Hepatocyte growth factor (HGF) and its receptor c-met perform a multitude of functions. However, despite the significant degree of study of HGF and c-met in numerous tissues and cell types, relatively few investigations have been performed on the lens. In the current study, therefore, the role of HGF and the receptor c-met in human lens epithelial cells was investigated.

METHODS. Anterior epithelium and capsular bags were prepared from human donor eyes and maintained in Eagle's minimum essential medium (EMEM) in a 5% CO2 atmosphere at 35°C. In addition, the human lens cell line FHL124, was routinely cultured and seeded onto glass coverslips (c-met immunodetection), 12-well plates (DNA and protein synthesis), and tissue culture dishes (migration). c-Met was detected by immunocytochemistry and fluorescence-activated cell scanning (FACS). HGF was measured using enzyme-linked immunosorbent assay (ELISA) techniques. Proliferation and protein synthesis were determined by [3H]thymidine and [35S]-methionine incorporation into DNA and proteins, respectively. Migration was assessed using a scratch-wound assay and time-lapse video microscopy.

RESULTS. HGF was detected at all stages of culture of capsular bags in protein-free medium. Moreover, c-met was present on the native epithelium and after mechanical trauma was seen to be upregulated. Immunolocalization and FACS analysis demonstrated c-met expression on FHL124 cells throughout the whole population. Furthermore, FACS analysis showed that serum-maintained cells sustained a higher level of receptor expression relative to serum-deprived cells. Additionally, HGF was found to stimulate proliferation, protein synthesis, and migratory responses.

CONCLUSIONS. c-Met receptors are expressed in native epithelium, capsular bag cultures, and FHL124 cells. Receptor is distributed across the entire cell population; however, this expression is environmentally and mechanically sensitive. HGF is also present in capsular bags at all stages of culture. In addition, HGF can stimulate migration, proliferation, and protein synthesis. It therefore appears that a multifunctional autocrine loop involving HGF and c-met is in place and could be important in the development of posterior capsule opacification. (Invest Ophthalmol Vis Sci. 2000;41:4216–4222)
both HGF and met in mammalian lens cells, but we are still not aware of HGF levels and receptor distribution in the lens.

A preliminary study into the functional role of HGF suggests that concentrations less than 20 ng/ml could facilitate a wound-healing response in the rabbit lens cell line NN1003A; however, higher concentrations (up to 312 ng/ml) impair the wound-healing process. This observation was significant, because wound healing has become increasingly important in lens cell biology, because of problems that develop after cataract surgery. Unfortunately, in between 20% and 30% of patients who undergo cataract surgery, a secondary loss of vision develop that necessitates further corrective surgery. Modern cataract operations give rise to a lens capsular bag, wherein a proportion of the anterior and the entire posterior collagen capsule remains in situ. Despite the rigors of surgery, a significant number of lens epithelial cells remain on the anterior capsule. These cells subsequently grow across all available lenticular surfaces including, importantly, the previously cell-free posterior capsule surface. In doing so, the cells encroach on the visual axis, and the ensuing cellular and capsular changes that result from this growth induce light scatter, diminishing visual quality. The act of surgery is in effect a wound, and this initiates a response. The early phases of this wound-healing process are influenced by the inflammatory response within the eye that give rise to elevated proteins in the aqueous humor; however, these effects are short lived. Posterior capsule opacification (PCO) in most cases does not become clinically relevant until months or years after surgery. However, the long-term development of PCO may be explained by recent findings that show lens cells can survive, grow, and maintain metabolic activity in protein-free medium for more than 100 days. Therefore, the lens cells themselves are likely to be major regulators of PCO progression. It is important to understand the autocrine systems that regulate lens cell growth and migration to further our understanding of PCO development. In turn this information could provide new opportunities and targets to prevent PCO.

In the present study we demonstrate that HGF can be detected in serum-free capsular bag cultures and that the receptor c-met is also expressed in these preparations. Complimentary human lens cell line data also show HGF can induce proliferation and migration. We therefore hypothesize that the HGF/c-met autocrine loop could contribute to wound healing and PCO.

METHODS

In Vitro Capsular Bags

The model previously described by Liu et al. was used. After removal of corneoscleral discs for transplantation purposes, human donor eyes, or isolated lenses, obtained from the East Anglian or Bristol Eye Banks were used to perform a sham cataract operation. This procedure was performed within 48 hours of enucleation. The resultant capsular bag was then dissected from the zonules and secured on a sterile 35-mm polymethylmethacrylate (PMMA) petri dish. Eight entomologic pins (D1; Watkins and Doncaster, Kent, UK) were inserted through the edge of the capsule to retain its circular shape. Capsular bags were maintained in 1.5 ml of nonsupplemented EMEM. Incubation was at 35°C in a 5% CO₂ atmosphere. The medium was sampled and replaced every 2 to 4 days. Ongoing observations were performed using phase-contrast microscopy.

Anterior Lens Epithelium

After removal from the eye, the lens was transferred to a 35-mm petri dish where it was placed anterior surface down. In some cases, lenses had been mechanically damaged with the intention of disrupting the epithelium. Using an insulin needle, the posterior capsule was punctured, and an incision made that separated the posterior capsule in to two halves. Two pins were then inserted at the ends of the incision. A small cut was then made near one of the pins before most of the posterior capsule was removed by curvilinear tear. This procedure was then repeated on the other half. Six additional pins were inserted through the peripheral to maintain stability. The fiber mass was then carefully removed, and remaining fibers were cleared using surgical forceps. The resulting preparations were then either prepared immediately for immunocytochemical evaluation or cultured in 1.5 ml of protein-free EMEM. Incubation was at 37°C in a 5% CO₂ atmosphere. The medium was sampled and replaced every 3 to 4 days. All lenses used were from donors aged more than 60 years.

FHL124 Cell Culture

FHL cells were generated from human capsule-epithelial explants. FHL124 cells were routinely cultured in EMEM supplemented with 10% fetal calf serum (FCS) and seeded on 12-well plates (DNA and protein synthesis); tissue culture dishes (migration) and glass coverslips (immunocytochemistry). In some cases cells were maintained in serum-deprived conditions for 48 hours.

Immunocytochemical Detection of c-Met

All reagents were from Sigma (Poole, UK) unless otherwise stated. Washes were for 3 × 10 minutes in phosphate-buffered saline (PBS). All preparations were fixed for 10 minutes in 4% paraformaldehyde in PBS. Nonspecific sites were blocked with normal goat serum (1:50 in 1% bovine serum albumin [BSA]-PBS). Anti-met (clone DO-24; Upstate Biotechnology, Lake Placid, NY) was diluted 1:100 and applied for 60 minutes. Alternatively, mouse ascites fluid, also diluted at 1:100, was used as a negative control. Preparations were then washed. c-Met was visualized with a secondary antibody (Alexa 488 conjugated; Molecular Probes, Eugene, OR). The stained preparations were again washed extensively before mounting (Vectashield; Vector, Peterborough, UK). Images were viewed with a fluorescence microscope (Eclipse E800; Nikon, Melville, NY).

c-Met Detection Using Flow Cytometry

FHL124 human lens cells cultured in a 25-cm² flask (Becton Dickinson Labware, Lincoln Park, NJ) maintained in 10% serum or under 48-hour serum-deprived conditions were trypsinized, neutralized with 10% FCS EMEM, and centrifuged (800 rpm for 5 minutes). The supernatant was removed, and cells were washed in 4 ml Dulbecco’s PBS (DPBS) and centrifuged (800 rpm for 2 minutes). The cells were then resuspended in 100 μl PBS containing anti c-met (Clone DO-24; diluted 1:100 in 1% BSA-PBS) or mouse ascites fluid (1:100), and incubated for 30 minutes on ice. Cells were then washed with PBS, centrifuged
(800 rpm for 2 minutes), and resuspended in PBS containing the secondary antibody (Alexa 488; 1:200) and incubated for 30 minutes on ice. At this point the cells were washed again, centrifuged (800 rpm for 2 minutes), and resuspended in 500 μl of 2% paraformaldehyde in PBS (pH 7.4). Cell fluorescence was detected using an Elite epic ESP system (Beckman & Coulter, UK).

**Ex Vivo Specimens**

In four cases donor eyes possessed capsular bags containing intraocular lenses (IOLs) that had been previously generated by cataract surgery. The capsular bag was dissected from the zonules and placed in homogenizing buffer for enzyme-linked immunosorbent assay (ELISA). All specimens showed signs of cell growth and of PCO development. No exclusion criteria were adopted in this study, because these specimens are extremely rare.

**HGF Estimation Using ELISA**

Capsular bags and FHL124 cells were placed in homogenizing medium (composition in millimolar: phosphate 6, KCl 100, NaCl 225, EGTA 1, EDTA 1, mercaptoethanol 10, N-ethylmalamide 10, phenylmethylsulfonyl fluoride [PMSF] 0.05, and E-64 0.005 and 1% vol/vol Tween 20, [pH 7.4]), before storage in a −70°C freezer. One hundred microliters of the homogenates and medium samples were analyzed for human HGF using commercially available ELISA kits (R&D, Abingdon, UK). The optical density of each well was determined using the multiwell plate reader (Victor; EG&G Wallac, Cambridge, UK) at a wavelength of 450 nm. The readings from the standard series were plotted with logarithmic axes, and the data from the samples were then applied to the graph giving the estimated level of growth factor in picograms per milliliter. The limit of sensitivity of the system is 40 pg/ml, and any sample below this level of growth factor in picograms per milliliter. The limit of sensitivity of the system is 40 pg/ml, and any sample below this level of growth factor in picograms per milliliter. The limit of sensitivity of the system is 40 pg/ml, and any sample below this level of growth factor in picograms per milliliter. The limit of sensitivity of the system is 40 pg/ml, and any sample below this level of growth factor in picograms per milliliter. The limit of sensitivity of the system is 40 pg/ml, and any sample below this level of growth factor in picograms per milliliter. The limit of sensitivity of the system is 40 pg/ml, and any sample below this level of growth factor in picograms per milliliter.

**Proliferation and Protein Synthesis Measurement by Radiolabel Incorporation**

One milliliter of a 1 × 10⁴-cells/ml suspension in 10% FCS-EMEM was added to each well of a 12-well tissue culture plate (Becton Dickinson Labware) and cultured for 48 hours. At this point, the medium was removed and replaced with serum-free medium (EMEM), and cultured for a further 48 hours. This medium was then removed and replaced with medium containing 0, 10, or 20 ng/ml HGF (n = 4 in each case) and cultured for a further 48 hours. During the final 4 hours of the culture period, the cells were exposed to both 1 μCi/ml ³⁵S-methionine and [³H]thymidine (Amersham, Amersham, UK), plus 1 μM cold thymidine (Sigma). At the end of culture, each well was washed twice with 1 ml EMEM to remove residual radioactive [³H]thymidine or ³⁵S-methionine. One milliliter of 5% trichloroacetic acid (TCA) was added to each well. After 30 minutes at room temperature, the TCA was removed and 1 ml of 250 mM NaOH was added to each well and left overnight at 4°C, before 0.5 ml of this NaOH was sampled. Scintillation fluid (10 ml, Hisafe Supermix; EG&G Wallac) was added to each sample and appropriate backgrounds. Measurements were obtained using a scintillation counter (EG&G Wallac).

**Migration Assay**

FHL124 cells were seeded on 35-mm tissue culture dishes and maintained in EMEM supplemented with 10% FCS. These cells were grown to form a monolayer before serum starving for 48 hours. At this point, a scratch wound was performed using a plastic pipette tip. The cells were then rinsed once with EMEM, before replacing the medium with CO₂-independent L15 medium containing different concentrations of HGF. Migration of cells into the wounded area was monitored using a digital camera (JVC, Tokyo, Japan) and Lucia image analysis software (Nikon, Melville, NY). Images were captured every 3 hours. The preparations were maintained at 37°C throughout.

**Statistical Analysis**

A t-test analysis was performed by computer to determine any statistical difference between groups (Excel; Microsoft, Redmond, WA). A 95% confidence interval was used to assess significance.

**RESULTS**

**Native Epithelium and Capsular Bag Preparations**

**Immunodetection of c-Met.** c-Met was detected in association with cells of the native epithelium (Fig. 1A). The receptor expression appeared to be across the entire epithelium and was largely distributed at the cell periphery. The degree of expression was low but could still be observed against background. However, after trauma to the lens epithelium by mechanical disruption, receptor expression appeared to be up-regulated (Figs. 1B, 1C). This observation was apparent both in cells growing across denuded areas of the anterior capsule (Fig. 1B) and in those colonizing the previously cell-free posterior capsule (Fig. 1C). In the case of cells that recolonized the anterior capsule, the receptor remained localized to the cell periphery (Fig. 1B). However, cells growing on the posterior capsule showed a diffuse staining across the cell, with a marginally higher density at the periphery (Fig. 1C).

**HGF Detection.** HGF levels in capsular bag preparations are presented in Figure 2. Analysis of capsular bag homogenates showed that HGF could be detected at all stages of culture in protein-free medium. Capsular bag homogenates showed no significant difference between HGF levels in non-cultured and day 8 capsular bags, but cultures maintained more than 100 days were significantly lower. Furthermore, in cases in which the donor had previously undergone a cataract operation, analysis of the capsular bag revealed HGF to be present at levels similar to those in long-term capsular bag cultures. In addition, significant amounts of HGF were also secreted into the culture medium during early phases of growth (Fig. 3). The total level detected in medium over the first 8 days of culture, sampled at 2-day intervals, was 733 ± 19 pg (n = 4). However, medium from long-term cultures was below detectable levels.

**Human Lens Cell Line**

Because the availability of human tissue is limited, it is useful to investigate specific systems by using cell lines as an experimental tool. We therefore used a human lens cell line, FHL124, to study c-met-mediated HGF events.
Immunodetection of c-Met. After a 48-hour period of serum deprivation, FHL124 cells continued to express c-met (Fig. 1D). Receptors were not predominately located at the cell borders but showed a diffuse expression pattern across the cell membrane (Fig. 1D). Moreover, it was observed that each cell expressed c-met to some extent. To further test this notion, a fluorescence-activated cell scanner (Coulter, Hialeah, FL) was used to detect levels of c-met-associated fluorescence of individual cells. With this technique, control cells (incubated with mouse ascites fluid) showed low levels of fluorescence, whereas the test population (incubated with anti c-met) showed a relatively high degree of fluorescence so that the data curve shifted completely to the right, thus showing expression in all the cells (Fig. 4). Additionally, the receptor expression on a per cell basis was found to be modified by culture conditions. Maintenance in EMEM supplemented with 10% FCS sustained a higher level of receptor expression relative to cells deprived of serum for 48 hours (Fig. 3). The distribution of the receptor on cells cultured under both conditions demonstrate a single gaussian distribution with a modal value of 1.2 and 2.5 for serum-deprived and serum-maintained cells, respectively.
HGF Detection. ELISA analysis of both cell homogenates (1 million cells/1 ml homogenizing buffer) and culture medium did not show any detectable amounts of HGF.

Functional Effects of HGF. After serum deprivation, HGF induced a dose-dependent enhancement of proliferation and protein synthesis in FHL124 cells as determined by incorporation of [3H]thymidine and [35S]methionine into newly synthesized DNA and proteins, respectively (Fig. 5). The maximum response observed was at 20 ng/ml; however, 10 ng/ml HGF also induced a significant response (t-test, P < 0.05). It should also be noted that FHL124 cells possess high levels of cellular activity under serum-free conditions and do not become quiescent quickly. The migratory activity of these cells in serum-free conditions was apparent using time-lapse video microscopy, and this was significantly enhanced by the addition of 10 and 20 ng/ml HGF (Fig. 6).

DISCUSSION

We have demonstrated that lens epithelial cells normally express c-met. Furthermore, after mechanical trauma, this ex-
expression is upregulated. In addition, the levels of HGF are initially upregulated after injury, and HGF is continually expressed throughout culture, although the levels decline with time. It therefore appears that the elements of an autocrine system are in place that could contribute to normal lens regulation and modulate the epithelial migration and growth that is associated with the development of PCO.

Earlier studies on human material have shown that primary lens cell cultures contain mRNA for c-met and HGF. However, these cultures were generated from young donors and maintained in serum-supplemented medium on plastic culture dishes. Additionally, c-met and HGF have been detected in the native rabbit lens epithelium using RT-PCR and Western blot techniques, but again this was in a young animal. In the present study we show that c-met and HGF protein are detectable in the native lens epithelium of aged human donors. Importantly, in this study, HGF was shown to be present in donor material from people who had previously undergone cataract surgery, although c-met was not examined in these preparations. The techniques previously adopted to study c-met in lens cells do not address the distribution of receptor; however, the immunocytochemical techniques used in the present study permit the receptor pattern to be observed. The native human epithelium shows a weak, but detectable, expression with the receptors distributed at the cell borders. This pattern of distribution is also maintained on cells growing across the anterior capsule after wounding; however, cells growing across the previously cell-free posterior capsule demonstrate a less ordered distribution. This difference is possibly due to variations between the posterior and anterior capsules, and furthermore, when cells are cultured on plastic, an even more diffuse receptor distribution is observed.

Of note, after mechanical trauma to the epithelium the receptor expression was upregulated. This observation is consistent with other data published in various tissue systems.

In experiments using the lens line FHL124, fluorescence-activated cell scanning (FACS) analysis showed that c-met expression could be influenced by culture conditions. These results showed that serum-maintained cells expressed higher levels of c-met than cells deprived of serum. Serum contains a multitude of factors that may modulate c-met expression. Elevation of protein in the eye can develop because of inflammation caused by mechanical injury—e.g., cataract surgery or in association with ocular disease. Modification to the environment could in turn alter c-met expression in the lens, which could alter the regulation and functional capacity of lens cells. Using the FACS system to study c-met expression in FHL124 cells can show the distribution within a population and permits quantification to take place. Therefore, it provides an excellent tool to investigate the influence of growth factors and underlying matrix on c-met expression.

Previous reports have shown that HGF levels can also be modulated by injury and factors associated with wound healing. The data presented in the present study also show that HGF is upregulated after injury. Analysis of medium samples show high levels of HGF at the first time point (day 2) after injury. Although the levels rapidly declined with time, the total amount of HGF secreted into the medium in the first 8 days of culture was significantly greater than the total amount detected in noncultured capsular bags. In addition, the amount detected in homogenates of day 8 capsular bags was not significantly different from their noncultured counterparts. Therefore, the total amount of HGF detected in the system over the initial 8-day period greatly exceeds HGF levels at the onset of culture. Consequently, these data clearly demonstrate that active synthesis of HGF can take place, under serum-free conditions, in response to trauma.

It is interesting that ELISA analysis of homogenates and culture medium of FHL124 cells did not show any detectable levels of HGF. In a previous study using a lens cell line, c-met mRNA was reported, but there were no data for HGF. The absence of HGF was possibly due to a loss of the gene in the transformation process, change of external environment, or potentially to the cells’ being cultured on plastic as opposed to native capsule with its full complement of matrix components. Studies using young human primary cells show mRNA for HGF is present. However, this analysis was performed on cells of early passage number. Work performed by Bloemendal has shown that lens epithelial cells removed from the capsule and cultured on plastic, exhibit diminished levels of α-crystallin with time in culture. Similar findings have also been reported with human lens cell lines.

Furthermore, when cells are cultured on plastic in serum-free medium they cannot survive as efficiently as cells grown on plastic, exhibit diminished levels of α-crystallin with time in culture. Similar findings have also been reported with human lens cell lines. Furthermore, when cells are cultured on plastic in serum-free medium they cannot survive as efficiently as cells grown on plastic, exhibit diminished levels of α-crystallin with time in culture. Similar findings have also been reported with human lens cell lines. Therefore, the matrix could play a role in the regulation of HGF and other proteins associated with lens cells.

Functional studies performed on the human lens cell line FHL124 showed that HGF could enhance protein synthesis, proliferation, and migratory rates. These processes are fundamental to cell growth and play integral roles in the wound-healing process. It therefore appears that both autocrine and paracrine activation of c-met by HGF may contribute to the development of PCO after cataract surgery.
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References