Insulin Growth Factor and Epidermal Growth Factor Trigger Mitosis in Lenses Cultured in a Serum-free Medium

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The mitogenicity of insulin, insulin growth factor (IGF), and epidermal growth factor (EGF) was evaluated on rabbit lenses cultured in medium KEI-4. IGF, the most highly purified of the insulin-like growth factors was a potent mitogen for mammalian lens epithelial cells. IGF and EGF triggered cell proliferation throughout the normally amitotic central and pre-equatorial region of the epithelium. The mitotic response elicited by IGF and EGF was dose dependent, was preceded by DNA synthesis, exceeded that engendered by equimolar insulin, and exhibited a chronology identical to that brought about by crystalline insulin. Lenses cultured in KEI-4 alone or in KEI-4 supplemented with growth hormone, proinsulin, the A and/or B chain of insulin, or MSA, another of the insulin-like growth factors belonging to the somatomedin family, did not show a mitotic response. Simultaneous exposure of the lens to IGF and EGF resulted in an increase in the total number of mitotic figures over that obtained with equimolar concentrations of IGF and EGF. Our results suggest that IGF or other insulin-like growth factors may be capable of regulating cell division in the mammalian lens in vivo.

That the lens epithelium responded to IGF and EGF may indicate that lens epithelial cells are subject to multiple hormonal interaction. Since growth factors appear to be cell type specific, information obtained from the rabbit lens epithelium should be useful in delineating the factors and conditions required for the growth of cultured human lens cells. Invest Ophthalmol Vis Sci 24:409-416, 1983

Investigations on the process of mitosis indicate that environmental factors modulate the entrance of cells into the cell cycle. This study is part of a continuing effort aimed at elucidating the environmental factors that are capable of regulating cell division in the mammalian lens.

Mitosis in the lens in vivo is normally confined to the peripheral region of the lens referred to as the germinative zone. However, as a result of mechanical, chemical, or immunologic insult or following repeated paracentesis, cells throughout the normally amitotic central region of the epithelium enter mitosis. A stimulation of cell division is also realized if the lens is cultured in a medium supplemented with serum or plasmoid aqueous humor, situations that expose the lens to complex mixtures of growth-promoting and -inhibiting factors. Insulin can initiate DNA synthesis and mitosis in lenses cultured in a completely defined serum-free medium. However, the concentration of insulin exceeded the level of the hormone in either primary or plasmoid aqueous humor. This suggests that peptides with structural and functional homologies to insulin could be more mitogenic toward the lens than insulin itself and might represent the type of peptide capable of regulating mitosis in the mammalian lens. Such a possibility is explored in the present study and is supported by the finding that mitosis in the amphibian lens in vivo is abolished by procedures that decrease the level of circulating insulin-like growth factors and is reinstated by the systemic administration of such peptides.

Certain polypeptides that exhibit insulin-like activity stimulate growth of mammalian cells in tissue culture. The insulin growth factors, originally termed nonsuppressible insulin-like activity, belong to a family of polypeptides called somatomedins that are ancestrally related to proinsulin. They are immunologically distinct from insulin, exhibit structural and functional homologies to insulin, bind to insulin receptors, and show extensive cross-immunologic reactivity with certain of the somatomedins. The complete amino acid sequence of human insulin growth factor (IGF) has been reported. Since certain lines of lens epithelial cells have receptors for...
insulin, epidermal growth factor (EGF), and fibroblast growth factor (FGF)\textsuperscript{16,17} and exhibit enhanced growth if these factors are present in a serum-containing medium, we also examined the mitogenicity of these peptides on lenses cultured in the absence of serum.

We report that IGF and EGF at nanogram concentrations stimulate cell division in lenses cultured in a serum-free medium. The response is dose-dependent, and each factor is more mitogenic toward the lens than crystalline insulin.

**Materials and Methods**

New Zealand White rabbits 10–12 weeks old were killed, the eyes enucleated, lenses isolated, and placed in Merriam-Kinsey tubes as previously described.\textsuperscript{5} Lenses were cultured in medium KEI-4 (see ref. 5 for formulation), or in KEI-4 containing one or more polypeptides. Gentamicin (Schering Corp., Kenilworth, NJ) was present at 0.05 mg/ml. KEI-4 is a serum-free medium that mimics the composition of rabbit aqueous humor. The effect of IGF was also determined on lenses cultured in minimal essential medium (MEM) or in medium 199 (Cat. No. 400-1100) (GIBCO, Grand Island, NY). The growth factors or peptides evaluated included: crystalline porcine insulin, porcine proinsulin, the A- or B-chain of porcine insulin (gifts of Dr. Ronald Chance, Lilly Research Laboratories, Indianapolis, IN); human IGF (36mIU/ml), a mixture of IGF-I and IGF-II (a gift of Dr. Rene Humbel, Biochemisches Institut der Universität Zurich, Zurich, Switzerland); bovine growth hormone (Miles Laboratories, Elkhart, IN [0.92 IU/mg]); human growth hormone (Calbiotech, La Jolla, CA) (22 IU/mg); and culture or receptor grade EGF, FGF, or multiplication stimulating activity (MSA) (Collaborative Research, Boston, MA). Reference preparations of EGF and FGF were provided by Drs. Stanley Cohen, Vanderbilt University, Nashville, TN and Denis Gospodarowicz, University of California, San Francisco, CA, respectively. The insulin, proinsulin, and A- or B-chain were prepared as previously described.\textsuperscript{5} The IGF stock was prepared in 0.5 M acetic acid and stored at 4°C. For working solutions, the IGF was diluted further with Earle’s balanced salt solution (GIBCO, Grand Island, NY) containing 2 mg/ml rabbit serum albumin (Sigma, St. Louis, MO). Lenses were also cultured in medium KEI-4 containing rabbit serum albumin at 10 μg/ml of culture medium, ie, the level of rabbit serum albumin present when IGF was used at 8.8 × 10\textsuperscript{-10} M. Growth hormone was diluted in medium KEI-4 prior to use. All peptides were present at 8.8 × 10\textsuperscript{-10} M unless otherwise noted.

The lenses were either continuously exposed to the growth factor or were exposed to the factor for the initial hour of culture, whereupon the medium was changed to KEI-4 alone for the remainder of the culture period. Lenses were fixed in 3:1 ethanol:acetic acid. Certain lenses were exposed to \textsuperscript{3}H]thymidine (μCi/ml; μCi/mM) at 30 hrs of culture and fixed at 36 hrs, a time of intensive DNA synthesis in lenses cultured in an insulin-containing medium.\textsuperscript{2} Whole mounts\textsuperscript{18} of the epithelial layer were prepared and processed for autoradiography,\textsuperscript{19} or the total number of mitotic figures per preparation was counted.

**Results**

Mitotic activity in lenses cultured in KEI-4 was principally confined to the peripheral region of the epithelium, which is thought to correspond to the site of cell division in the lens in vivo. A low level of mitosis was noted in the equatorial region of all the cultured lenses. Cells residing in the pre-equatorial and central region of lenses cultured in KEI-4 were essentially amitotic (Fig. 1A). In contrast, epithelia from lenses cultured in KEI-4 plus IGF exhibited DNA synthesis and mitosis throughout the pre-equatorial and central region of the epithelium (Figs. 1B, D).

The magnitude of the proliferative response engendered by IGF is shown in Figure 1D which depicts the number of nuclei that have incorporated \textsuperscript{3}H]thymidine following a 6-hr exposure to the isotope. Approximately 50% of the nuclei are labeled. In marked contrast, cells residing in the pre-equatorial and central regions of lenses cultured in KEI-4 alone (Fig. 1C) did not enter DNA synthesis. The effect of various concentrations of IGF on the magnitude of cell division in the cultured lens is shown in Figure 2. IGF at 8.8 × 10\textsuperscript{-12} M was not mitogenic, however, at all concentrations above 4.8 × 10\textsuperscript{-11} M the proliferative response was dose dependent. IGF was mitogenic toward the lens epithelium when present at 8.8 × 10\textsuperscript{-11} M (0.66 ng/ml).

In order to gain some understanding of the specificity of IGF-induced mitosis, lenses were cultured in medium KEI-4 plus compounds that are related structurally or functionally to the insulin-like growth factors. Since the production of IGF in vivo is regulated partially by somatotrophin, it was also evaluated. The addition of proinsulin, the A- and/or B-chain of insulin, or MSA to medium KEI-4, all at 8.8 × 10\textsuperscript{-10} M, did not stimulate mitosis; neither human nor bovine growth hormone induced a program of cell proliferation. Indeed, whole mount preparations from lenses that had been cultured for 52 hrs in medium KEI-4 plus proinsulin, the A- and/or B-chain of insulin, MSA, or human or bovine growth hormones were essentially amitotic throughout the entire
Fig. 1. Photomicrographs from the central region of the epithelium of the cultured rabbit lens. Lenses were cultured in medium KEI-4 or in KEI-4 containing IGF at $8.8 \times 10^{-10}$ M. Lenses were fixed at 52 hrs and examined for mitosis; or were exposed to $[^{3}H]$-thymidine from 30-36 hrs of culture, fixed at 36 hrs, and prepared for autoradiography. Whole mount preparations of a lens cultured in KEI-4 alone (A) or in KEI-4 plus IGF (B). Autoradiogram from a lens cultured in KEI-4 alone (C). Autoradiogram from a lens cultured in KEI-4 plus IGF (D). (Bar = 75 μm.)

Fig. 2. Lenses were cultured in KEI-4 containing various concentrations of IGF, fixed at 52 hrs, whole mounts of epithelium were prepared, and the total number of mitotic figures per lens was counted. Values represent the mean ± standard deviation ($n = 3$).

central and pre-equatorial regions of the epithelium and resembled preparations from lenses cultured in KEI-4 alone. At least five lenses were examined for each peptide. A mitotic activation was not obtained in lenses cultured for 52 hrs in medium KEI-4 containing 10 μg/ml rabbit serum albumin (RSA). FGF at $8.8 \times 10^{-10}$ M did not elicit a reproducible mitotic response.

The chronology and magnitude of cell division in lenses exposed to IGF is shown in Figure 3. Lenses cultured in medium KEI-4 or in KEI-4 supplemented with IGF showed a low level of mitosis during the initial 40 hrs of culture. Lenses cultured in KEI-4 plus IGF for 52 hrs exhibited mitosis throughout the central and pre-equatorial regions of the epithelium, a situation not realized in lenses cultured in KEI-4 alone for 52 hrs (Figs. 1, 3). Lenses cultured in MEM plus IGF or in medium 199 plus IGF also exhibited an activation of mitosis throughout the central region of the lens epithelium.

Since insulin is known to stimulate cell proliferation in lens epithelial cells, the mitogenicity of insulin was compared to that of IGF. IGF was more mitogenic toward the lens epithelium than equimolar in-
Fig. 3. Rabbit lenses were cultured in medium KEI-4 alone (○ --- ○) or in KEI-4 containing IGF at $8.8 \times 10^{-10}$ M (• --- •). Lenses were fixed at 0, 3, 7, 24, 40, and 52 hrs of culture, whole mounts were prepared, and the total number of mitotic figures per lens was counted. Values represent the mean ± standard deviation (n = 4).

sulin (Table 1). This result does not appear to be attributable to the presence of RSA since the mitogenicity of insulin was not increased by the presence of 10 µg/ml RSA.

Epidermal growth factor was also a potent mitogen; culture and receptor grades were equally mitogenic. The addition of EGF to medium KEI-4 engendered a mitotic response that was accompanied by a slight disorganization of the epithelial monolayer (Fig. 4A). The disorganization, which was more pronounced in the cells in the pre-equatorial region of the lens, is thought to be a result of cellular migration. In contrast, the proliferation induced by IGF was not accompanied by marked cellular disorganization. The mitotic response triggered by EGF was preceded by DNA synthesis (Figs. 4B, C), was dose dependent (Fig. 5), and exhibited a time course identical to that of IGF (Fig. 3). EGF was mitogenic toward the lens when present at 0.067 ng/ml ($10^{-11}$ M).

![Fig. 4](image-url)

Table 1. Comparison of the effect of insulin and insulin growth factor on cell division in the cultured rabbit lens

<table>
<thead>
<tr>
<th>Lens pairs</th>
<th>Insulin</th>
<th>Insulin growth factor</th>
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<tbody>
<tr>
<td>1</td>
<td>2,028</td>
<td>8,816</td>
</tr>
<tr>
<td>2</td>
<td>1,837</td>
<td>7,936</td>
</tr>
<tr>
<td>3</td>
<td>2,161</td>
<td>9,724</td>
</tr>
<tr>
<td>4</td>
<td>1,594</td>
<td>7,916</td>
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Table 1. Comparison of the effect of insulin and insulin growth factor on cell division in the cultured rabbit lens

**Legend:**
- **A:** Whole mount preparation of a lens cultured in KEI-4 plus EGF for 52 hrs; **B:** Autoradiogram from a lens cultured in KEI-4 alone; **C:** Autoradiogram from a lens cultured in KEI-4 plus EGF.
In the preceding experiments, the lenses were exposed to medium KEI-4 containing IGF or EGF for the entire culture period. We next determined if the activation of mitosis brought about by EGF or IGF was dependent upon the continued presence of the growth factor. If lenses were placed in medium KEI-4 containing EGF for the initial hour of culture and then cultured for the remaining 51 hrs in KEI-4 alone, the number of mitotic figures per whole mount preparation paralleled that obtained for lenses continuously exposed to EGF (Fig. 6). Lenses cultured in KEI-4 plus IGF for the initial hour of culture and examined for mitosis at 52 hrs showed a significant increase in the number of mitotic figures per whole mount preparation over lenses cultured for 52 hrs in KEI-4 alone. The magnitude of the mitotic response realized in lenses cultured in KEI-4 plus IGF for the first hour of culture, however, was reduced relative to that found in lenses continuously exposed to IGF (Fig. 6). Moreover, the mitotic response noted in lenses exposed to EGF for the initial 60 min of culture exceeded that brought about by an initial 60-min exposure to IGF.

In many culture systems, cells are thought to respond to sets of mitogenic factors. In view of the mitogenicity of IGF and EGF on the lens epithelium, we determined the magnitude of the proliferative response in lenses exposed to both mitogens (Table 2). Simultaneous exposure of lenses to EGF and IGF resulted in an increase in the number of mitotic figures per lens that exceeded that obtained with equipolar concentrations of EGF (Table 2) or IGF.

Table 2. Effect of IGF and EGF on cell division in the cultured rabbit lens

<table>
<thead>
<tr>
<th>Lens pairs</th>
<th>Total number of mitotic figures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 × [EGF]</td>
</tr>
<tr>
<td>1</td>
<td>7,127</td>
</tr>
<tr>
<td>2</td>
<td>3,910</td>
</tr>
<tr>
<td>3</td>
<td>6,183</td>
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</tbody>
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Lenses were cultured in KEI-4 containing EGF and IGF with both factors at 8.8 × 10⁻¹⁰ M, or in KEI-4 containing EGF at 1.8 × 10⁻⁸ M (2 × EGF). Lenses were fixed after 52 h of culture and the total number of mitotic figures per whole mount preparation was counted.
Lenses cultured for 52 hrs in medium KEI-4 containing IGF at $1.8 \times 10^{-9}$ M ($n = 3$) had a total of $4,794 \pm 1,124$ mitotic figures, which is substantially below the value obtained for lenses cultured in the presence of both mitogens at $8.8 \times 10^{-10}$ M (Table 2). Whole mount preparations from lenses exposed to both mitogens exhibited cellular disorganization similar to that depicted in Figure 3.

Discussion

Earlier we demonstrated that insulin triggered cell division in the cultured lens. Later, studies in our laboratory indicate that IGF-I and IGF-II are equipotent in stimulating mitosis in the rabbit lens in organ culture, a situation that is also obtained in lenses exposed to a relatively pure preparation of somatomedin C. The mitotic response elicited by IGF is dose dependent and exceeded that engendered by equimolar insulin. The data suggest that IGF or other insulin-like growth factors might represent the in vivo analog of insulin. The activation of cell division occurred with concentrations of IGF that appear to be within the physiologic range. As a point of reference, the concentration of insulin-like growth factors in human serum is 300 and 180 ng/ml respectively. Relative to other model systems, IGF stimulates DNA synthesis in cultured chick embryo fibroblasts and induces proliferation and differentiation in rat myoblasts. Other reports indicate that human embryo fibroblasts and other cultured mammalian cells do not undergo mitosis in response to somatomedin C alone. These investigators conclude that somatomedin C is not capable of triggering the initial sequence of events leading to division in certain mammalian cells, but is required for their progression into DNA synthesis. Our report clearly establishes that an insulin-like peptide can initiate cell division in cultured mammalian cells.

Lenses cultured in KEI-4 supplemented with MSA, a molecule closely related to IGF, did not show a mitotic activation. In view of the high concentration of MSA in fetal rat serum relative to adult rat serum, Moses et al. suggested a role for MSA in fetal growth. Among other possibilities, our results suggest that MSA, a factor produced by fetal rat liver cells, is unable to trigger mitosis in rabbit lens epithelial cells, or that the response of the lens to specific insulin-like growth factors varies as a function of age. In this respect, it is known that donor age is a crucial variable for the establishment and growth of lens epithelial cell lines. The influence of insulin-like growth factors, including MSA on lens epithelial cells from different aged animals is under investigation.

Relative to the control of cell division in the lens in vivo, it is known that hypophysectomy abolishes cell proliferation in the frog lens. The pituitary is thought to facilitate growth by mediating the synthesis and/or release of insulin-like growth factors from other tissues. The most salient finding implicating insulin-like growth factor in the control of cell division in the lens in vivo is the demonstration that administration of somatomedin C to the hypophysectomized frog restores cell proliferation in the lens. Whether somatomedin C acts directly or indirectly on the amphibian lens in vivo, or singly or in concert with other factors to mediate the initiation or progression of lens cells into mitosis is unknown.

Pituitary ablation brings about a slight decrease in the growth of the rat lens. An increase in the number of dividing cells and a concomitant decrease in the number of colchicine arrested mitoses occurs in the lens of the hypophysectomized rat. These findings might indicate that insulin-like growth factors do not influence cell division in the mammalian lens. However, the level of insulin-like growth factors in mammals is not determined solely by the pituitary but is subject to regulation by both insulin and adequate levels of nutrition. Although cultured mammalian lens epithelium divide in response to insulin-like growth factors, the role of these factors on the mammalian lens in vivo remains to be demonstrated.

In other ocular tissues, several reports, summarized by Reddan indicate that hypophysectomy, a procedure known to lower the plasma level of insulin-like growth factors, results in an actual regression of new blood vessel formation associated with diabetic retinopathy.

Fibroblast growth factor did not bring about a reproducible stimulation of mitosis in the cultured lens. Other investigators reported that FGF is mitogenic toward cultured lens epithelia when added to medium containing fetal calf serum. The FGF used in this investigation was mitogenic toward rabbit lens cells cultured in a serum-containing medium.

Epidermal growth factor stimulates DNA synthesis and mitosis in lenses cultured in a serum-free me-
medium and in rabbit lens epithelia cultured in a medium supplemented with fetal calf serum. The values for EGF in mouse and human plasma are 1 and 2–4 ng/ml, respectively. The chronology of cell proliferation in EGF-treated lenses is identical to that noted with IGF. Although EGF is a potent mitogen, the physiologic significance of this peptide remains to be established. In apparent contrast to the present findings, Gospodarowicz et al reported that EGF did not stimulate proliferation of cultured bovine or rabbit corneal or lens epithelial cells, but did initiate mitosis if the corneal epithelium was combined with the corneal stroma. As shown in the present study, the epithelial cells of the lens in organ culture, wherein the cells lie immediately subjacent to their natural basement membrane, entered mitosis upon exposure to EGF. Rabbit lens cells cultured on laminin or fibronectin, components of the extracellular matrix, increase in number in a serum-free medium supplemented with EGF. These findings indicate that the response of lens cells to mitogens can be regulated in part by components of the extracellular matrix.

In most culture systems, EGF must be present for 6 to 8 hrs to stimulate DNA synthesis. In the present study, a 1-hr exposure of the lens to EGF or IGF resulted in an increase in mitotic activity. It would appear that the sequence of events triggered by EGF is completed within the initial hour of culture. Additional studies using EGF antibodies should provide information on these possibilities. Although a 1-hour exposure of the lens to IGF resulted in an increase in mitosis, the magnitude of the response was decidedly lower than that brought about by a 1-hr exposure of the lens to EGF. The cellular mechanism responsible for this remains to be determined. Of additional interest is the finding that simultaneous exposure of the lens to IGF and EGF evoked a mitotic response substantially greater than that obtained by either peptide alone. Since we have no evidence indicating that the time on onset of DNA synthesis and mitosis is different in the presence of both mitogens, the increase in the number of mitotic figures has been tentatively ascribed to an increase in the number of cells that have been stimulated to enter the cell cycle. Since the cells respond to more than one peptide, lens epithelia, like their counterparts in other tissues, may be subject to multiple hormonal interactions.

Through a judicious selection of growth factors and substrata it should be possible to establish long-term cultures of lens epithelial cells in a serum-free medium. Since a single peptide can trigger mitosis in lenses cultured in a serum-free medium, this system is suitable for determining the growth limiting components in the medium that act in concert with specific polypeptides to initiate the mitotic response. Moreover, since growth factors appear to be cell type specific, information obtained from rabbit lens epithelia should be useful in delineating the factors and conditions required for the establishment of continuous lines of human lens epithelial cells. Such studies should permit a further understanding of the extracellular and intracellular events regulating growth, development, repair, and senescence in mammalian lens epithelia.

Key words: lens, mitosis, epithelial cells, insulin growth factor, somatomedins, epidermal growth factor, organ culture, serum-free medium

References