Effects of Growth Factors on Proliferation and Differentiation in Human Lens Epithelial Cells in Early Subculture

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Purpose. A successful method to subculture human lens epithelial (HLE) cells that retain their intrinsic characteristics is of great importance. This study examines the effects of four different growth factors on proliferation and differentiation in HLE cells in early subcultures.

Methods. Specimens of HLE cells were obtained from infants. First- or second-passage cells were cultured in the presence of 10⁻² to 10² ng/ml acidic and basic fibroblast growth factor (aFGF, bFGF), epidermal growth factor (EGF), or insulin-like growth factor-I (IGF-I). Cell proliferation was determined from cell number, and fiber differentiation was assessed from the time of appearance, the number of lentoids formed, and the expression of γ-crystallin.

Results. Cell proliferation was increased by EGF, bFGF, and IGF-I at concentrations greater than 10⁻¹ ng/ml; the most effective concentration was 10 ng/ml. The effect of aFGF on proliferation appeared only at a concentration of 10² ng/ml. EGF, bFGF, or IGF-I at 10 ng/ml affected the time of appearance and the number of lentoids formed within 5 to 7 days. In contrast, lentoids were observed after 42 days without the addition of growth factors. Lentoid formation was accompanied by the expression of γ-crystallin.


A successful method to subculture human lens epithelial (HLE) cells that retain their intrinsic characteristics is of great importance for the study of the normal physiology of these cells and for investigations on the etiology of cataract formation. There are numerous studies in which several mammalian epithelial cells have been cultured successfully for long periods of time.¹⁻³ In contrast to the observations in animal lens epithelial cells, HLE cells in tissue culture lose their growth potential and their ability to differentiate into fiber cells over a limited number of passages.⁴⁻⁶ Previous studies from this laboratory have shown that HLE cells from infants can be grown successfully through several passages, but their proliferative potency declines gradually.⁷ In addition, attempts in many laboratories to culture lens epithelial cells from eye bank lens or senile cataract have been less successful and cannot be subcultured.⁸⁻¹¹

In vitro fiber differentiation can be assessed from the appearance of lentoids, which have the characteristics of lens fibers, gap junction formation, and the expression of γ-crystallin and membrane protein (MP-26).¹²,¹³ Spontaneous in vitro fiber differentiation in which lentoids arise from monolayer cultures has been noted in most long-term cultures with HLE cells obtained from children and in early subcultures.¹⁴,¹⁵ Also, fiber differentiation was observed in these short-term cultures when the cells were grown on cell-substrate adhesion-free surfaces at high density.¹⁶

Fiber differentiation and crystallin expression in lens epithelial cells of animals have been shown to be affected by several kinds of growth factors,¹⁷⁻²⁴ some of which have been isolated from the retina, the vitreous humor, and other ocular structures.²⁵⁻³⁰ Growth factor is defined as a peptide that regulates cell proliferation and differentiation similar to hormones.³¹ An epidermal growth factor (EGF) receptor has been characterized in rabbit lens epithelial cells using ¹²⁵I-labeled EGF.³² Also,
the effects of fibroblast growth factor (FGF) on rat lens epithelial cells have been studied.\textsuperscript{20-22} This growth factor has been found to regulate cell proliferation, migration, and fiber differentiation in a concentration-dependent manner.\textsuperscript{21} This capacity of FGF to modulate cell growth and fiber differentiation decreases in cells obtained from older rats.\textsuperscript{22} The effects of eye-derived growth factor\textsuperscript{17,18} and retinal extracts\textsuperscript{20} on mammalian lens cells have been attributed to FGF because anti-FGF antibody almost consistently prevents fiber differentiation induced by retinal extracts.\textsuperscript{22} Other growth factors reported to affect cell proliferation and differentiation on lens epithelial cells in culture are insulin-like growth factor (IGF)\textsuperscript{23,26} and platelet derived growth factor.\textsuperscript{24}

In contrast to the numerous studies with various mammalian cells cited, there are no reports of the effects of growth factors on HLE cells. In this study, we describe the quantitative effect of four different growth factors on proliferation in HLE cells in early subcultures, and we report the enhancement of in vitro differentiation of HLE cells as evidenced by lens epithelial cell differentiation.

**MATERIALS AND METHODS**

**Culture of Human Lens Epithelial Cells**

Specimens of HLE cells were obtained from 20 infants 3 to 9 months old who underwent surgery for retinopathy of prematurity. Informed consent was obtained from parents or legal guardians, and Oakland University’s institutional human experimentation committee approval was granted. Tenets of the Declaration of Helsinki were followed. The method for establishing primary cultures has been described previously.\textsuperscript{3} Briefly, the pieces of capsule and epithelium obtained during surgery were washed once with Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-free phosphate-buffered saline and collected with a microsuction pipette. After microscopic examination, small fragments of capsule (1 to 2 mm square) with epithelial cells attached were placed in a 60-mm culture dish (Falcon; Becton Dickinson, Oxnard, CA) as explants.

Cells from primary culture or first-generation subculture were dissociated with trypsin–EDTA solution (0.05% trypsin–0.02% ethylenediaminetetraacetic acid solution; Gibco, Grand Island, NY), and inoculated at 70 ± 5 cells per mm\textsuperscript{2} of cell density in each culture dish or protein-binding biopore membrane (Millicell-HA; Millipore, Bedford, MA).

Cultures were incubated in a 5% CO\textsubscript{2} atmosphere at 36.5°C. Antibiotic-free Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 20% fetal bovine serum (Gibco) was used as the standard medium. This medium has been shown to be optimal for maintenance of HLE cells in culture.\textsuperscript{7,39,34}

**Growth Factors**

Human EGF (Sigma, St. Louis, MO), human FGF-acidic (aFGF; Sigma), human FGF-basic (bFGF; Sigma), and human IGF-I (IGF-I; Pepro Tech, Rocky Hill, NY) were added to the standard medium. During the culture period, the medium containing growth factor was replaced three times per week.

**Effect on Proliferation**

To examine the effect of growth factors on cell proliferation, second-passage cells were selected because proliferative potency of the cells had already decreased\textsuperscript{7} so that any stimulation with added growth factors could be observed readily. Proliferation was assessed by counting the cell number. Ten batches of HLE cells from 10 infants were used; the cells from the same batch were used for each experiment and for each growth factor. The cells were seeded in three wells for each culture period and each concentration of growth factor using Multiwell tissue culture plate (12-Well; Becton Dickinson, Lincoln Park, NJ). For counting of the cells at four different time intervals with five different concentrations of each growth factor and controls, a total of 72 wells had to be cultured. The experiments were repeated at least three times for each growth factor using different batches of cells. To determine appropriate concentrations, growth factors were added to the medium at concentrations of 10\textsuperscript{-2}, 10\textsuperscript{-1}, 1, 10, 10\textsuperscript{2} ng/ml (1.7 X 10\textsuperscript{-12} to 1.7 X 10\textsuperscript{-8} M of EGF; 1.3 X 10\textsuperscript{-12} to 1.3 X 10\textsuperscript{-8} M of IGF-I; and 0.6 X 10\textsuperscript{-12} to 0.6 X 10\textsuperscript{-8} M of aFGF and bFGF). Cells were dissociated with trypsin–EDTA solution and counted using trypan blue and a hemocytometer at various time periods from 1 day through 4 weeks after incubation. The number of viable cells was counted three times for each well.

To determine the quantitative proliferative potency, the maximum population doubling level (PDL\textsubscript{max}) was calculated.\textsuperscript{9,35} PDL\textsubscript{max} is expressed as:

$$
PDL_{\text{max}} = \log_{10}(N_{\text{max}}/N_{0})/\log_{10}2$$

where $N_{\text{max}}$ = maximum number of cells, and $N_{0}$ = initial number of cells.

**Effect on Differentiation**

For studies of fiber differentiation, we used first-passage cells because previous experiments indicated that these cells had a greater capacity than cells in later passages to undergo spontaneous in vitro differentiation;\textsuperscript{33} the cells in second- or third-passage failed to form lentoids at least during the 40- to 50-day culture period. Differentiation was assessed from lentoid for-
mation, appearance of gap junctions in the lentoids, and the expression of \( \gamma \)-crystallin. Ten batches of HLE cells derived from 10 infants were used; four batches were used for the observations of lentoid formation, and the other six were used for ultrastructural and immunocytochemical studies. The cells on 60-mm dishes were prepared for invert phase-contrast microscopy and transmission electron microscopy (TEM); cells on 12-mm protein-binding membrane (Millicell-HA) were used for immunogold labeling electron microscopy. The growth factors that affected cell proliferation were added to the standard medium. The concentration of EGF, bFGF, and IGF-I was 10 ng/ml (1.7 \( \times 10^{-9}, \) 0.6 \( \times 10^{-9}, \) and 1.3 \( \times 10^{-9} \) M, respectively); \( \alpha \)-FGF was 10\(^2 \) ng/ml (0.6 \( \times 10^{-8} \) M).

Lentoid formation was observed under a phase-contrast microscope and quantified by counting the total number of lentoids in the entire culture dish; the cells from the same batch were used for each experiment, and at least two culture dishes were prepared for each growth factor in one experiment. This experiment was repeated four times using four different batches of cells.

Transmission electron microscopy on monolayer cultures was carried out in a fashion similar to the one we previously described.\(^{16} \) Briefly, cells were cultured for 14, 28, or 42 days and were rinsed several times with serum-free Dulbecco's modified Eagle's medium and fixed with 1% osmium tetroxide solution in 0.1 M cacodylate buffer (pH 7.4) for 3 minutes. After fixation with 2.5% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer for 3 hours, tissues were refixed with 1% osmium tetroxide solution for 60 minutes. After each fixation, tissues were washed several times with cacodylate buffer. They were dehydrated through graded ethanol and embedded in Poly/Bed 812 (Polysciences, Washington, PA). Sections approximately 60 nm thick were cut with a diamond knife, stained with 2% uranyl acetate and Reynolds' lead citrate solution, and examined with an ISI-LEM 2000 (International Scientific Instruments, Milpitas, CA) or a Phillips EM 410 (Phillips, Mahwah, NJ) transmission electron microscope.

For immunogold labeling of \( \gamma \)-crystallin, tissues along with protein-binding membranes (Millicell-HA) were fixed in 2% paraformaldehyde and 0.25% glutaraldehyde in phosphate buffer (pH 7.4) for 1 hour on day 14 of cultivation. After dehydration through graded ethanol, tissues were embedded in L-R White resin (Polysciences), sectioned, and mounted on nickel grids. Sections were blocked with 10% goat serum in 20 mM Tris-buffered saline with 0.1% bovine serum albumin (BSA-TBS, pH 7.4) for 1 hour, and then incubated with rabbit anti-human \( \gamma \)-crystallin antibody (1:100 dilution in BSA-TBS, pH 7.4) for 3 hours. This antibody has been shown to react with \( \gamma \)-crystallin but not with \( \alpha \)- and \( \beta \)-crystallin.\(^{7,16} \) After washing three times in BSA-TBS for a total of 30 minutes and reblooding in goat serum in BSA-TBS for 1 hour, the sections were treated in goat anti-rabbit immunoglobulin containing 15 nm colloidal gold (Amersham, Arlington Heights, IL; 1:100 dilution in BSA-TBS, pH 8.2) for 1 hour. The grids were washed three times in BSA-TBS for a total of 45 minutes, and they were dipped in distilled water for 15 seconds and dried; they were then stained with uranyl acetate and examined by TEM. Nonimmune rabbit serum instead of \( \gamma \)-crystallin antibody was used as a negative control.

**RESULTS**

**Cell Proliferation**

During the course of this study, it became apparent that the cells from different batches had varied growth potential so that the results obtained from different batches could not be pooled and compared. Therefore, a valid comparison could be made only between controls and experimental data derived from the same batch of cells.

The results of a typical experiment are shown in Figure 1. All four growth factors studied affected cell proliferation, and the effect of each growth factor on cell number was concentration dependent. The increase in cell number was statistically significant after 5 days of cultivation and at a concentration greater than 10\(^{-1} \) ng/ml of EGF, IGF-I, and bFGF; the most effective concentration was 10 ng/ml (Student's \( t \)-test, \( P < 0.001, n = 3 \) for each culture period and each concentration). The effect of \( \alpha \)-FGF on proliferation appeared only at a concentration of 10\(^{-2} \) ng/ml (Student's \( t \)-test, \( P < 0.005, n = 3 \) for each culture period and each concentration). In addition to the increase in cell number, the data in Figure 1 also show an enhanced growth rate as indicated by the steeper growth curves.

PDLmax is one of the quantitative mathematical models to determine cell proliferative potency.\(^{9,35} \) Table 1 shows PDLmax of each concentration of the four growth factors investigated in typical batches of cells. PDLmax was significantly higher with IGF-I and EGF compared to their controls at a concentration of 10\(^{-2} \) ng/ml, whereas bFGF and \( \alpha \)-FGF required concentrations greater than 10\(^{-1} \) and 10\(^{-2} \) ng/ml, respectively (Student's \( t \)-test, \( P < 0.001 \) for EGF, IGF-I, and bFGF, \( P < 0.005 \) for \( \alpha \)-FGF, \( n = 6 \) for each culture period and each concentration).

Similar results to those shown in Figure 1 and Table 1 were obtained in other experiments in which cells from different batches were used (data not shown).
FIGURE 1. The effect of growth factors on cell proliferation. (a) EGF, (b) IGF-I, (c) aFGF, (d) bFGF. Growth factors were added to the standard medium (○) at concentrations of $10^2$ (●), 10 (○), 1 (■), $10^{-1}$ (△), $10^{-2}$ (▼) ng/ml. Cell proliferation was stimulated with more than $10^{-1}$ ng/ml of b-FGF, EGF, IGF-I ($P < 0.001$), and $10^2$ ng/ml of a-FGF ($P < 0.005$). n = 3 for each culture period and each concentration. Student’s t-test was used. Standard deviations are omitted to simplify these graphs.

Cell Differentiation

In addition to the effect on cell proliferation, the growth factors had an influence on the time of appearance and the number of lentoids formed. Figure 2 shows phase-contrast micrographs of first-passage cells with 10 ng/ml of EGF, bFGF, and IGF-I and $10^2$ ng/ml of aFGF. With EGF, cell size became smaller than control cells, cell aggregations appeared on day 3 after cultivation, and lentoids formed on day 5. The cells surrounding the lentoid became larger than the cells in the early culture period. With bFGF or IGF-I, cell aggregation first appeared on day 5, and lentoid formations were observed on day 7. With aFGF, cell aggregation appeared on day 7, and no lentoids were seen until day 14. The time course and the number of lentoids formed in the presence of growth factors were determined by counting the total number of lentoids present in the entire 60-mm culture dish at different culture periods. In a typical experiment, as shown in Figure 3, three culture dishes were prepared for each growth factor. Lentoids appeared from day 5 with EGF, day 7 with bFGF and IGF-I, and day 14 with aFGF. The number of lentoids increased gradually and reached 451 ± 21 with EGF, 250 ± 15 with bFGF, 70 ± 7 with IGF-I, and 22 ± 1 with aFGF on day 42. In contrast, only 4 ± 0 lentoids were observed after 42 days without the addition of growth factors. The number of lentoids formed in cells from different batches varied between each culture period and with each growth factor, but the time course showed similar results for all batches of cells (data not shown).

The ultrastructure of lentoids enhanced with EGF is shown in Figure 4. Lentoids consisted of multilayers of enlarged cells that had few cytoplasmic organelles on day 14. On day 28, the cytoplasm was very finely granular and uniform in density, with concomitant

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<tr>
<th>Growth Factors</th>
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<tr>
<td></td>
<td>$10^2$</td>
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<tr>
<td>EGF</td>
<td>2.74 ± 0.00*</td>
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<tr>
<td>IGF-I</td>
<td>1.92 ± 0.04†</td>
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<tr>
<td>aFGF</td>
<td>2.48 ± 0.01†</td>
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<tr>
<td>bFGF</td>
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EGF = epidermal growth factor; IGF = insulin-like growth factor; aFGF = acidic fibroblast growth factor; bFGF = basic fibroblast growth factor. The method to calculate PDLmax is given in the text. Briefly, maximum number of cells for each concentration and each growth factor was used for calculating PDLmax. Two different batches of cells, one for EGF and IGF-I and the other for aFGF and bFGF, were used in this experiment. Values are means ± SD. n = 6. Statistical analysis performed using Student’s t-test.

* P < 0.001; † P < 0.005.
FIGURE 2. The effect of growth factors on lentoid formation as seen by phase-contrast microscopy. (a) The standard medium, (b) EGF, (c) IGF-I, (d) aFGF, and (e) bFGF. Arrows indicate lentoids, and arrowhead shows cell aggregation.

loss of cytoplasmic organelles and the appearance of interdigitations or interlocking processes. In some areas, typical gap junctions were observed under higher magnification. Similar observations were made with bFGF and IGF-I (data not shown). In contrast, there were only normal epithelial cells, even on day 28, and interdigitations, gap junctions, and homogeneous cytoplasms appeared after 42 days of cultivation in the standard medium.

Figure 5 is a transmission electron microscopic view of lentoid sections labeled with immunogold for γ-crystallin. Even in the lentoid formed on day 14, an early stage of fiber differentiation, many gold particles were observed in the cytoplasm. In contrast, no gold particles were observed in the negative control, and there was little reaction against γ-crystallin in monolayers of HLE cells (nonlentoid).

DISCUSSION

The major purpose of this investigation was to examine the possible effects of a number of growth factors in HLE cells to establish a more successful culture system than currently available. All the growth factors selected for this study are known to stimulate proliferation of cells of ectodermal origin.31

The results of this study clearly demonstrate that a number of growth factors enhance the proliferative potency of HLE cells in culture and stimulate fiber differentiation, as evidenced by the time course and the number of lentoids formed. These findings are similar to the observations made in a number of cells derived from lenses of various animals.17–25 Our observations in HLE cells have been made on early subcultures that had a low but definite proliferative potency. Because of the limited number of cells that can be derived from each specimen and the varied proliferative potency of HLE cells, the results of cell proliferation enhanced by growth factors could only be statistically compared with the data obtained from the same batch of cells. Based on population doubling criteria (PDLMmax), EGF was most effective in promoting cell proliferation, and the effect was dose dependent. The

FIGURE 3. The effect of growth factors on the time course and number of lentoids formed. Lentoids appeared on day 5 with 10 ng/ml of EGF (O), day 7 with 10 ng/ml of IGF-I (A) and bFGF (•), and day 14 with 10² ng/ml of aFGF (▲). The lentoid number increased gradually, and the mean number reached 451 with EGF, 250 with bFGF, 70 with IGF-I, and 22 with aFGF on day 42. In contrast, four lentoids were observed after 42 days in the control culture (○). Standard deviations are given in the text.
FIGURE 4. The effect of growth factors on cell differentiation as seen by transmission electron microscopy. Lentoid formation stimulated with 10 ng/ml of EGF consisted of multilayered, elongated cells that had few cytoplasmic organelles on day 14 (b). On day 28, interdigitations (arrowheads) and fine granular cytoplasms with few organelles were observed (c); at higher magnification (d); gap junctions (arrows) were clearly evident. In control, there were only normal epithelial cells even on day 28 (a).

effect of the other growth factors, bFGF and IGF-I, was also concentration dependent. These findings are similar to those with bFGF on rat lens epithelial cells.21

We used 20% fetal bovine serum in the standard medium because HLE cells require at least 5% of fetal bovine or calf serum to be alive or to show any growth; the optimum serum concentration is 15% to 20%.33,34 Even at the optimum concentration of serum, the proliferative capacity of HLE cells is very low compared with many animal lens epithelial cells.1-11 However, the addition of growth factors used in this study significantly enhanced cell proliferation. To the extent that serum is thought to be associated with some growth factors of undetermined nature and unknown quantity, there is a possibility that our results may represent the combined effects of the added growth factors and those that might be present in serum or other unknown factors in serum. In many tissues, growth factors have been shown to have multiple effects; they have been found to be mitogenic and inhibitory as well as to promote cell survival.31 At the highest concentration used—10^7 ng/ml—these growth factors had little effect on PDLo, and growth curves compared with the effects noted with 1 ng/ml of EGF, and 10^-1 ng/ml of bFGF or IGF-I. It has been reported that cell proliferation in rat lens epithelial cell culture is optimized by lower concentrations of bFGF than fiber differentiation.31 In our study, only one concentration of each growth factor, which was determined as the optimum for cell proliferation, was used for fiber differentiation. It is possible that the number of lentoids formed may increase with higher concentrations of growth factors if the human lens system behaves similarly to the rat lens epithelium, but this remains to be established.

In vitro fiber differentiation of HLE cells in this study was assessed from morphologic characteristics of lentoids as well as γ-crystallin expression. In contrast to the spontaneous fiber differentiation in controls, without the addition of growth factors, in which lentoids are few and appeared after long periods of culture, EGF, aFGF, bFGF, and IGF-I affected both
FIGURES. Immunogold labeling of γ-crystallin. On day 14, many gold particles were observed in lentoids formed in the presence of 10 ng/ml of bFGF (a). In contrast, no gold particles were found in lentoid, in which nonimmune serum instead of γ-crystallin antibody was used (b). There was little reaction with γ-crystallin antibody in the monolayers of the cultured cells with the standard medium (c). N = cell nucleus; asterisks = cellular membrane.

the time of appearance and the number of lentoids formed. The time course of the morphologic changes of these lentoids included the decrease of cytoplasmic organelles, the formation of interdigitations or interlocking of cellular membranes, the appearance of a very finely granular and uniform density structure with concomitant loss of cytoplasmic organelles, and the presence of abundant gap junctions. These findings are consistent with the differentiation of lens epithelial cells into fiber-like structures, and the results clearly indicate that, in addition to their stimulatory effect on cell proliferation, growth factors also enhance lens cell differentiation. Although the presence of gap junctions in lentoids as seen by TEM is consistent with fiber differentiation, the identity of these structures with gap junctions in situ remains to be established by other criteria. This ability of growth factors to stimulate or accelerate lentoid formation may prove useful in studying the HLE cell differentiation in vitro. Although previous studies on animal lens cells have shown that growth factors enhance cell differentiation, no detailed morphologic characteristics of the fibers formed were presented that could be compared with in situ structures.

In the current study, γ-crystallin, a marker protein for fiber differentiation, was detected in lentoids on day 14 of culture when the morphologic characteristics showed only loss of cytoplasmic organelles. These results suggest that γ-crystallin expression is induced at an early stage of fiber differentiation, i.e., before the appearance of interdigitations or gap junctions.
Growth factors Effects on Cultured Human Lens Epithelial Cells

Growth factors have been detected in human ocular media. Furthermore, human ocular media consisted of a mixture of growth factors. At least in rat lens epithelial cell cultures, IGF-I was shown to enhance bFGF-induced fiber differentiation. Whether multiple growth factors influence cell differentiation in HLE cells is unknown and remains a subject for further study.

Key Words
- cell proliferation, epidermal growth factor, fiber differentiation, fibroblast growth factor, \( \gamma \)-crystallin expression, gap junction, human lens epithelial cells, insulin-like growth factor, lentoid

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References

28. Tripathi RC, Borisuth NSC, Tripathi BJ. Detection, quantification, and significance of basic fibroblast


