Hepatocyte Growth Factor, Keratinocyte Growth Factor, and Other Growth Factor–Receptor Systems in the Lens

Jian Weng,* Qianwa Liang,† Rajiv R. Mohan,† Qian Li,* and Steven E. Wilson†

Purpose. To examine the expression and function of hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), epidermal growth factor (EGF) and other growth factor–cytokine-receptor systems in lens epithelial cells.

Methods. Reverse transcription–polymerase chain reaction (RT–PCR) and Northern blot analysis were used to examine the expression of messenger RNAs in primary cultured rabbit and human lens cells and in ex vivo rabbit lens tissue. Protein expression and the effect of HGF and KGF on crystallin expression in lens epithelial cells were evaluated by immunoprecipitation and Western blot analysis. The effect of exogenous HGF, KGF, and EGF and of the coculture of lens epithelial cells with corneal endothelial cells on the proliferation of rabbit lens cells in a Transwell system was determined by cell counting.

Results. Messenger RNAs and proteins of HGF and KGF were expressed in primary rabbit lens epithelial cells and in ex vivo rabbit lens epithelial tissue. Human lens cells also expressed the mRNAs. Other growth factors and receptor messenger RNAs were also expressed. Hepatocyte and keratinocyte growth factors, and coculture with corneal endothelial cells stimulated proliferation of rabbit lens epithelial cells. In first-passage rabbit lens cells, HGF, KGF, and EGF increased the expression of alpha and beta crystallins.

Conclusions. Hepatocyte and keratinocyte growth factor–receptor systems are expressed in lens cells. HGF and KGF are not expressed in epithelial cells in such tissues as skin, cornea, and lacrimal gland in which fibroblastic and epithelial cells interact in the formation of an organ. Expression of these growth factors in the lens may have evolved because the lens cells are relatively isolated within the anterior chamber of the eye. Our results suggest, however, that growth factors released by the corneal endothelium also could modulate lens functions (aquecrine interactions). Invest Ophthalmol Vis Sci. 1997;38:1543–1554.

Growth factors modulate such functions as proliferation, motility, differentiation, and apoptosis in lens epithelial cells.1–3 Acidic fibroblast growth factor (FGF-1), basic fibroblast growth factor (FGF-2), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF) have been implicated in the regulation of lens cell functions.1–3

The anatomic position of the lens within the anterior segment presents a regulatory milieu that is unique to the eye. Because the adult lens is avascular and lacks innervation, all of the regulatory signals must be derived from the lens cells themselves or from signals delivered through the aqueous humor. All of the nutritional requirements of the lens are provided by the aqueous humor, and it therefore seems plausible that important regulatory signals could also be delivered through this medium.

In results of recent studies on the expression and function of corneal hepatocyte growth factor (HGF) and of keratinocyte growth factor (KGF), we were surprised to discover that corneal endothelial cells pro-
duce HGF and KGF. These two growth factors have been identified in many tissues as paracrine modulators of stromal–epithelial interactions secreted by fibroblastic cells to regulate epithelial cell functions. What functions could be served by endothelial-derived HGF and KGF? Is it possible that these growth factors could be released from the endothelium to modulate the functions of epithelial-derived lens tissue that is continuously exposed to the aqueous humor? We have speculated previously that these types of growth factor– or cytokine-mediated communications might occur between different cell types exposed to the aqueous humor; and we have termed these hypothetical regulatory mechanisms aquocrine interactions because this mode of communication is not defined adequately by terms that are currently available to describe growth factor–cytokine function (that is, autocrine, paracrine, endocrine, and juxtacrine). This study examined the expression of the HGF and KGF growth factor–receptor systems in the lens and the effect of HGF, KGF, and cocultured corneal endothelial cells on the proliferation of lens cells. The expression of other growth factor–receptor system messenger ribonucleic acids (mRNAs) in the human and rabbit lens was also evaluated.

METHODS

Cell Culture, Ex Vivo Tissue, and Proliferation Assays

Primary cultures of human cells and rabbit lens epithelial cells and of rabbit corneal endothelial cells were established by previously described methods from explants in Dulbecco’s minimal essential medium (DMEM; Gibco, Gaithersburg, MD) with 10% fetal bovine serum (Gibco). Human cells were derived from fresh eyes of donors less than 2 years old in compliance with the Helsinki Accord on Human Rights. Rabbit cells were obtained from 7-week-old New Zealand White rabbits. Rabbit lens epithelial cell cultures had a heterogeneous morphology at low passage, with some cells resembling epithelial cells and others fibroblastic in appearance. After a 2-week exposure to growth factors, rabbit epithelial cell cultures were used for experimental procedures.

Rabbit Lens Epithelial Cells

Coculture of first-passage rabbit lens epithelial cells with rabbit corneal endothelial cells was performed in six-well Transwell plates (Falcon, Lincoln Park, NJ) with a 0.45-μM pore size. Two × 10⁴ first-passage rabbit lens cells per well were plated in the bottom of the well with a monolayer of first-passage rabbit corneal endothelial cells on the Transwell membrane insert. Two milliliters of KDM were added to the wells and changed at 3-day intervals. Cells were counted with a Coulter counter (Model Zf; Coulter Electronics, San Diego, CA). At each time point, five 5-μl aliquots of each sample were added to a Coulter counter, and the mean of five measurements was recorded.

Proliferation experiments were performed by plating 2 × 10⁴ first-passage rabbit cells per well in six-well cluster plates (Falcon, Lincoln Park, NJ). Twenty-four hours after seeding, cells were transferred into 2 ml of keratinocyte-defined medium (KDM: modified MCDB 153, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, and 0.15 mM Ca²⁺ without EGF; Clonetics, San Diego, CA) without growth factors, serum, or other extracts. Growth factors to be tested were added to the medium.

At the time of addition, growth factors were diluted in sterile, phosphate-buffered saline (PBS) with 0.2% gelatin (Sigma, St. Louis, MO) to provide 5 μl of additional volume to each well and to obtain the final concentration of growth factor. Each growth factor was tested in six wells in each experiment. Five microliters of PBS with 0.2% gelatin as a vehicle was added to control flasks. Media were changed at 3-day intervals with the addition of fresh growth factors.

Human recombinant double-chain HGF was obtained from Genentech (South San Francisco, CA), human EGF from Collaborative Biomedical Products (Bedford, MA), and human KGF from Bachem (Philadelphia, PA). Each of these human growth factors stimulates proliferation of rabbit corneal epithelial cells (Wilson SE, unpublished data, 1994), demonstrating the cross-species efficacy of the growth factors. Concentrations of HGF and KGF were at 5 ng/ml and EGF was at 10 ng/ml, because these concentrations are optimal for a wide variety of cell types. Aliquots of stock solutions of the growth factors were placed in siliconized microtubes and were stored at ~80°C to minimize denaturation caused by repeated freezing and thawing. Coculture of first-passage rabbit lens epithelial cells with rabbit corneal endothelial cells was performed in six-well Transwell plates (Falcon, Lincoln Park, NJ) with a 0.45-μM pore size. Two × 10⁴ first-passage rabbit lens cells per well were plated in the bottom of the well with a monolayer of first-passage rabbit corneal endothelial cells on the Transwell membrane insert. Two milliliters of KDM were added to the wells and changed at 3-day intervals. Cells were counted with a Coulter counter (Model Zf; Coulter Electronics, San Diego, CA). At each time point, five 5-μl aliquots of each sample were added to a Coulter counter, and the mean of five measurements was recorded.
**Table 1. Primers and Expected Amplification Sizes**

<table>
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<tr>
<th>Modulator</th>
<th>Size mRNA/Genomic</th>
<th>Upstream Primer</th>
<th>Downstream Primer</th>
<th>Accession Numbers</th>
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<tr>
<td>H and R β actin</td>
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<td>AGGCCAACCGCGAGAAGATGACC</td>
<td>GAAGTCCAGGGCGAGCTAGCAC</td>
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<td>GAGTACTGTGCAATTAAAACATGC</td>
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<td>H FGF-1</td>
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</table>

H and R = primers that will amplify human and rabbit mRNA, respectively; Size = expected size of the PCR amplification from mRNA and genomic DNA; Unk = sequences in which the genomic sequence is not known. > = amplifications in which the upstream and downstream primers are known to be in separate exons although the exact size of the genomic amplification is not known. Accession numbers refer to the Genbank accession numbers assigned to each sequence used in designing primers.

* Additional primer pairs for KGF receptor and HGF receptor were designed for ex vivo rabbit lens samples.
† Primers for FGF receptor-1 yield three alternative mRNAs yielding amplification products of 800 base pairs for the β amino-terminal motif, 1000 base pairs for the γ amino-terminal motif, and 1100 base pairs for the α amino-terminal motif.
‡ Primers for TGF β receptor I were designed from the mouse sequence.
§ Primers for TGF β receptor II identify two alternative mRNAs. The 543 base pair transcript corresponds to TGF β receptor II mRNA with a 25 amino acid insertion relative to the better characterized TGF β receptor II mRNA.

HGF = hepatocyte growth factor; KGF = keratinocyte growth factor; EGF = epidermal growth factor; FGF = fibroblast growth factor; TGF = transforming growth factor; IL-1 = interferon-1; PDGF = platelet-derived growth factor.
Hialeah, FL). Growth factors and the effect of endothelial cells were tested in three independent experiments.

Errors were expressed as the standard error of the mean. Statistical comparisons were performed with the analysis of variance Dunn's test. P < 0.05 was considered statistically significant.

**Reverse Transcription–Polymerase Chain Reaction**

Total cellular RNA was isolated from primary cultures of rabbit and human lens epithelial cells and complementary DNA (cDNA), synthesized as previously described. The quality of cDNA synthesis was monitored through the amplification of beta actin. Only cDNA yielding beta-actin amplifications of the expected size for beta-actin mRNA, without contamination with a genomic beta-actin amplification product of a larger size, was used for experimental amplification. Polymerase chain reactions were performed with a temperature cycler (MJ Research, Watertown, MA) according to a previously described method. A modified hot-start method was used in which anti-Taq polymerase antibody (Clontech, Palo Alto, CA) was added to the reactions according to the manufacturer's instructions to prevent initiation of amplification until after the reactions were raised to the antibody-denaturing temperature of 70°C. Amplification products of PCR were run on agarose gels, as previously described. Primers for the human and rabbit growth factors and receptors are provided in Table 1. Human primers were designed from human Genbank sequences, excepting primers that amplified human transforming growth factor beta (TGF-β) receptor type I mRNA, which were designed from the mouse sequence (Table 1). Human primers were tested with rabbit liver cDNA to determine whether they would amplify the rabbit sequence with an annealing temperature of 50°C. The original human KGF and HGF receptor primers would not amplify rabbit cDNA. Therefore, alternative primers that amplified the rabbit sequence at an annealing temperature of 50°C were designed from the mouse (KGF) and human (HGF) receptor sequences. In addition, second sets of PCR primers for rabbit HGF and rabbit KGF receptors were designed (Table 1). Human and rabbit PCR products of the expected sizes were cut from agarose gels, cloned into the II Cloning Vector (Invitrogen, San Diego, CA), and sequenced (Sequenase 2.0; United States Biochemical, Cleveland, OH) according

![Image 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933424/ on 10/16/2017)

**FIGURE 1.** Epidermal growth factor, hepatocyte growth factor, and keratinocyte growth factor messenger RNA expression in human lens cells. Primary cultures of human lens epithelial cells from young donors were evaluated for the production of messenger RNAs of these factors by reverse transcription–polymerase chain reaction. Beta-actin expression was also evaluated as a positive control: 1 and 2 represent two different primary lens epithelial cell cultures for each primer pair; C is a simultaneous control reaction without added complementary DNA target for each polymerase chain reaction primer pair. Amplification bands of the expected size (Table 1) were detected for each growth factor receptor and for beta actin (arrow). Note that in the amplifications of hepatocyte growth factor receptors an alternative band (approximately 430 base pairs) is present just above the expected band (arrow). This alternative band corresponds to an alternative hepatocyte growth factor receptor messenger RNA that codes for a truncated form of the receptor. The smaller amplification products in hepatocyte growth factor receptor 1 were artifacts in the polymerase chain reaction. A 100-base-pair marker with sizes in base pairs (BP) is indicated.

![Image 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933424/ on 10/16/2017)

**FIGURE 2.** Epidermal growth factor receptor, hepatocyte growth factor receptor, and keratinocyte growth factor receptor messenger RNA expression in human lens cells. Primary cultures of human lens epithelial cells from young donors were evaluated for the production of messenger RNAs by reverse transcription–polymerase chain reaction. Beta-actin expression was also evaluated as a positive control: 1 and 2 represent two different primary lens epithelial cell cultures for each primer pair; C is a simultaneous control reaction without added complementary DNA target for each polymerase chain reaction primer pair. Amplification bands of the expected size (Table 1) were detected for each growth factor receptor and for beta actin (arrow). Note that in the amplifications of hepatocyte growth factor receptors an alternative band (approximately 430 base pairs) is present just above the expected band (arrow). This alternative band corresponds to an alternative hepatocyte growth factor receptor messenger RNA that codes for a truncated form of the receptor. The smaller amplification products in hepatocyte growth factor receptor 1 were artifacts in the polymerase chain reaction. A 100-base-pair marker with sizes in base pairs (BP) is indicated.
were specific.

Mannheim, Indianapolis, IN), according to a pre-

isolated using an mRNA Isolation Kit (Boehringer

Total cellular RNA was isolated using TRIzol (BRL,

mRNA in Rabbit Lens Cells

growth factor receptor type II were detected in human

FIGURE 3. Basic fibroblast growth factor, fibroblast growth
factor receptor-1, fibroblast growth factor receptor-2, transforming
growth factor beta-1, transforming growth factor beta receptor type I, and transforming growth factor beta receptor type II messenger RNA expression in human lens cells. Primary cultures of human lens epithelial cells from young donors were evaluated for the production of messenger RNAs of these factors by reverse transcription–polymerase chain reaction: 1 and 2 represent two different primary lens epithelial cell cultures from different donors for each primer pair; C is a simultaneous control reaction without added complementary DNA target for each polymerase chain reaction primer pair. Amplification bands of the expected size (Table 1) were detected for each growth factor and receptor (arrow). The fibroblast growth factor receptor-1 (fgfr) primers yielded three alternative polymerase chain reaction products that correspond to alternative fibroblast growth factor receptor-1 transcripts. The 1100-base-pair amplification product corresponds to the alpha amino-terminal motif of fibroblast growth factor receptor-1 containing three extracellular immunoglobulin G-like disulfide loops. The 800-base-pair product corresponds to the beta amino-terminal motif of fibroblast growth factor receptor-1 containing two extracellular immunoglobulin G-like disulfide loops. Finally, the 1000-base-pair amplification product corresponds to the gamma amino-terminal motif of fibroblast growth factor receptor-1. This motif does not contain a known signal sequence for membrane translocation and is thought to represent an intracellular form of fibroblast growth factor receptor-1. Similarly, two alternative transcripts for transforming growth factor beta receptor type II were detected in human lens cells. Within a lane the ratio of the alternative amplification products should be similar to the ratios of the target messenger RNAs in the complementary DNA. Therefore, the alternative transforming growth factor beta receptor type II messenger RNAs appear to be expressed at approximately equal levels in cultured human lens cells. A 100-base pair marker with sizes in base pairs (BP) is indicated.

Northern Hybridization to Detect Hepatocyte Growth Factor and Keratinocyte Growth Factor mRNA in Rabbit Lens Cells

To the manufacturer's protocols, to verify that they were specific.

Northern Hybridization to Detect Hepatocyte Growth Factor and Keratinocyte Growth Factor mRNA in Rabbit Lens Cells

Total cellular RNA was isolated using TRIzol (BRL, Gaithersburg, MD). Polyadenylic acid; A+ RNA was isolated using an mRNA Isolation Kit (Boehringer Mannheim, Indianapolis, IN), according to a previously described method. RNA size markers and 9 μg poly(A)+ RNA per lane were resolved on 1.2% agarose formamide gels. The RNA was transferred to Duralon–UV (Stratagene, San Diego, CA) using a vacuum blotter (Pharmacia, Piscataway, NJ) and cross-linked to the Duralon–UV with the UV–Stratalinker (Stratagene).

Probes for HGF and KGF were generated by PCR from rabbit corneal epithelial cells or from the total cellular RNA of stromal fibroblast cells, using the specific primers (Table 1). Amplification products of the expected size were cut from agarose gels, cloned into the PCR II Cloning Vector, and sequenced according to the manufacturer's protocols. Sequences were confirmed to be specific for the mRNA for HGF and KGF. Each sequence was compared with all known sequences in the Genbank and EMBL databases using the Mac Vector 5.0 System (IBI, New Haven, CT). No other nucleic acid sequences have been reported that would be expected to hybridize to the HGF or KGF probes under the conditions used for Northern blot analysis.

Purified radiolabeled DNA probes with a specific activity of approximately 2 × 10⁶ cpm/μg were prepared with the cloned HGF and KGF sequences, using the random primer-labeling kit Rediprime and 32P-dCTP (Amersham Life Sciences, Arlington Heights, IL) according to the manufacturer's protocol. Probe for the DNA size markers was prepared by 32P-labeling lambda genomic DNA. A total of 5 × 10⁶ cpm of probe was used in each hybridization. Hybridization was per-

FIGURE 4. Platelet-derived growth factor alpha, glucocorticoid receptor, interleukin-1 alpha, and interleukin-1 receptor type 1 messenger RNA expression in human lens cells. Primary cultures of human lens epithelial cells from young donors were evaluated for the production of the messenger RNAs of these cell factors by reverse transcription–polymerase chain reaction: 1 and 2 represent two different primary lens epithelial cell cultures from different donors for each primer pair; C is a simultaneous control reaction without added complementary DNA target for each polymerase chain reaction primer pair. Amplification bands of the expected size (Table 1) were detected for each growth factor and receptor (arrow). A 100 base pair marker with sizes in base pairs (BP) is indicated.
FIGURE 5. Epidermal growth factor, epidermal growth factor receptor, hepatocyte growth factor, hepatocyte growth factor receptor, keratinocyte growth factor, and keratinocyte growth factor receptor messenger RNA expression in rabbit lens cells. Primary cultures of rabbit lens epithelial cells were evaluated for the production of messenger RNAs of these factors by reverse transcription–polymerase chain reaction: 1 and 2 represent two different primary lens epithelial cell cultures for each primer pair; C is a simultaneous control reaction without added complementary DNA target for each polymerase chain reaction primer pair. Amplification bands of the expected size (Table 1) were detected for each growth factor and receptor (arrows). There were alternative polymerase chain reaction products with the epidermal growth factor primers that were attributable to the large number of messenger RNAs that have sequence similarity to epidermal growth factor. The expected product for epidermal growth factor, however, was detected and confirmed by nucleic acid sequencing. A 100 base pair marker with sizes in base pairs (BP) is indicated.

Immunoprecipitation and Western Blot Analysis to Detect Growth Factor Proteins

The soluble protein fraction used for immunoprecipitation of HGF or KGF was isolated from rabbit primary lens cells, ex vivo rabbit lens tissue, and rabbit corneal stromal fibroblast cells. Immunoprecipitation of HGF from the cytosolic protein fraction and Western blot analysis under nonreducing conditions were performed as previously reported using the 1A3.1.2 anti-HGF antibody that has been previously characterized.8 The 1A3.1.2 antibody was used at a final concentration of 5 × 10^-4 mg/ml in Western blot analysis. Protein molecular weight standards (Amersham Life Sciences) were run simultaneously on the Western blot analysis gels (4% to 20% gradient polyacrylamide; Novel Experimental Technology, San Diego, CA).

Western Blot Analysis to Evaluate the Effect of Growth Factors on Crystallin Expression

Fourth-passage rabbit lens cells from a single batch of rabbit eyes were subcultured into KDM without growth factors, serum, or other extracts and exposed to vehicle solution only and to 20 ng/ml EGF, 10 ng/ml KGF, or 10 ng/ml double-chain HGF. The cytosolic protein fractions were isolated after 1, 5, or 7 days of exposure to vehicle solution or to growth factors, as previously described.17 Gels were run under reducing conditions with SDS.17 One hundred micrograms of

FIGURE 6. Hepatocyte growth factor, keratinocyte growth factor, epidermal growth factor, hepatocyte growth factor receptor, keratinocyte growth factor receptor, and epidermal growth factor receptor messenger RNA expression in ex vivo rabbit lens tissue. Growth factor and receptor results are shown in (A) and (B), respectively. Ex vivo rabbit lens tissue was evaluated for the production of the growth factor and receptor messenger RNA by reverse transcription–polymerase chain reaction: L1 and L2 represent two different ex vivo lens tissue specimens from different animals for each primer pair; Li is a positive control reaction performed with complementary DNA from rabbit liver; C is a simultaneous control reaction without added complementary DNA target for each polymerase chain reaction primer pair. Amplification bands of the expected size (Table 1) were detected for each growth factor and receptor (arrows). M indicates a 100 base pair marker with sizes in base pairs indicated. Alternative bands for epidermal growth factor and hepatocyte growth factor receptor were determined to be artifacts of polymerase chain reaction.
RESULTS

Messenger RNAs of EGF, HGF, and KGF were detected by PCR in primary cultured human lens epithelial cells (Fig. 1). Hepatocyte growth factor mRNA was detected using two different sets of PCR primers. Receptor mRNAs for EGF, HGF, and KGF were also produced by primary cultured human lens epithelial cells (Fig. 2). In addition to the well-characterized mRNA transcript, a previously reported alternative HGF receptor transcript corresponding to a variant receptor that is truncated in the intracytoplasmic domain was detected (Fig. 2).

Basic FGF and FGF receptor-1 mRNAs were detected in human lens epithelial cells (Fig. 3). Three previously reported alternative transcripts for FGF receptor-1 that are resolved by these PCR primers were detected in human lens epithelial cells; FGF receptor-2 mRNA was not detected. Messenger RNA for TGF-β1 and mRNAs for TGF-β receptor type I and TGF-β receptor type II were detected in human lens cells.

Two alternative transcripts for TGF-β receptor type II mRNA were expressed at approximately equal levels. Messenger RNAs for platelet-derived growth factor alpha, the glucocorticoid receptor, interleukin-1 alpha, and interleukin-1 receptor type I were also detected in primary cultured human lens cells (Fig. 4).

Detected in primary cultured rabbit lens epithelial cells (Fig. 5) and in fresh ex vivo rabbit lens tissue (Figs. 6A, 6B) were mRNAs for EFG, HGF, KGF, and their receptors. Messenger RNA was detected by RT-PCR for both HGF receptors and KGF receptors with two different sets of PCR primers (Table 1). The PCR obtained with the second set of primers for each of these receptors is shown in Figure 6B.

All human and rabbit PCR products were confirmed to be specific by nucleic acid sequencing.

Northern blot analysis was performed to confirm that HGF and KGF mRNAs are produced in rabbit lens epithelial cells. Two HGF transcripts (approximately 4.4 kb and 1.8 kb in size) were detected in rabbit lens epithelial cells and in positive-control rabbit corneal stromal fibroblast cells (Fig. 7A). Two KGF transcripts (approximately 5.1 kb and 2.1 kb in size) were detected in rabbit lens cells and in positive-control rabbit corneal fibroblast cells. (Fig. 7B).

FIGURE 8. Immunoprecipitation and Western blot analysis. (A) Keratinocyte growth factor and hepatocyte growth factor proteins were detected in cultured rabbit lens epithelial cells. Proteins of the expected size for keratinocyte growth factor (28 kDa) and hepatocyte growth factor (90 kDa) were detected in primary cultured rabbit lens epithelial (L) and corneal stromal fibroblast (SF) cells. (B) hepatocyte growth factor, hepatocyte growth factor receptor, and keratinocyte growth factor proteins were detected in ex vivo rabbit lens specimens. Proteins of the expected size for hepatocyte growth factor (90 kDa), hepatocyte growth factor receptor (180 kDa), and keratinocyte growth factor (28 kDa) were detected. Alternative bands in the ex vivo hepatocyte growth factor and hepatocyte growth factor receptor blots were derived from immunoglobulin.
FIGURE 9. Primary cultures of human (A) and rabbit (B) lens epithelial cells. Rabbit lens epithelial cells in a Transwell culture without rabbit corneal endothelial cells (C) or with rabbit corneal endothelial cells (D) after 2 weeks of culture. There was a much larger number of epithelial cells in the Transwell culture with corneal endothelial cells (Fig. 11) after 2 weeks in coculture. The matted background in C and D is generated by photographing through the Transwell membrane.

Western blot analysis was performed to detect HGF, HGF receptors, and KGF protein in primary cultured rabbit lens epithelial cells and in ex vivo rabbit lens tissue. Primary rabbit corneal fibroblasts were used as positive controls, because they are known to produce HGF and KGF. Both HGF and KGF proteins were detected in cultured rabbit lens epithelial cells (Fig. 8A) and in ex vivo rabbit lens tissue (Fig. 8B). Hepatocyte growth factor receptor protein was detected in ex vivo rabbit lens tissue (Fig. 8B).

First-passage rabbit lens epithelial cells (Fig. 9B) were stimulated (Fig. 10) to proliferate by EGF and HGF, but not by KGF. First-passage rabbit lens epithelial cells were also stimulated to proliferate by a confluent monolayer of first-passage rabbit corneal endothelial cells in a Transwell culture (Figs. 9C, 9D, 11).

Increased expression of alpha- (Fig. 12A) and beta- (Fig. 12B) crystallin proteins was stimulated by EGF, KGF, and HGF in cultured rabbit lens epithelial cells compared with that in control vehicle, detected by Western blot analysis. The difference was especially prominent for alpha A crystallins 1 day after exposure but could also be noted at each time point, with each growth factor, for alpha A, alpha B, and beta crystallins.

DISCUSSION

Messenger RNAs (mRNA) coding for HGF, KGF, EGF, and their receptors; basic FGF; FGF receptor-1; TGF-β1; TGF-β receptor type I; TGF-β receptor type II; platelet-derived growth factor alpha; interleukin-1α; interleukin-1 receptor type I; and the glucocorticoid receptor were detected by RT-PCR in primary cultures of human lens epithelial cells from donors newborn to 2 years old. Messenger RNAs, coding for EGF, HGF, KGF and their receptors, were also detected in rabbit primary lens epithelial cells. Importantly, we confirmed that mRNAs, coding for HGF, KGF, EGF, and their receptors, were detectable in ex vivo rabbit lens tissue, proving that expression of these mRNAs is not an artifact of tissue culture. Other investigators have demonstrated production of growth factors, cytokines, and their receptors in lens epithelial cells, including TGF-β, acidic FGF, basic FGF, insulin-like growth factor-1, and EGF receptors. Thus, lens epithelial cells transcribe mRNAs for a large number of growth factors and receptors that may modulate functions of lens cells.

Both HGF and KGF have been best characterized as paracrine mediators expressed by fibroblastic cells to modulate such functions as proliferation, motility, and differentiation in the epithelial cells of many tissues. Results of recent studies showed that HGF and KGF were produced in corneal endothelial cells. Although the HGF and the KGF produced by these cells could have autocrine effects on endothelial cells or paracrine effects on other cells of the cornea, we...
FIGURE 10. Effect of epidermal growth factor, hepatocyte growth factor, and keratinocyte growth factor on the proliferation of first-passage rabbit lens epithelial cells. The effect of each growth factor and the control was tested in 12 wells. Epidermal growth factor (10 ng/ml) and hepatocyte growth factor (5 ng/ml) increased the proliferation of rabbit lens epithelial cells. The increase was statistically significant (**) compared with the increase in control cells (epidermal growth factor, $P < 0.0001$; hepatocyte growth factor, $P = 0.002$). The effect of epidermal growth factor was significantly greater than that of hepatocyte growth factor ($P < 0.0001$). Keratinocyte growth factor at 10 ng/ml was not different from vehicle (con). There was a trend toward inhibition of proliferation of rabbit lens epithelial cells by keratinocyte growth factor, but the decrease was not statistically significant ($P = 0.16$). The results in three experiments were similar for each growth factor.

We hypothesized that HGF and KGF produced by the corneal endothelium could regulate functions of the epithelial lens cells through the aqueous humor. We have termed such communications *aquecrine* interactions and have hypothesized that these interactions may occur not only between corneal endothelial cells and lens cells, but also between endothelial cells, trabecular meshwork cells, and other cells that are immersed in the aqueous humor. Communication by aquecrine mechanisms from endothelial cells to lens cells would necessarily occur retrograde to the direction of flow of the aqueous humor. Fluorophotometry has demonstrated, however, that the diffusion of soluble factors from the cornea to the lens and even to the vitreous humor can occur.

We were surprised to detect HGF and KGF mRNA by RT-PCR in primary cultured human and rabbit lens epithelial cells and ex vivo rabbit lens tissue. Because HGF and KGF are not normally produced by epithelial cells, it was important to confirm whether the HGF and KGF mRNA transcripts detected by RT-PCR were produced at physiologically significant levels and whether they were translated into proteins. We used rabbit lens epithelial cells for the Northern and Western blot analysis experiments because we did not have access to enough human lens tissue to generate the quantities of first-passage human lens epithelial cells needed for these experiments. Messenger RNAs for HGF and KGF were detected in rabbit lens epithelial cells by Northern blot analysis. The sizes of the transcripts were identical to those detected in rabbit corneal stromal fibroblasts (Fig. 7). Two different sizes of mRNA for both HGF and KGF were detected. Alternative sized bands for HGF and KGF that code for the corresponding proteins have been detected previously in other systems. The significance of the alternatively sized mRNAs is unknown, but in human cells both the larger and smaller HGF transcripts code for the full-length HGF precursor protein. Proteins of HGF and KGF of the expected sizes were also detected in rabbit lens epithelial cells (Fig. 8A) and in ex vivo rabbit lens epithelial tissue (Fig. 8B) by immunoprecipitation and Western
The expected approximately 21-kDa alpha A and 22-kDa alpha B crystallins were detected, and the expression of both was increased by epidermal growth factor, keratinocyte growth factor, or hepatocyte growth factor. The origin of a somewhat larger band (Unk) is unknown. (B) A 28-kDa beta-crystallin protein (horizontal arrow) was detected and its expression was also increased by epidermal growth factor, keratinocyte growth factor, or hepatocyte growth factor. A slightly larger band (diagonal arrow) of unknown origin is very faint.

blot analysis. The HGF receptor protein of the expected size was also detected in ex vivo rabbit lens tissue (Fig. 8B). These data demonstrate that rabbit lens cells produce HGF, HGF receptors, and KGF. One possibility is that lens epithelial cells developed the capacity to produce HGF and KGF as the lens evolved into a relatively isolated epithelial structure suspended within the anterior segment of the eye. Hepatocyte and keratinocyte growth factors could function within lens cells by autocrine or paracrine mechanisms. These data, however, do not eliminate the possibility that HGF and KGF secreted into the anterior chamber by the corneal endothelium also modulate lens epithelial cell functions. In fact, the results of the Transwell experiments performed in this study demonstrate that a diffusible factor released from corneal endothelial cells stimulates lens epithelial cell proliferation. Future studies using neutralizing antibodies will be needed to determine whether the diffusible factor released by the corneal endothelial cells is HGF or KGF.

What functions of lens epithelial cells are regulated by HGF and KGF? Double-chain, mature HGF stimulates the proliferation of first-passage rabbit lens epithelial cells. It is important that double-chain HGF be used, in that single-chain precursor HGF has no effect on cultured lens epithelial cells (Wilson SE, unpublished data, 1994). Whether lens-cell produced HGF is processed into the active growth factor could not be determined in the current study. Human KGF had no effect on the proliferation of rabbit lens cells, although there was a trend toward inhibition. Human KGF stimulates the proliferation of rabbit corneal epithelial cells (Wilson SE, unpublished data, 1994), and it is therefore unlikely that the species difference between the growth factor and cultured cells accounts for the lack of stimulation. The expression of alpha and beta crystallins in first-passage rabbit lens epithe-
The effect of each of the growth factors was most prominent for alpha crystallin after 1 day of exposure. There was, however, increased expression of alpha and beta crystallin in cultured rabbit lens cells with HGF, KGF, and EGF compared with that expressed by the vehicle solution after 1, 3, or 7 days of exposure. Epidermal growth factor has been shown previously to increase production of gamma crystallin in human lens epithelial cells. We were unable to obtain an antibody that reliably detected rabbit gamma crystallin to evaluate the effect of HGF or KGF on gamma-crystallin expression.

Alternative splicing of mRNA is a common mechanism used by cells to generate growth factor and cytokine receptors with varying functions. Alternative receptors generated from a specific genomic sequence may have differences in ligand specificity, intracellular and extracellular localization, phosphorylation, tyrosine kinase activity, and intracellular substrate interaction that modulate the effects produced by the growth factor, cytokine, or hormone ligand. In the current study, alternative mRNAs coding for HGF receptor-1, FGF receptor-1, and TGF-β receptor type II were detected by RT-PCR. The alternative HGF receptor mRNA has been shown to code for a receptor having HGF ligand-binding specificity with a truncated intracytoplasmic domain. Whether this alternative receptor functions as a dominant negative inhibitor of HGF stimulation or activates alternative signal transduction pathways is under investigation. The alternative FGF receptor-1 mRNAs code for receptor variants having differing extracellular domains (Fig. 3). The functions of these alternative FGF receptors have not been well characterized. Although the TGF-β receptor type II variant we detected in lens cells had been previously reported, its function remains unknown.

Thus, the HGF and KGF growth factor–receptor systems appear to function locally within the lens. Future investigations into endogenous growth factor, cytokine, and receptor expression in lens cells and the functions that they regulate should lead to a better understanding of the development, homeostasis, and pathophysiology of the lens. In addition, investigations into communication between cells of the anterior segment of the eye should provide new insights into the physiology and pathophysiology of the eye.

Key Words
cell-cell interactions, crystallins, differentiation, hepatocyte growth factor, keratinocyte growth factor, lens, proliferation

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References


