Enhancement of Differentiation of Human Lens Epithelium in Tissue Culture by Changes in Cell–Substrate Adhesion

Tatsuo Arita,* Li-Ren Lin, Stanley R. Susan, and Venkat N. Reddy

Differentiation of human lens epithelial (HLE) cells cultured in vitro was drastically accelerated when the cells were cultured on cell–substrate adhesion-free surfaces. Spontaneous differentiation of the lens epithelial cells in monolayer cultures could be recognized with the appearance of lentoid bodies after 40–50 days if maintained without further passage. Although dissociated HLE cells reconstituted into monolayers consistently on the haptotactic substrates (either gold-coated biopore membrane or regular plastic dishes), the cells from the same batches exclusively formed cell aggregates when cultured on either biopore membrane or agarose-coated plastic dishes (nonhaptotactic). The cells on nonadhesion substrate first aggregate, then synchronously develop into lentoids by the 10th day of culture. The differentiation of HLE cells into lentoid structures with lens-fiber characteristics was documented by both ultrastructural and biochemical markers, such as loss of cytoplasmic organelles, formation of gap junctions, and the expression of γ-crystallin and MP26. The system, in which differentiation of epithelial cells can be induced predictably in a short period of time, provides an excellent model for the study of differentiation and gene expression in human lens cells cultured in vitro. Invest Ophthalmol Vis 31:2395–2404, 1990

Since Hamada and Okada and Eguchi and Kodama first reported in vitro differentiation of human lens epithelial (HLE) cells in tissue culture, many efforts have been made to find conditions to obtain better growth and differentiation. In contrast to the considerable success with animal lens cells, HLE cells lose their growth potential and ability to differentiate consistently. Until recently only two early reports showed formation of lentoids in HLE cultures. Using young human lens cells, we also reported that HLE cells can be cultured through several passages in which spontaneous differentiation occurred after long periods of time. The spontaneous in vitro differentiation of HLE cells, in which lentoids arose from monolayer cultures, was observed in most long-term cultures. The number of lentoids, however, was limited, and most of the cultured cells retained their epithelial cell shape even after the cultures were maintained for more than 1 yr (manuscript in preparation). Although this system with heterogeneous cell populations demonstrates that HLE cells can differentiate in vitro, its usefulness is limited by the fact that spontaneous differentiation is not consistent and that long periods of time are required for lentoid formation. Therefore, we directed our efforts toward developing a culture system in which lentoids could be produced consistently over a shorter period of time.

In a preliminary report, we showed that in vitro differentiation of young HLE cells can be enhanced by culturing them on cell–substrate adhesion-free surfaces. When the cells were prevented from adhering to the substrate, the inoculated cells exclusively formed lentoids.

We now describe in greater detail the procedures for establishing an in vitro HLE system which is highly reproducible. Cell differentiation takes place predictably, and the lentoids have the essential characteristics of lens fiber cell differentiation. This method, in which homogeneous population of differentiated cells (lentoids) can be produced, is not only applicable to cells derived from young human lenses but also to epithelia obtained from lenses of older patients ranging in age from 40–73 yr. We also describe the morphologic characteristics of differentiated cells and the expression of γ-crystallin and MP26, marker proteins for lens fiber differentiation.

From the Eye Research Institute of Oakland University, Rochester, Michigan.

Supported in part by NEI Research Grants EY00484 and EY05230 (Core Grant for Vision Research).

*Current address: Department of Ophthalmology, Kumamoto University Medical School, Kumamoto 860, Japan.

Reprint requests: Venkat N. Reddy, PhD, Eye Research Institute of Oakland University, Rochester, MI 48309-4401.
Materials and Methods

Tissue Culture

Substrates: Low protein-binding biopore membrane (Millicell CM; Millipore, Bedford, MA) with no coating of any cell attachment material and agarose (Sigma, St. Louis, MO) coated plastic culture plates (Falcon; Becton & Dickinson, Oxnard, CA) were selected for the substrates as adhesion-free or nonhaptotactic surface. Agarose-coated plastic plates were prepared by coating with a sterile warm 1% agarose, poured to make a thin layer covering the plastic surface. The agarose-coated culture plates were preincubated with culture medium before inoculating the cells.

Low protein-binding membrane with a gold coating as the cell attachment material and uncoated regular plastic culture plates were used for the control monolayer culture as adhesive or haptotactic surface. The gold coating of the Milipore membrane was done in a Polaron sputter coater (Waterford, UK) using argon gas as the ionizing plasma and completed when the color of the membrane became slightly gray from the coated gold. The gold-coated membrane chamber was sterilized under ultraviolet light in a laminar flow hood. To facilitate infiltration of the culture medium into the gold-coated membrane, a few drops of 70% ethanol were applied to the membrane. The membrane was then washed with phosphate-buffered saline (PBS) before adding the medium.

Harvesting of cells: The HLE cells harvested from subcultures of young human lenses and from primary cultures of older lenses were used for the experiments. Specimens of young HLE cells were obtained from patients who underwent surgery for retinopathy of prematurity. The methods for establishing primary and subsequent subcultures were described previously.13 Specimens of older human lens cells were obtained from five donor eyes ranging in age from 40–73. Every culture was set up independently for each eye within 48 hr after the death of the donor. All subsequent manipulations were done under sterile conditions. After the removal of the entire cornea and iris tissues and rinsing with PBS, the edge of the anterior lens capsule was punctured continuously with a 27-G needle tip similar to the anterior capsulotomy under binocular microscope. Then the anterior capsule with lens epithelium was peeled off from the lens cortex and cut into several pieces with Vannas scissors. In some instances lenses were released from zonules before the isolation of the capsule, in which case the lens was placed in a petri dish for removal of the capsule and epithelium. The pieces of capsule and epithelium were placed in a culture dish (Falcon) as explants and incubated in 5% CO₂ incubator at 36.5°C. Antibiotic-free Eagle’s minimum essential medium (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (Gibco) was used as the standard medium.

Primary cultures from older HLE cells were harvested in the same fashion as the young HLE cells.13,14 The cells were dissociated with 0.057% trypsin and 0.53 mM ethylenediaminetetraacetic acid solution, washed with culture medium and centrifuged at 1000 rpm for 4 min. They were then resuspended in culture medium and cultured on appropriate substrate.

Ultrastructure

For transmission electron microscopy (TEM), cultures were first exposed to osmium vapor for 30 min. (Lentoids in culture media were placed with an osmium crystal container in a small plastic chamber which was sealed with a masking tape for 30 min. The idea to use osmium vapor to prevent possible artifacts caused by aldehydes was suggested to T.A. by Dr. Kazushige Hirosawa of The Institute of Medical Sciences, The University of Tokyo, Japan.) The osmium-fixed tissues were washed several times with serum-free culture medium and fixed for 2–3 hr in half-strength Karnovsky’s solution (2% formaldehyde and 2.5% glutaraldehyde). After rinsing with Hank’s solution, they were postfixed in 1% osmium tetroxide solution for 60 min. They underwent dehydration through graded alcohols and infiltration with propylene oxide, and then the cultures were embedded in EM bed-812 (CMX, Fort Washington, PA). Thin sections were cut with a diamond knife, stained with 5% uranyl acetate and Reynold’s lead solution, and examined with an ISI-LEM 2000 or a Phillips EM 410 transmission electron microscope.

For scanning electron microscopy (SEM) the procedures of fixation and dehydration of the cells were the same as for TEM. The specimens were then critical point dried using liquid CO₂ as the transitional fluid, coated with gold in a Polaron sputter coater using argon gas as the ionizing plasma and viewed with a ISI-DS 130 scanning electron microscope.

Immunogold Labeling of Lens Proteins

For immunogold labeling of γ-crystallin and MP26, lentoids were fixed in 1% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer (pH 7.4), embedded in L-R white resin, sectioned with a diamond knife, and mounted on nickel grids. Sections were etched in saturated sodium metaperiodate for 60 min followed by three washings in distilled water for a total of 25 min. After blocking with serum “blocking
solution” (10% goat serum in PBS, 0.5 M NaCl, and 0.05% Tween 20) for 30 min, the sections were treated with either anti-γ-crystallin (1:20 dilution in PBS or 10% goat serum) or anti-MP26 serum (1:50 dilution in PBS or 10% goat serum in PBS).

After primary antibody reaction, the sections were washed three times in “rinsing solution” (PBS, 0.5 M NaCl, and 0.05% Tween 20) for a total of 20 min and again treated with blocking solution for 15 min before labeling with colloidal gold. For this, the grids were incubated for 50 min in Janssen goat anti-rabbit (GAR-Au) (Janssen Life Sciences, Beerse, Belgium) containing 15 nM colloidal gold (the stock solution was diluted 1:20 in PBS). When the incubation with the colloidal gold was complete, the grids were washed three times in the rinsing solution for a total of 45 min, then with dripping distilled water for 15 sec and dried. The grids were stained with uranyl acetate and lead citrate and examined with an electron microscope.

**Results**

Figure 1 is a schematic representation of the behavior of HLE cells on haptotactic (adhesive) and non-haptotactic surfaces. The cells inoculated onto haptotactic surface attached to the surface of the culture dish and then formed a regularly arranged monolayer consistently. These HLE cells usually stayed as epithelium with no lentoid formation for more than 40–50 days. In contrast, the cells inoculated onto nonhaptotactic substrate aggregated and developed into lentoids without a monolayer in approximately 10 days.

Typical lentoids obtained from HLE cells on low protein-binding biopore membrane are shown in Figures 2A–2C. The lentoids formed on agarose surface are shown in Figure 2D. Figure 2E is a monolayer formed on a regular plastic culture dish. Usually lentoids had a dark core under phase-contrast microscope. The cortices of these lentoids were transparent and some of them had so-called bottle cells3 (Fig. 2A). In some cases, typical lentoids were recognized under the phase-contrast microscope in less than 1 week (Fig. 2B). Subcultured HLE cells also formed lentoids in a manner similar to those from dissociated primary cultures (Fig. 2C). Although lentoids grown on either biopore membrane or agarose-coated dishes could be clearly seen under a phase-contrast microscope, the epithelial monolayer formed on gold-coated biopore membrane could not be seen because of the translucent nature of the membrane. However, the monolayer could be visualized by SEM. Figure 3A shows SEM of a lentoid grown on noncoated biopore membrane with complete absence of any monolayer epithelial cell growth. Figure 3B is an epithelial monolayer of the cells cultured on gold-coated biopore membrane.

To test whether lentoid formation could be induced in HLE cells from adult lenses, primary cultures from five different lenses from patients ranging in age from 40–73 yr were grown for 30 days to 2.5 months. Unlike the young HLE cells, the growth of the older cells was much less active so that some cultures were not fully confluent (60–80%) at this time. All of the HLE cells harvested from such primary cultures that were inoculated onto nonadhesive substrates exclusively formed lentoids without any monolayers within 10 days analogous to the behavior of young HLE cells. Figure 2A is an example of lentoids formed from primary lens cultures of a 40-year-old patient. The same batch of cells plated on an agarose-coated dish formed lentoids (Fig. 2D), while those on a plastic dish formed a monolayer (Fig. 2E). Thus, the nonhaptotactic surface acts as an external trigger for differentiation of HLE cells and that differentiation can be induced equally in young and old cells. In one experiment, the lentoids from HLE cells
Fig. 2. Examples of the lentoid. Phase-contrast micrographs. (A1) and (A2) stereo-micrograph of typical lentoids. Note lentoids with smooth surface and with so-called bottle cells (arrows). (B) Lentoids at sixth day of culture from older HLE cells. (C) Lentoids from young HLE cells that were sub-cultured five times. (A), (B), and (C) are lentoids developed on low-protein binding biopore membrane. No HLE monolayer was observed. HLE cells from the same batch grown on agarose-coated surface (D) or on plastic dish (E) showed exclusive lentoid formation on agarose coated dish and a monolayer on a regular plastic culture dish (original magnification x120).

Fig. 3. Scanning electron micrograph of HLE cells cultured on biopore membranes. (A) Lentoids with no monolayer on noncoated membrane (non-haptotactic). (B) HLE monolayer with no lentoid on the membrane with gold-coating as cell attachment material (haptotactic). The same batch of cells were cultured on the two surfaces.
from the lens of a 68-year-old patient were cultured for an extended period of time. The size of the lentoids gradually increased so that at 110 days of culture, their diameter was nearly three to four times that of a 10-day old lentoid (Fig. 4).

The ultrastructure of a typical lentoid examined by TEM is shown in Figure 5. A number of cells were found to be elongated and regularly arranged. The cytoplasm was very finely granular and uniform in density with concomitant loss of cytoplasmic organelles and the appearance of ball and socket-like interdigitations or interlocking processes of neighboring cells. These ultrastructural characteristics noted in lentoids are also typical of lens fibers. In some areas, under higher magnification, TEM also revealed typical gap junctions (Fig. 5B). The innermost cells of the lentoid were rich in organelles and basement membrane-like material accumulated in the center of the lentoids (Fig. 5C).

One of the criteria for mammalian lens cell differentiation is the appearance of marker protein γ-crystallin. This crystallin, absent in the epithelium, is believed to be expressed in differentiated fibers. Therefore, the expression of this crystallin was examined in lentoid, using immunogold techniques as described. Figure 6 is a TEM of a lentoid section labeled with immunogold for γ-crystallin. Both polyclonal and monoclonal antibodies to γ-crystallin were used. The former was obtained by injecting the low-molecular-weight fraction of human lens into rabbits, and the monoclonal antibody was to a human γ-crystallin preparation. For comparison, a section of rat lens cortex was simultaneously stained as a positive control. The gold particles may be seen throughout the cytoplasm of the human lentoid and rat lens fibers. Immunogold labeling with anti-γ-crystallin serum was much more intense in the cytoplasm than in the nuclei, cell membrane, or in extracellular area of the photomicrograph. Some variation in labeling intensity of cytoplasm in the two adjacent cells may reflect the difference in γ-crystallin content of each cell. Essentially similar results were obtained with either polyclonal and monoclonal antibodies to γ-crystallin. A more extensive study on the expression of γ-crystallin in HLE cells will be reported later (manuscript in preparation).

Another characteristic of differentiated lens cells is the appearance of the so-called intrinsic cell membrane protein MP26. It was, therefore, interesting to determine if lentoid bodies represent terminal differentiation and express the marker protein for fiber cell differentiation. Figure 7 is a section of human lentoid labeled with immunogold for MP26. The gold particles appear to be localized primarily along the cell membrane (arrows). No labeling was observed in the negative control in which nonimmune serum was used (Fig. 7B). A higher magnification of the cell membrane between two cells of the lentoid clearly showed the localization of gold particles along the cell membranes.

These results provide conclusive evidence that the human lens epithelial cells in culture can be made to undergo differentiation in a consistent manner and that lentoid formation represents fiber cell differen-
Fig. 5. Transmission electron micrographs of typical lentoids grown on nonhaptotactic surface. (A) Lentoids consisted of regularly arranged elongated cells with finely granular cytoplasm. Note the loss of cytoplasmic organelles, interdigitating processes of adjoining cells, and a series of gap junctions (arrow). Bar indicates 1 μm. (B) A higher magnification representing a gap junction (arrow) seen in lentoids. (C) Center of the lentoid. Innermost cells have nucleus (Nu) and more cytoplasmic organelles than overlying differentiated cells. Basement membrane-like material accumulation* is seen at the center of the lentoid. Bars indicate 0.5 μm.

Our results show that restriction of cell-substrate adhesion acts as an exogenous trigger for fiber differentiation of HLE cells in vitro. In contrast to the spontaneous formation of lentoids in monolayer cultures\textsuperscript{1,2,13} in which lentoids are few in number and appear after long periods of culture, the differentiation of HLE cells into lentoids can be achieved consistently and efficiently in a relatively short time by growing the cells on a nonhaptotactic surface. That we can obtain lentoids exclusively without an heterogeneous population of epithelial cells and differentiated fibers (lentoids) is advantageous and makes it possible to undertake detailed investigations on the mechanism of differentiation and gene expression in HLE cells. We can also examine changes associated with differentiation and pathologic processes such as cataract formation. The system is useful because epithelial cells from older human lenses can also be made to undergo differentiation, equally efficiently under similar experimental conditions.
The morphologic changes and the expression of biochemical markers for fiber cell differentiation we observed in the sections of lentoids are similar to those seen in differentiated lens fibers in situ. An in vitro culture system in which undifferentiated HLE monolayers and differentiated HLE lentoids develop separately in the same culture dish, sharing the same culture media, provides a unique model for studies of lens fiber differentiation in vitro under well-controlled conditions. It has been suggested that an ideal system for such studies in tissue culture should have the following characteristics: (1) differentiation can be recognized by comparable morphologic changes that are observed in situ; (2) specific protein markers should arise during the course of the morphologic changes; and (3) it should be possible to maintain the epithelial cells in culture in an undifferentiated state and to induce differentiation when desired. It is apparent that the system we describe satisfies a number of these criteria.

Although in vitro differentiation of HLE cells, enhanced by changes in cell-substrate adhesion, is homologous to the in vivo differentiation of lens (as judged by morphology and the expression of marker proteins), there are some differences between the lens and lentoid. One of these is cellular polarity. The lens
in situ develops from the lens vesicle in which cell polarity is primarily basal-side out. On the other hand, lentoids originate from randomly aggregated single cells, and the cells in lentoids have a reverse polarity in which basal side is inward, leading to the accumulation of basement membrane-like material at the center of the lentoids. Therefore, lentoids may be viewed as an inside-out structure compared with the lens in situ. Thus, lentoid is not a true miniature of the lens but a collection of cells homologous to cell population similar to the lens in situ.

Yet another difference between lentoid and in situ lens fibers is in the cell-substrate relationship. During differentiation in situ, which usually begins in the equatorial zone of the lens, the cells maintain contact with the substrate, i.e., the lens capsule. However, in the lentoid, the cell-substrate adhesion is lost. No mitotic figures were found in lentoids. It appears that

Fig. 7. Expression of MP26 in lentoids. Transmission electron micrograph of a lentoid section stained with immunogold for MP26. Bars indicate 1 μm. (A) Gold particles (arrow) are localized along the cell membrane. Cell cytoplasm, nuclei, or extracellular regions are distinctly less labeled with gold particles. (B) Negative control of lentoid section of corresponding area of (A). (C) A higher magnification view. Note gold particles (arrow) along cell membrane.
dynamic changes in the relationship between cell and substrate adhesion presumably obligate lens epithelial cells to differentiation and not to proliferation. It is interesting to note that there are certain similarities in the differentiation induced on nonhaptotactic surfaces in this study and the in vitro differentiation of lens fibers previously noted in other studies. Spontaneous lentoid formation usually arises in a multilayered area in the monolayer culture of the chick-lens and human-lens epithelial cells. In the case of a rat-lens organ culture, differentiation was observed after the multilayering of the lens epithelium on the capsule.

The acceleration of the "piling up" or the "aggregation" of potential lens cells which were dedifferentiated from the original phenotype was also one of the steps during transdifferentiation from chick retinal pigment epithelium or from retinal glial cells from chick embryos to lentoids. Thus these systems also appear to involve changes in cell-substrate adhesion. However, the mechanism by which the freeing of the cells from substrate adhesion accelerates the process of differentiation is not known.

The finding that lentoids express γ-crystallin, a characteristic marker protein for mammalian lens fiber, is similar to earlier findings of Eguchi and Kodama. This confirms our previous observation that its presence in lentoid was demonstrated by immunofluorescence with anti-γ-crystallin. The expression of MP26 in lentoids from a mammalian lens is the first report to our knowledge in which this lens plasma membrane protein has been shown in differentiated HLE cells in vitro. Our data also demonstrate (Fig. 7) that MP26 is localized primarily along the cell membrane of the lentoids. Whether this plasma membrane protein is associated specifically with gap junctions remains to be ascertained by further studies. Previous reports of the presence of MP26 in lentoids are from a non-mammalian system. The expression of MP26 has been documented in fiber-like structures of lentoids produced in chick embryo lens cells in vitro. Also, this plasma membrane was observed in lentoids formed as a result of transdifferentiation of retinal glial cells from chick embryo into lens-like phenotype.

Finally, our technique for enhancing differentiation in HLE cells is also applicable to lens cells from other species. Preliminary studies have shown that lentoid-like bodies are formed when dissociated cells from a rabbit lens and ciliary epithelia and human retinal pigment epithelium were cultured on nonhaptotactic surfaces. Thus the system may be advantageous for studying both differentiation and transdifferentiation in other cell types. However, it remains to be established whether lentoid–like bodies from ciliary epithelium and human retinal pigment epithelium represent transdifferentiation of these cells into phenotypic lens cells. These studies will be reported in a future communication.

**Key words:** lentoids, human lens epithelium, tissue culture, differentiation, cell–substrate adhesion, γ-crystallin, MP26

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**Acknowledgments**

The authors thank Drs. Samuel J. Zigler, Jr, of the NEI and Larry Takeimoto of Kansas State University for the antisera for γ-crystallin, Dr. Joseph Horwitz of Jules Stein Eye Institute for MP26 antiserum, Dr. Michael Trese of ERI, Oakland University, and William Beaumont Hospital, Royal Oak, MI, for his cooperation in obtaining specimens of HLE. This study was completed while Dr. Arita was a visiting research scholar from the Department of Ophthalmology, Kumamoto University, Kumamoto, Japan.

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