Growth and Differentiation of Human Lens Epithelial Cells In Vitro on Matrix

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PURPOSE. To characterize the growth and maturation of nonimmortalized human lens epithelial (HLE) cells grown in vitro.

METHODS. HLE cells, established from 18-week prenatal lenses, were maintained on bovine corneal endothelial (BCE) extracellular matrix (ECM) in medium supplemented with basic fibroblast growth factor (FGF-2). The identity, growth, and differentiation of the cultures were characterized by karyotyping, cell morphology, and growth kinetics studies, reverse transcription-polymerase chain reaction (RT-PCR), immunofluorescence, and Western blot analysis.

RESULTS. HLE cells had a male, human diploid (2N = 46) karyotype. The population-doubling time of exponentially growing cells was 24 hours. After 15 days in culture, cell morphology changed, and lentoid formation was evident. Reverse transcription-polymerase chain reaction (RT-PCR) indicated expression of αA- and βB2-crystallin, fibroblast growth factor receptor 1 (FGFR1), and major intrinsic protein (MIP26) in exponential growth. Western analyses of protein extracts show positive expression of three immunologically distinct classes of crystallin proteins (αA-, αB-, and βB2-crystallin) with time in culture. By Western blot analysis, expression of p57KIP2, a known marker of terminally differentiated fiber cells, was detectable in exponential cultures, and levels increased after confluence. MIP26 and γ-crystallin protein expression was detected in confluent cultures, by using immunofluorescence, but not in exponentially growing cells.

CONCLUSIONS. HLE cells can be maintained for up to 4 months on ECM derived from BCE cells in medium containing FGF-2. With time in culture, the cells demonstrate morphologic characteristics of, and express protein markers for, lens fiber cell differentiation. This in vitro model will be useful for investigations of radiation-induced cataractogenesis and other studies of lens toxicity. (Invest Ophthalmol Vis Sci. 2000;41:3898–3907)

In vivo studies of lens embryogenic development and the differentiation of lens epithelial cells into lens fiber cells from a large number of species have contributed to our current understanding of the lens and its response to toxic insults.1,2 In situ hybridization of lens tissue sections and genetic engineering have provided enormous insight into transcriptional regulation of crystallin genes.3,4 There are limitations, however, in experimental designs involving molecular biologic endpoints for lens epithelial cells at the boundary where lens fiber cell differentiation begins. It would be of particular interest to have a source of lens-specific transcriptional factors. Crystallin gene expression in the lens is known to be differentially expressed, both temporally and spatially, in the developing lens.5 Surgical access to obtain individual cohorts of cells is delicate. The volume of tissue obtained is small and in many cases requires a high multiplicity for the acquisition of sufficient material for the study of gene expression by other molecular techniques.6

Tissue culture models of lens epithelial cells therefore have been long sought for the purpose of investigating crystallin gene expression and the mechanisms of response to toxic insult. In addition, the molecular mechanisms controlling lens fiber cell differentiation are not completely known. Evidence implicates a role for apoptotic machinery in the enucleation of terminally differentiated lens fiber cells.7 However, the study of apoptosis in the human lens in vivo can be hampered by the trauma of tissue acquisition.8

Significant progress has been made in optimizing lens epithelial cell culture conditions during the past several years to promote lens fiber cell differentiation.9,10 Because there are species differences between animal and human lens epithelial cell physiology, considerable effort has been directed to culturing human lens epithelial (HLE) cells. Several groups have now succeeded in culturing HLE of prenatal or neonatal origin and in immortalizing HLE cells.10–15 Using these model systems, various groups have demonstrated morphologic and biochemical traits associated with lens fiber cell differentiation.10,12,13,15–17 There is a dearth of information comparing the lens biochemistry of normal versus immortalized animal cell lines, but abnormalities in crystallin expression in immun-
talized lens cell cultures from transgenic mice of the αPyLT1 lineage have been reported.18

In this article, we report the establishment of a nonimmortalized prenatal HLE primary cell culture on bovine corneal endothelium (BCE)-derived extracellular matrix (ECM). The methods used allow the HLE cultures to proliferate, and to demonstrate morphologic and molecular markers for lens fiber cell differentiation. We have characterized this primary culture with respect to its human lenticular identity and differentiation properties with time in culture. Comparisons have been made between the prenatal HLE and the immortalized neonatal HLE B-3 cell lines. Despite some limitations in the uniformity of differentiation, the HLE model system has features that allow quantitative investigation of underlying toxic insults to differentiating lens cells in vitro. These studies are much more difficult to address in vivo at the boundary of epithelial and differentiating lens cells and are likely to be impossible to obtain with immortalized cell lines because of their altered cell cycle control.19

METHODS

Cell Cultures

The HLE cells were established as a primary culture from an 18-week prenatal lens. The donor had no history of ocular abnormalities.11 Institutional approval by the University of California, San Francisco (UCSF) Committee for the Protection of Human Subjects was obtained, and the tenets of the Declaration of Helsinki were followed in the acquisition of the donated tissue. From a stock of vials of HLE cells frozen at passages 2 and 3, we expanded our frozen stock of HLE cells by culturing them on ECM derived from BCE cells, as described later, and returning to the freezer several hundred vials of cells up to and including passage 9. For the current studies, we have used cells generated up to passage 10, beyond which time subculturing became growth arrested. Immortalized HLE B-3 cells, kindly provided by Usha P. Andley (Washington University School of Medicine, St. Louis, MO) were used for comparisons in our work. These cells were transformed by infection with an adeno virus 12-simian virus (SV)40 and have been passed to 76 population doublings. We used cells from passages 13 through 18 for the currently reported work.

Fresh bovine eyes were obtained from a local abattoir. The methods for securing animal tissue were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of Matrix from BCE Cells

Primary cultures of BCE cells were prepared using the method originated by Gospodarowicz and III20 and modified by Song and Lui.21 We made some additional modifications to the protocol. The primary corneal endothelial explants were dissected from bovine eyes and trypsinized, and cells were incubated at 37°C in 10% CO2 in growth medium (DME-H16; low glucose; UCSF), 15% fetal bovine serum (FBS; Hyclone, Logan, UT), 5 ng/ml basic fibroblast growth factor (FGF-2), 300 μg/ml glutamine (Gibco, Grand Island, NY), 50 μg/ml gentamicin (Sigma, St. Louis, MO), and 2.5 μg/ml amphotericin B (ICN Biomedicals, Inc., Aurora, OH). FGF-2 was purified from a bovine pituitary source.22 To minimize disturbing the primary cultures, FGF-2 (in 0.005% bovine serum albumin [BSA]) was added (final concentration, 5 ng/ml) to the existing medium in dishes on days 2 and 4. On days 6 and 8, spent medium was removed and gently replaced with fresh growth medium. On day 10, BCE primary cultures were expanded in a 1:5 split to passage 1 (P1). Dishes were rinsed twice with phosphate-buffered saline (PBS) without Ca2+ and Mg2+, rinsed with 2 ml STV (0.05% trypsin and 0.02% versene in Saline A; UCSF) for less than 30 seconds, and trypsinized with 500 μl STV for 1 minute. STV was quickly neutralized with 4.5 ml of growth medium. This cell suspension was mixed gently, and 1 ml was inoculated into each 100-mm plastic dish containing 9 ml growth media. The P1 cultures were fed with the same regimen described for the primary cultures. On day 10, P1 cultures were subcultured in ECM medium (growth medium with 4% dextran) to make ECM. Cells from three 100-mm dishes were inoculated into 500 ml of ECM medium. One hundred-mm, 60-mm, and 35-mm dishes were set up with 10 ml, 4 ml, or 2 ml of inoculum, respectively. ECM cultures were grown and fed as described for primary cultures. After the ECM cultures were absolutely confluent with no visible holes in a stained sample (this variably occurs between days 7 to 10), the cultures were held confluent for 3 full days before the ECM harvest (days 10 to 13). To harvest the ECM the cells were treated with 0.02 M ammonium hydroxide for 5 minutes at room temperature and washed five times with ice-cold PBS with Ca2+ and Mg2+. The ECM-coated plates were stored at 4°C in PBS with 2.5 μg/ml amphotericin B and 50 μg/ml gentamicin and used within 6 months. The matrix was washed free of antibiotics before use.

Growth and Cell Culture Conditions

The HLE cells grew on ECM in DME-H16, with 15% FBS, 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, and 5 ng/ml FGF-2 at 37°C in 10% CO2. HLE cells required very gentle 50-second trypsinization at 37°C with STV after a rinse with Mg2+- and Ca2+-free PBS. Once trypsin was neutralized, cells were gently mixed, and counted. The cultures received a fresh-medium replacement every other day. Growth curve kinetics indicated the cells had a 50% plating efficiency at 24 hours after subculture. Cells adhered very strongly to the ECM, with 80% of viable cells attached after 15 minutes. Cells were plated rapidly and distributed evenly to avoid nonuniform cell density problems. Once these cells became confluent, they became growth arrested.

HLE B-3 cells were grown at 37°C, 10% CO2, in minimum essential medium (MEM) from Sigma (St. Louis, MO) containing 20% FBS and antibiotics but without supplemental FGF-2. The HLE B-3 cells were grown at 37°C in 10% CO2 on tissue culture plastic without matrix and were subcultured by trypsinization twice weekly.

For the measurement of cell growth of each cell line, cells were diluted to 1 × 103/ml and either 2 ml (in 35-mm dishes) or 4 ml (in 60-mm dishes) was plated with cell densities of 3 to 4 × 103/cm2. At an initial time point within an hour of plating and at intervals thereafter, a minimum of two identical petri dishes were trypsinized and cell counts made with a particle cell counter (Coulter, Hialeah, FL). The SEM of cell counts was determined, and the growth curve data from three to five
separate experiments were plotted as a function of time in culture.

**Chromosome Analysis and In Situ Hybridization for Human Phenotype**

Exponentially growing HLE cells at passage 6 were subjected to 0.05 μg/ml colcemide for 1 hour to arrest cell division at metaphase. Cells were then harvested and suspended in 0.075 M KCl at 37°C for 20 minutes. Cells were fixed slowly with 3:1 methanol-acetic acid and dropped onto polished glass microscope slides. Cells were stained with 0.25 μg/ml 4,6-diamidino-2-phenylindole (DAPI) and analyzed by inverse DAPI G banding. Chromosomal analysis of 10 metaphase spreads was performed using Skyview software (Applied Spectral Imaging, Inc., Carlsbad, CA). Chromosomes were hybridized with spectral red-labeled human placental Cot-1 DNA to confirm that the cells were of human origin.

**Lentoid Scoring**

The numbers of lentoid bodies were counted in 10 fields of 200× magnification, for exponentially growing and confluent HLE cultures grown in 100-mm diameter petri dishes. Lentoids were identified as being roughly spherical and highly refractive multicellular clusters of at least 20 μm in diameter, as has been similarly illustrated by others. Lentoids rose above the surrounding flat HLE cells and were thus difficult to photograph in focus. The SEM number of lentoids counted was determined.

**Reverse Transcription–Polymerase Chain Reaction (RT-PCR)**

Total RNA was isolated from human lens epithelial cells using a standard acid guanidium phenol chloroform (AGPC) method. The quality and yield of recovered RNA was assessed by measuring absorption at 260 and 280 nm. Total RNA was reverse transcribed per the manufacturer’s instructions into single-stranded cDNA using a reverse transcription kit (Superscript II; Gibco). One to 5 μg of total RNA was incubated with 0.5 μg oligo(dT)12–18 primer at 70°C for 10 minutes and immediately denatured of 1 minute at 95°C and 1 minute at 60°C. The Mg²⁺ concentration was supplemented to 2 mM in the final reaction mixture. For FGFR1, the PCR conditions were essentially the same as that for αA-crystallin, without the additional supplement of Mg²⁺ in the reaction mixture. For GAPDH we used an initial denaturation of 5 minutes at 94°C, followed by 35 cycles of 45 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C followed by a final extension of 5 minutes at 72°C. For αA-crystallin, the PCR program was 2 minutes at 95°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C followed by a final extension of 5 minutes at 72°C.

**PCR Conditions and Primers**

The primer pair for αA-crystallin is upstream primer, 5′-ATGAGCGTGACATCCAG-3′; downstream primer, 5′-GGCTGCTATCTAA-3′ (reference accession no. U05569). The expected product size is 754 bp. The primer pair for β2-crystallin is: upstream primer, 5′-GGAAGGCGGACAGGT-3′; and downstream primer, 5′-GTGGAGGGGTGAAGG-3′ (reference accession no. L10035). Expected product size is 434 bp. The primer pair for GAPDH is upstream sequence, 5′-TGAAGGTGGGTTACCCGC-3′; and downstream sequence, 5′-CATGTTGGGCACTGCCACCAC-3′ (Clontech, Palo Alto, CA). Expected product size is 983 bp. The primer pair for FGFR1 is upstream sequence, 5′-CGAGCTCCTGGTGGATTCGAT-3′; and downstream sequence, 5′-GTGTAACCAGCAGTGATATAG-3′ (reference accession no. M60485). Our PCR conditions included the use of PCR beads (Amersham Pharmacia, Piscataway, NJ). MgCl₂ (1.5 mM) was used in a standard 25-μl reaction mixture, unless otherwise stated. For β2-crystallin, the PCR program was 2 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C followed by a final extension of 5 minutes at 72°C. For αA-crystallin, the PCR program was 2 minutes at 95°C, followed by 35 cycles of a two-step procedure of 1 minute at 95°C and 1 minute at 60°C. The Mg²⁺ concentration was supplemented to 2 mM in the final reaction mixture.

**Table 1. Antibodies Used for Immunofluorescence and Western Analysis**

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Blocking Solution</th>
<th>Dilution/Source</th>
<th>Host</th>
<th>Secondary Antibody Dilution/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies Used for Immunofluorescence</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP26</td>
<td>0.5% Casein in PBS</td>
<td>1:170/Antiseria Chepelsky</td>
<td>Mouse</td>
<td>1:100/Alexa 594*</td>
</tr>
<tr>
<td>γ-Crystallin</td>
<td>0.5% Casein in PBS</td>
<td>1:100/Antiseria Zigler</td>
<td>Rabbit</td>
<td>1:100/Alexa 488*</td>
</tr>
<tr>
<td>Western Blot Analyses</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>5% nonfat Milk</td>
<td>1:500/N350†</td>
<td>Mouse</td>
<td>1:5,000/NA9340†</td>
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<tr>
<td>p57Kip2</td>
<td>2% BSA</td>
<td>1:200/NA-36†</td>
<td>Mouse</td>
<td>1:2,000/NA9340†</td>
</tr>
<tr>
<td>αA-Crystallin</td>
<td>2% BSA</td>
<td>1:1,000/SC-102§</td>
<td>Rabbit</td>
<td>1:10,000/SC-2005§</td>
</tr>
<tr>
<td>αB-Crystallin</td>
<td>2% BSA</td>
<td>1:3,000/Antiseria Horwitz</td>
<td>Rabbit</td>
<td>1:30,000/NA9340†</td>
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<tr>
<td>β-Crystallin</td>
<td>2% BSA</td>
<td>1:3,000/Antiseria Zigler</td>
<td>Rabbit</td>
<td>1:30,000/NA9340†</td>
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<td>E2F1</td>
<td>2% BSA</td>
<td>1:1,000/SC-251§</td>
<td>Mouse</td>
<td>1:10,000/SC-2005§</td>
</tr>
</tbody>
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* Molecular Probes, Eugene, OR.
† Amersham, Arlington Heights, IL.
‡ Oncogene Research Products, Boston, MA.
§ Santa Cruz Biotechnology, Santa Cruz, CA.
Immunohistochemistry

For immunofluorescence experiments, monolayer cultures on ECM were fixed with 4% paraformaldehyde. Fluorescent stains used were phalloidin-tetramethyl rhodamine isothiocyanate (TRITC; Sigma) at 5 μg/ml in PBS, for visualization of filamentous actin; DiOC6 (Molecular Probes, Eugene, OR) at 1 μg/ml in PBS, which stains all cell and organelle membranes, and DAPI (Sigma) at 0.25 μg/ml, for examination of nuclear material. For primary and secondary antibodies, see Table 1. All experiments were replicated at least once, and controls for each primary and secondary antibody were screened to confirm the absence of nonspecific immunofluorescence.

Western Blot Analysis

Total proteins were isolated from cultures rinsed twice with PBS and treated on ice with extraction buffer (2% sodium dodecyl sulfate [SDS], 100 mM Tris-HCl [pH 6.8], 30% glycerol, protease inhibitor cocktail tablets [Boehringer–Mannheim, Mannheim, Germany], 5 mM phenylmethylsulfonyl fluoride [PMSF], and 1:200 aprotinin [Sigma]). Cell lysates were pooled and frozen immediately at −80°C. Protein concentration in the supernatant was measured using a kit (Lowry DC Assay Kit; Bio-Rad, Hercules, CA). For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), 30 μg of total protein was loaded in a 1.0-mm thick gel. A 17% gel was used for separating proteins with molecular weight (MW) less than 40 kDa and 7.5% for proteins with MW greater than 40 kDa. Gels were transferred onto a membrane (Immobilon-P; Millipore, Bedford, MA) at 20 V and 4°C overnight (approximately 16 hours) in transfer buffer (20% methanol, 0.1% SDS, 20 mM Tris [pH 8.3], and 150 mM glycine). For Western blot analysis, membranes were rinsed in 0.5% Tween-Tris–buffered saline (TBS; 100 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.5% Tween-20) before being blocked. Primary and secondary antibodies were diluted in their corresponding blocking solutions (Table 1) and incubated at 2 and 0.5 hours, respectively, at room temperature. All secondary antibodies were conjugated to horseradish peroxidase (HRP). Proteins were detected by ECL (Amersham, Arlington Heights, IL) on film (Hyperfilm; Amersham, Arlington Heights, IL). Quantitation of the gel bands was completed using an image analyzer (Alpha Image; Alpha Innotech, San Leandro, CA). Molecular weight markers included the following: human recombinant FGF-2, SK-Hep1 total cell lysate, RPN 756 (Amersham), bovine lens extracts, and α- and β-purified crystallin standards (Sigma).

RESULTS

We have presented growth curves for both HLE cells grown on BCE-derived matrix and HLE B-3 cells grown on tissue culture plastic (Fig. 1). After a 2-day period of recovery from subcultivation, the HLE cultures proliferated exponentially and reached confluence at 3 to 4 × 10^5 cells per cm². HLE cells at passage 6 grew at a reproducible population-doubling time of approximately 24 hours, nearly identical with the growth rate of the HLE B-3 cell line between passages 13 and 18. Both grew to a confluent state, although the HLE grew to a slightly higher density because of the organized alignment of the cells. Both cell lines have been maintained at confluent densities for up to 4 months with feeding. The proliferation potential of HLE cells on ECM diminishes significantly after passage 10, and ceases if the cultures are allowed to reach confluence before subcultivation. Karyotyping (illustrated in Fig. 2) and in situ hybridization with human Cot-1 DNA (data not shown) confirmed the
HLE cells were of human origin and not contaminated with bovine cells. All cells were diploid with $2N = 46$ chromosomes. Cell morphology changed during cultivation. Lentoid bodies were present in HLE cultures at day 15 in culture (Fig. 3) but were not observed in HLE B-3 cultures. Several days after confluence was achieved, patches of parallel cells began to appear, and phalloidin-staining of actin fibers and DAPI nuclei revealed parallel alignment of the cells as long, narrow cells. Figure 4 compares the morphology of DAPI- and phalloidin-stained confluent HLE and HLE B-3 cells. This fluorescence micrograph indicated that the HLE had morphologic characteristics of elongated nuclei and filamentous actin fibers that were more similar to lens fiber cell differentiation in vivo than was evident with the HLE B-3 cell line that showed rounded cell nuclei and disorganized actin filaments. Finally, use of dual fluorescence staining for nuclear and cytoplasmic compartments (Fig. 5) revealed evidence for enucleation of intact HLE cells at day 15.

Immunofluorescence histochemistry was completed in situ with exponentially growing and 47-day confluent HLE cultures to look for markers of lens fiber cell differentiation. Figure 6 is a photograph montage depicting three pairs of immunohistochemistry studies on lens epithelial cells (Figs. 6a, 6b, 6c), and on 6-week confluent differentiating lens cultures (Figs. 6d, 6e, 6f). Figures 6a and 6d were stained with DAPI and phalloidin-TRITC, 6b and 6e were probed with a polyclonal human anti-MIP26, and 6c and 6f were probed with human anti-γ-crystallin antibody. Note that identical lens cell fields were visualized in 6e and 6f. The confluent cultures showed significant evidence of γ-crystallin and MIP26 that was not evident in the younger, epithelial cultures. Western blot analysis has confirmed the specificity of the α-crystallin antisera (discussed later), and experiments are in progress to examine the specificity of the MIP26 and γ-crystallin antisera.

We also investigated the expression of several protein markers for lens fiber cell differentiation in our HLE model system with RT-PCR or Western blot analysis. They include lens crystallin proteins (αA-, αB-, and β-crystallin), MIP26, and the cyclin-dependent kinase inhibitor (CDKI) p57KIP2 that is expressed when the cells exit the cell cycle during development and terminal differentiation. Our initial observations are that p57KIP2, αA-, and βB2-crystallin proteins appeared early in the differentiation process, whereas αB- and MIP26 were expressed later. The following figures present some of the evidence we have for expression of these markers. Figure 7 summarizes digitized RT-PCR data on the expression of αA- and βB2-crystallin and FGFR1. Lanes 1 and 3 in the first two scans represent day 3 in culture (exponential), and day 15 (confluent). The data are semiquantitative, in that equal amounts of RNA from the same set of samples were reverse transcribed.
and subjected to PCR. Equal volumes were loaded in the gel, as determined by digitized GAPDH levels, in all studies of each growth state. Gel band intensity suggests higher expression of $\alpha$-A- and $\beta$B2-crystallin and FGFR1 in exponentially growing cells than at confluence. According to Weng et al., this set of FGFR1 primers should yield three products, 1100 bp, 1000 bp, and 800 bp, corresponding to the three transcripts. Our results from PCR amplification show a single product at 800 bp. Figure 8 summarizes our Western analyses of differentiation proteins as a function of days in culture. The data show that a steady level of $\alpha$A-crystallin was present at all cell ages. $\beta$-crystallin was not present at day 6 in culture but increased steadily over time until day 21. Figure 8 also shows the Western blot analysis of the $\beta$-crystallin expression. Quantitative densitometry indicated that there was an increased prevalence of 22-kDa $\beta$B2-crystallin with time in culture from day 6 (lane 1) to day 12 (lane 2), to day 19 (lane 3), and at day 28 (lane 4).

Quantitative densitometry completed on a p57 KIP2 Western blot of total protein from lens cultures of different ages from day 3 to day 25, shows a significant increase in p57 KIP2 expression as the cultures reach confluence, and the levels stay

**Figure 5.** A 15-day HLE culture sample, fixed and dual stained with DiOC6 and DAPI, showed evidence of enucleation.

**Figure 6.** Photograph montage depicting three pairs of immunohistochemistry studies on lens epithelial cells (a, b, and c), and 6-week confluent differentiating lens cells (d, e, and f). Cultures in (a) and (d) were dual stained with phalloidin-TRITC and DAPI. Cultures in (b) and (e) were probed with a polyclonal human anti-MIP antibody and those in (c) and (f) were probed with human anti-$\gamma$-crystallin antibody.

**Figure 7.** Gene-specific amplification of cDNA in the analysis of mRNA expression by RT-PCR amplification of $\alpha$A- and $\beta$B2-crystallin, FGFR1, and the housekeeping gene GAPDH of exponential 5-day (lane 1) and 15-day confluent (lane 3) HLE cultures. Lanes 2 and 4 are the corresponding negative controls where reverse transcriptase was excluded in the RT reaction to check for genomic DNA contamination in the samples. A positive GAPDH control (+) was provided by the manufacturer.
At later times in culture, Western blot analysis of the prevalence of E2F1 and pRB with time in culture for the HLE. The data show that E2F1 had greater prevalence in exponential growth of HLE cells and at day 20 show a shift downward to a lower MW. pRB, however, may have a slightly lesser prevalence in exponentially growing cells, which appeared to stay high thereafter with time in culture.

DISCUSSION

As with any in vitro model system, there are both advantages and potential limitations to the use of a model within the framework of the scientific questions asked. Our goal was to develop and characterize a system amenable to the investigation of the specific molecular mechanisms underlying radiation-induced cataractogenesis. We sought a method that could complement existing information gleaned from studies of radiation-induced cataractogenesis in vivo, both from animal work and from clinical experience. We therefore have studied how reproducibly lens cell growth and differentiation traits could be achieved with the method of culture on BCE-derived matrix and feeding with FGF-2.

Few published studies have characterized the molecular basis of lens development in the human eye. Instead the focus of most work has been rodent or chicken lens. Animal surrogates of both normal and engineered genotypes have added fundamentally important information to our understanding of lens development, differentiation, and response to toxic insults. Use and influence of the αA-crystallin promoter have dominated the creation of transgenic mice in which there is lens-specific overexpression of certain factors. Concern has been expressed, however, regarding the use of these transgenic mice as models for the study of cataractogenesis and for the potential limitations of the predominant use of this single promoter. Genetically engineered cell cultures also have limitations but may help elucidate the complex and interacting signal transduction pathways under study in mammalian cell cycle control and DNA repair systems. There is a need to compare the results obtained from each of these approaches to document the significance of the method used to the answers obtained.

HLE cells have relatively low proliferative potency compared with epithelial cells from other species including chicken, rat, rabbit, canine, or bovine. It has been reported that HLE grown out onto plastic petri dishes from an 18-week prenatal source formed lentoid bodies and showed evidence of differentiation, but grew slowly and only achieved a sixth passage after 6 months in culture. HLE cells immortalized by viral infection or transfection grow well in vitro, with steady population-doubling capability.

HLE cell growth and differentiation traits could be achieved with the method of culture on BCE-derived matrix and feeding with FGF-2.
on lens capsule in a protein-free medium has been reported to be capable of sustaining lens cell survival and proliferation over periods of several weeks. There is also a recent report of the generation of two nontransfected HLE cell lines from capsule explants on microporous membranes supplemented with growth stimulants.

However, although each of the HLE models described herein has potential advantages for the investigation of specific research questions, there are potential problems for their use in radiation studies. For example, immortalized cells have been reported to have altered cell cycle control in response to radiation. The use of cell lines with nondiploid chromosome numbers could present an altered radiation damage and repair profile compared with a normal diploid cell line. Reproducible access to human lens capsule material could be a problem for radiation studies if the age of the donor alters the status of cytokines or other growth factors associated with the capsule. The use of the BCE-derived matrix system to grow prenatal HLE has features that make it more amenable to radiation studies.

There are several unique aspects of the HLE cell model reported here. The expression of mRNA and protein markers of lens differentiation in HLE cells appears in exponential growth as reported by others culturing HLE on plastic, but before the dramatic morphologic alignment and elongation of the nuclei. The dual evidence for protein markers of differentiation, and the morphologic evidence of elongation is a novel aspect to our system that to our knowledge has not been reported for cultured HLE. The cell elongation observed is similar to that observed in cultured chicken lens cells stimulated with FBS or insulin and in rat lens epithelial cells cultured as explants, on glass slides, and on Matrigel–Engelbreth–Holm–Swarm (EHS) matrix (Collaborative Research, Bedford, MA) supplemented with FGF-2. Bovine lens epithelial cells elongate after 20 passages in culture. Elongation of cultured bovine lens cells can also be induced by dexamethasone or by an extract of the bovine retina. Stress fibers present in parallel stress fibers appear. Several investigators have indicated that crystallin synthesis per se is by no means a prerequisite for elongation-associated morphologic differentiation.

Another unique aspect of our HLE model is the demonstration of p57Kip2 expression. The p57Kip2 gene encodes an inhibitor of several G1 cyclin–cyclin-dependent kinase activities, which negatively regulates cell cycle progression and is associated with exit from the cell cycle in lens fiber cell differentiation in vivo. Mice without p57Kip2 have altered lens cell proliferation and differentiation, but still express γ-crystallin and MIP26. We observed expression of p57Kip2 protein in HLE during exponential growth and an increased expression at confluence, which is consistent with in vivo data.

Recently it was shown that the expression of one of the FGF receptors, FGFR1, was closely associated with the onset of lens fiber differentiation. In addition, FGFR1 mRNA was found to be upregulated in primary cultures of lens epithelial cells by either serum or exogenous FGF-2, whereas the mRNA encoding FGFR2 and FGFR3 were not. Expression of a truncated FGFR1 results in defective lens development in transgenic mice.

Characterization of Human Lens Cells on Matrix

We used RT-PCR to measure the transcription of mRNA for marker proteins for lens fiber differentiation. Total RNA extracts from HLE cells in exponential (day 5) or confluent (day 15) growth was studied. Figure 9 shows that, similar to some published accounts with immortalized HLE cells, the nontransformed HLE cells on matrix also expressed α- and βB2-crystallin and FGFR1 transcripts in exponential growth. The PCR products of αA-crystallin show a band of the expected 750-bp size. Additional amplicons at 400 bp and 600 bp may represent alternative splice variants. We also show that, in our model system, there was diminished expression at day 15 in culture. Our results are consistent with a recently published report that fiber cells are transcriptionally and translationally competent until the time of organelle loss.

MIP26 is a protein with a significant prevalence whose specific function in the lens fiber cell membrane is still not completely known but is thought to involve cell–cell communication. The spatial distribution of MIP26 protein in rat lens is reported to be present throughout the fiber cell membrane compartments, both functional and nonfunctional. In our system, MIP26 and γ-crystallin immunofluorescence were absent from lens cell cultures at day 5, but were evident at day 47 (Fig. 6). Other cultured lens cell models do not show evidence for either protein.

Western analyses of total proteins extracted from HLE cells at different times in culture (Fig. 8) showed a prevalence of p57Kip2 protein in exponentially growing HLE and a factor of greater than two increase at day 15 in culture. The Western analyses also showed a steady level of αA-crystallin and increasing levels of αB- and βB2-crystallin. Our results are not in complete agreement with published data on the transcription of αA- and αB-crystallin in mouse embryos. Robinson and Overbeek show that αB-crystallin precedes the expression of αA-crystallin during murine ocular development, the pattern of αA-crystallin is upregulated, and αB-crystallin expression is downregulated during prenatal fiber cell differentiation. In cultured chicken lens epithelial cells αA- and αB-crystallin mRNA were significantly upregulated after time in confluence at the time of lentoid development. HLE B-3 cells do not express αA-crystallin after passage 11. Immortalized HLEsRA 01/04 express very low levels of α- and β-crystallin and aldose reductase at the protein level. The upregulation of αB-crystallin in other tissues has been associated with stress factors or pathologic conditions. Figure 9 shows, however, that HLE cells held in confluence showed a shift in the expression pattern of E2F1 protein that is consistent with differentiation. Cell cycle control of differentiating lens cells has had only limited investigation. Significant recent work has indicated that E2F and pRB families may contribute to growth arrest as lens epithelial cells differentiate into fiber cells.

One limitation of HLE culturing on BCE-derived matrix is that the described characteristics do not occur homogeneously. With time in culture after confluence, patches of cells began showing evidence of these morphologic changes, and more occurred within periods of up to 4 months. Examination of cell morphology in the dense cultures became more difficult because the cells were so compact. After this time, the required change of media on alternate days caused the condition of the ECM to deteriorate and detach.

In summary, we are reporting a method to cultivate HLE on BCE-derived matrix supplemented with FGF-2 that allows...
prenatal cells to begin differentiation in vitro, and represents a mixed cohort of cells at the bow of the lens in vivo. Although future work is needed to establish the generality of the differentiation observations described for prenatal HLE cell sources, especially those grown in other laboratories, this report can be used as a basis for comparison. We believe the HLE model on matrix is amenable to experimental investigations of mechanisms of radiation-induced cataractogenesis, and experiments are in progress.

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