9,17} For example, Ibaraki et al\textsuperscript{8} showed that cellular outgrowths from the capsulorhexis can be seen on the anterior surface of the IOL 1 week after surgery. Again, this is in accord with the observations of Rakic et al\textsuperscript{10} made on their in situ capsular bag preparation, in which BrDU labeling of anterior epithelial cells followed a day or so after labeling of the bow region cells. The edge of the rhexis rapidly adhered to the surface of the IOL, and this process also occurs in vivo.\textsuperscript{3} The attachment of the anterior capsule also was apparent in the absence of an IOL, where it adhered to the posterior capsule. In such preparations, more detail was revealed by immunofluorescent studies. These clearly showed the complexity of the adherence zone, with cells growing radially inward across the posterior capsule, outward across the front surface of the anterior capsule, and upward at the zone itself (Fig. 2c). This zone probably plays an important role in setting up the elastic tension forces within the capsule because it will obviously act as an anchor zone, limiting the movement of the capsule under tension. The areas free to move in the current model were primarily the posterior and anterior surfaces of the bag, where wrinkling occurs. In vivo the outer edges of the capsule are still attached by the suspensory ligaments, and the tension forces developed are so strong that lateral movement of the IOL (decentralization) and even ligament rupture can occur.\textsuperscript{3}

Wrinkling of the posterior surface is important optically because significant light scattering areas are produced in this way, and the clinical importance of this process has been recognized for some time.\textsuperscript{2} Some interesting feedback effects must occur that make cells congregate along such stress lines. Immunofluorescent staining reveals that not only are nuclei lined up within the wrinkle, they are largely absent in areas immediately adjacent. Similarly, actin stress fibers tend to be lined up either along or at right angles to the wrinkle, and the vector effect of such stress forces, small though they may be in a single cell, will be enhanced considerably when lined up in the same direction in many cells. The net effect of such forces is seen clearly in the inward curvature of the capsule.
margins in the current model and in the shrinkage of the capsule observed in the ring support system developed by Nagamoto and Bissen−Miyasima. It has been suggested from in vivo studies that it is not simply the presence of the cell monolayers, either on the posterior capsule or on the IOL, that causes the major optical problems, but rather the later pearl formation and wrinkling of the posterior capsule. Both of these latter changes are important in visual degradation, although the relative importance of either one is a matter for discussion. However, both follow on the resilient growth of human lens cells after extracapsular surgery. The current model, involving culture over a relatively short time, reproduces many of the changes seen in vivo, including resilient cell growth, wrinkling of the posterior capsule, and tensioning of the capsular bag. It will be possible to extend the period of study and to investigate whether de novo synthesis of lens-specific proteins does occur late in culture, but it already provides an accurate and accessible means with which to develop strategies to limit cell growth within the capsular bag.

**Key Words**
cataract, cell growth, epithelial cells, human, lens, posterior capsule opacification

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**References**