ORIGINAL ARTICLES – Laboratory science

Effects of the cytokines on the proliferation of and collagen synthesis by human cataract lens epithelial cells

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Abstract

***Aims*—To assess the effects of the cytokines, interleukin-1 (IL-1), IL-1 receptor antagonist (IL-1ra), transforming growth factor-β 2 (TGF-β2) and basic fibroblast growth factor (b-FGF), on the mitosis of and collagen synthesis by lens epithelial cells (LECs) of human cataracts.**

***Methods*—The anterior lens capsule with attached LECs was obtained by capsulotomy during cataract surgery and cultured. The cultures at 2 to 3 weeks before confluency were used for the experiments. To quantify the mitosis and collagen synthesis, the incorporation of ^{3}H-thymidine and ^{3}H-proline, respectively, into the LECs was measured by a scintillation counter at 48 hours and 24 hours, respectively, after addition of the cytokine at various concentrations into the incubation medium.**

***Results*—IL-1 and b-FGF increased the mitosis and collagen synthesis significantly, but IL-1ra significantly decreased the mitosis while leaving the collagen synthesis intact. TGF-β2 decreased the mitosis significantly, but increased the collagen synthesis significantly.**

***Conclusion*—These cytokines may play an important role in an autocrine or paracrine pathway in the proliferation of residual LECs after cataract surgery. Elucidation of the role of these cytokines may lead to the development of new therapies for the prevention of secondary cataract.**

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Residual lens epithelial cells (LECs) proliferate in a defective lens capsule after cataract surgery, resulting in secondary cataract. They can also undergo fibrous metaplasia and produce collagen, resulting in capsular fibrosis that represents a form of secondary cataract. We reported previously that cultured human cataract LECs obtained by anterior capsulotomy during cataract surgery produce interleukin-1 (IL-1),[1] IL-6,[2] IL-8 (unpublished data), basic fibroblast growth factor (b-FGF),[3] and transforming growth factor-β (TGF-β) (unpublished data) in the incubation medium. Cytokines act generally in an autocrine or paracrine manner on the target cells. In this paper, we examined the effect of the cytokines, IL-1, IL-1 receptor antagonist (IL-1ra), TGF-β2 and b-FGF, on the proliferation of and collagen synthesis by human cataract LECs in culture.

Materials and methods

As an indicator of the mitosis rate of the LECs and their collagen synthesis rate, the uptake of ^{3}H-thymidine and ^{3}H-proline by the cells was measured. The uptake by the LECs when a cytokine at various concentrations was added to the culture was compared with that of the controls without addition of any cytokine.

CULTURE OF HUMAN CATARACT LECS

Human cataract LECs were cultured as previous described.[1] Briefly, a circular piece of the anterior capsule with LECs attached was obtained by capsulotomy during cataract surgery and cultured directly without dispersion of the cells. After circular capsulorhexis, the piece of capsule, about 5 mm in diameter, was touched with an irrigation/aspiration tip and withdrawn from the eye by aspiration. The piece of capsule was held with fine forceps and washed thoroughly with irrigating solution. Each piece of anterior capsule was placed immediately into a well of a 48 well, multiwell plate containing 0·5 ml of Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), penicillin G at 100 U/ml, and streptomycin sulphate at 100 μg/ml and then cultured in 100% humidity at 37°C in a 5% carbon dioxide atmosphere. The medium was changed weekly. The specimens from patients aged between 55 and 69 years were used for the entire experiment.

CELL HARVEST IN THE TIME COURSE AND DETERMINATION OF THE NUMBER OF VIABLE CELLS IN CULTURE

To determine the cell harvest in the time course, the number of viable cells in each culture was counted (see below) in a separate

group of cultures at the beginning (n=8), 1 (n=8), 2 (n=6), 2½ (17 days) (n=6), 3 (n=6), 3½ (24 days), 4 (n=6), and 5 weeks (n=4) of culture. Before the cell count procedure, each culture was photographed under an inverted phase contrast microscope (MFA 20/100, Nikon, Tokyo, Japan) with a video camera. To obtain the entire culture area in a glance, an objective with a magnification ×2 was used. The microphotographs recorded on video tape were incorporated into the device for automated morphometry of corneal endothelial cells using an image analyser[4] to determine the cell culture area. After obtaining the number of viable cells and the value of cell area in these cultures, the regression equation between them for the cultures at 2 to 3½ weeks was determined for the estimation of the number of viable cells in each culture that was used for the subsequent experiment.

COUNT OF VIABLE CELLS

At each end of the culture, the number of viable cells was counted in each well according to our previously reported procedure.[1]

The incubation medium in each well was removed, followed by a wash with 0·5 ml of calcium free and magnesium free phosphate buffered saline (PBS(−)). The cells in each well were then incubated with 0·4 ml of a mixture of 0·25% trypsin and 0·02% ethylene diaminotetra-acetic acid (EDTA) sodium for 10 minutes at 37°C. After repeated pipetting to remove the cells from the capsule and the plastic wall of the well, the entire cell suspension was transferred into a small glass test tube, taking care to leave as few cells as possible in the well. The cells were stained by adding 0·1 ml of a mixture of 0·05% crystal violet and 2·1% citric acid, followed by one drop of formalin. Only viable cells could be stained by this procedure. The number of viable cells in the cell suspension from each well was determined, in triplicate, in a Fuchs-Rosenthal cell chamber for counting erythrocytes, and the mean value was used to obtain the total number of viable cells in each sample.

UPTAKE OF ^{3}H-THYMIDINE BY HUMAN CATARACT LECs

The mitosis of LECs was assessed by measurement of ^{3}H-thymidine incorporation into the cells. The cultures at 2 to 3 weeks, which showed no confluency and were still proliferating, were used. Before the procedure, the culture area in each well was determined in order to read the number of viable cells from the regression equation described.

Addition of the cytokine

An aliquot of the culture medium was replaced with 0·5 ml culture medium containing the cytokine at various concentrations. Accordingly, human recombinant IL-1α (0·5 μg/ml, Genzyme, Cambridge, MA, USA) was added at the concentration of 1, 10, and 100 pg/ml and 1 ng/ml; human recombinant IL-1ra (1 mg/ml, Otsuka Pharmaceutical, Tokushima, Japan) at 1, 10, and 100 μg/ml; human recombinant TGF-β2 (1 ng/ml, Austral Biologicals, San Ramon, CA, USA) at 10 and 100 pg/ml and 1 ng/ml; or human recombinant b-FGF (1 ng/ml UBI, Lake Placid, NY, USA) at 1, 10, and 100 ng/ml.

Five to six cultures were used at each concentration. No cytokine was added to six cultures which served as the controls.

^{3}H-thymidine labelling and mitosis assay

After incubating for 48 hours at 37°C in a 5% carbon dioxide atmosphere following cytokine addition, cells were labelled with 0·2 μCi/ml of ^{3}H-thymidine (1 mg Ci/ml) for 16 hours; ^{3}H-thymidine incorporation into the cells was measured by a standard procedure.[5]

The radioactivity of ^{3}H-thymidine was expressed as disintegrations per minute (dpm)/10^4 cells using the value of the viable cells at the end of the culture, which was determined from the regression equation described.

UPTAKE OF ^{3}H-PROLINE BY HUMAN CATARACT LECs

Collagen synthesis was assessed by ^{3}H-proline incorporation according to the method of Agelli *et al*[6] with some slight modifications.

Addition of the cytokine

The medium was removed and washed with PBS(−) twice and replaced with the proline-free Eagle's MEM (controls) or proline-free Eagle's MEM containing the cytokine at various concentrations. Accordingly, human recombinant IL-1 at concentrations of 10 and 100 pg/ml and 1 ng/ml; human recombinant IL-1ra at 1, 10, and 100 μg/ml; human recombinant TGF-β2 at 100 pg/ml and 1 and 10 ng/ml; or human recombinant b-FGF 2 at 1, 10, and 100 ng/ml was added. Five to six cultures were used for each concentration. No cytokine was added to six cultures that served as the controls. These cultures were then incubated overnight.

^{3}H-proline labelling

For radioactive labelling of the collagens synthesised by LECs, 2 μCi/ml ^{3}H-proline (1·04 T Bq/mM, Amersham, Tokyo, Japan), 100 μg/ml L-ascorbic acid, and 100 μg/ml β aminopropionitrile (Daiichi Chemicals Inc, Tokyo, Japan) were added to the medium and then the cells were incubated for a further 6 hours. The medium was then removed and kept in a polyethylene tube. The cells were washed with PBS(−) and then dissolved by 0·5 N sodium hydroxide for 10 minutes and put into the same tube as the medium. Twenty μl of bovine serum albumin (BSA) (2 mg/ml) were added as a carrier to the tube. The entire contents were diluted with 0·1 M acetic acid and then distilled water to remove

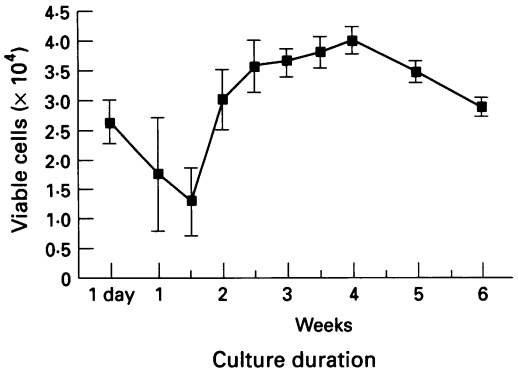

Figure 1 Cell harvest in the time course of cultured human lens epithelial cells obtained during cataract surgery. Each value indicates the mean number of viable cells with standard deviation.

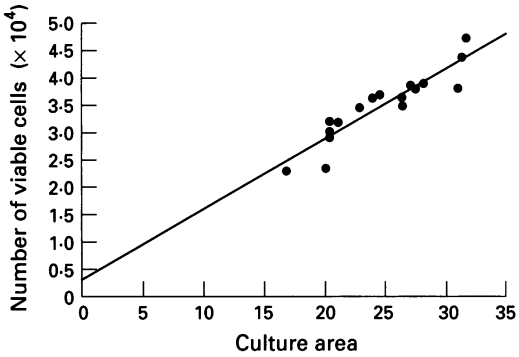

Figure 2 The regression equation between the number of viable cells and the culture area at 2 to 3½ weeks of culture.

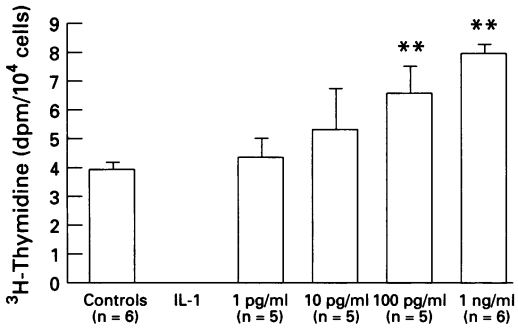

*Figure 3 The effects of IL-1 on ³H-thymidine uptake by human cataract lens epithelial cells. **Indicates p<0·01.*

the unincorporated ³H-proline and then dried and frozen.

Collagen synthesis assay

The frozen material was dissolved in 900 µl of a solution containing 50 mM Tris-HCl (pH 7·4), 5 mM $CaCl_2$, and 40 µl of 62·5 mM N-ethyl-maleimide (NEM); the solution was divided equally into two tubes. Collagenase solution containing 100 µl of 25 mM $CaCl_2$, 62·5 µl of 0·5 M Tris buffer, 200 µl of 62·5 mM NEM, and 50 µl of collagenase stock solution (3000 units/ml 50 mM Tris (pH 7·4)) (Sigma, St Louis, MO, USA) was added into one tube, which was then incubated at 37°C for 2 hours. The solution in the tube that served as control received distilled water instead of the collagenase solution and was incubated for 2 hours at 37°C. Both samples were then cooled with ice and then 400 µl of 10% trichloroacetic acid were added to each to

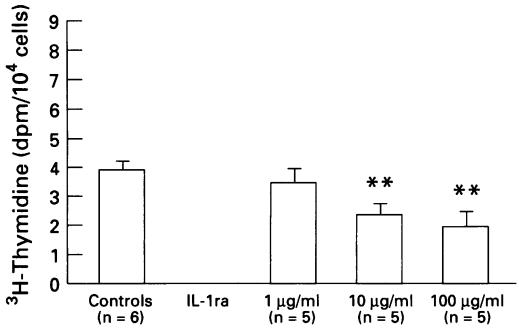

*Figure 4 The effects of IL-1ra on ³H-thymidine uptake by human cataract lens epithelial cells. **Indicates p<0·01.*

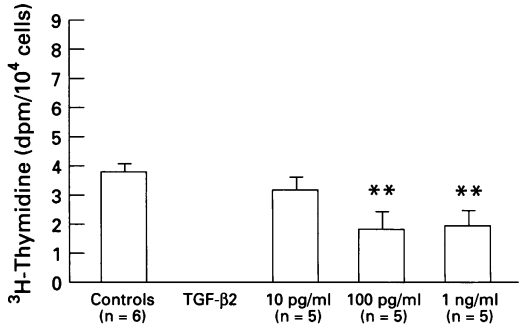

*Figure 5 The effects of TGF-β2 on ³H-thymidine uptake by human cataract lens epithelial cells. **Indicates p<0·01.*

precipitate the proteins. Each sample was then centrifuged at 14 000 rpm for 5 minutes. Radioactivities in the supernatants were measured by a liquid scintillation counter (B460CD, Packard Japan, Tokyo, Japan). The pellets were dissolved with 200 µl of 0·5 N sodium hydroxide; their radioactivity was measured to quantify radiolabelled non-collagenous proteins.

Collagens synthesised by LECs were quantified by subtracting the radioactivity in the supernatants incubated without collagenase from that in the supernatants incubated with collagenase. The amount of synthesised collagens was expressed as dpm/10^4 cells using the value of the viable cells at the end of the culture, which was determined from the regression equation described.

STATISTICS

One way ANOVA (Scheffe's method) was used to study dose dependent differences within the different cytokine groups.

Results

CELL HARVEST IN THE TIME COURSE AND DETERMINATION OF THE NUMBER OF VIABLE CELLS IN CULTURE

Figure 1 shows the cell harvest in the time course of culture. The number of initially viable cells decreased at 1 and 1½ weeks of culture with a large standard deviation (SD), but increased towards 4 weeks with the decreasing SD and then decreased slightly at 5 and 6 weeks of culture. The regression equation between the number of viable cells and the culture area for the cultures at 2 to 3½ weeks was y=0·126x+0·375 (y=number of viable

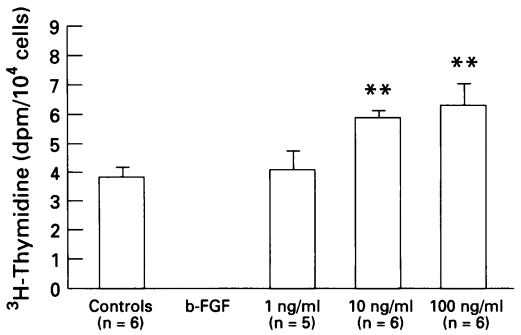

*Figure 6 The effects of b-FGF on ^{3}H-thymidine uptake by human cataract lens epithelial cells. **Indicates $p<0.01$.*

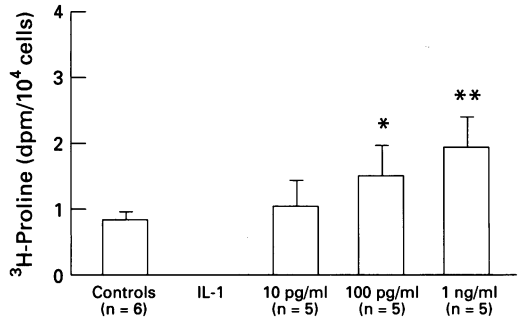

*Figure 7 The effects of IL-1 on ^{3}H-proline uptake by human cataract lens epithelial cells. *Indicates $p<0.05$; **indicates $p<0.01$.*

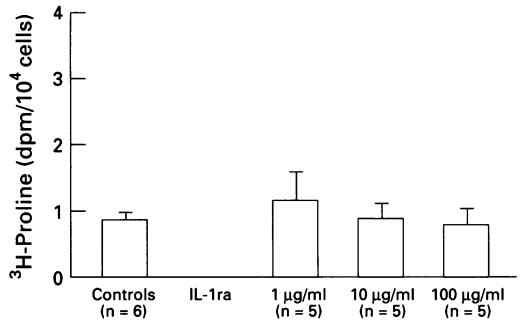

Figure 8 The effects of IL-1ra on ^{3}H-proline uptake by human cataract lens epithelial cells.

cells, x=culture area) with r (coefficient of correlation)=0·92 (Fig 2).

^{3}H-THYMIDINE INCORPORATION AND MITOSIS ASSAY

The uptake of ^{3}H-thymidine was significantly increased by IL-1 at 100 pg/ml and 1 ng/ml ($p<0.01$ for both) (Fig 3), while it was decreased significantly by IL-1ra at 10 and 100 μg/ml ($p<0.01$ for both) (Fig 4). The uptake was significantly decreased by TGF-β2 at 100 pg/ml and 1 ng/ml ($p<0.01$ for both) (Fig 5) and increased by b-FGF at 10 and 100 ng/ml ($p<0.01$ for both) (Fig 6).

^{3}H-PROLINE INCORPORATION

The uptake of ^{3}H-proline was significantly increased by IL-1 at 100 pg/ml ($p<0.05$) and 1 ng/ml ($p<0.01$) (Fig 7). IL-1ra did not affect the uptake significantly (Fig 8). The uptake was significantly increased by TGF-β2 at 1 ng/ml ($p<0.05$) and 10 ng/ml ($p<0.01$) (Fig 9) and by b-FGF at 10 ng/ml ($p<0.05$) and 100 ng/ml ($p<0.01$) (Fig 10).

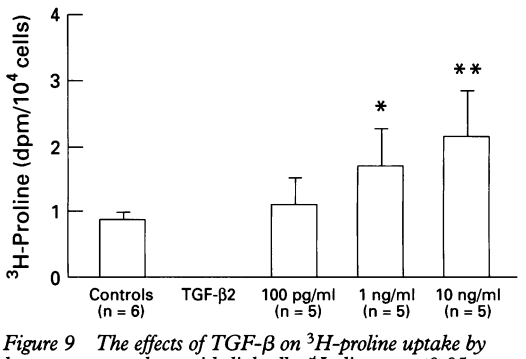

*Figure 9 The effects of TGF-β on ^{3}H-proline uptake by human cataract lens epithelial cells. *Indicates $p<0.05$; **indicates $p<0.01$.*

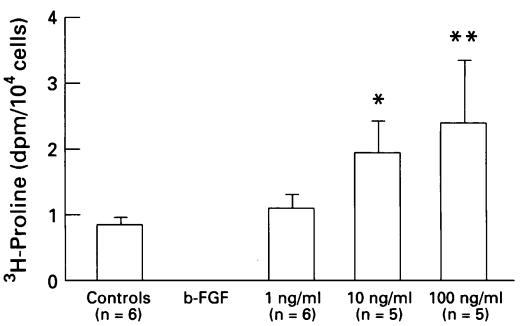

*Figure 10 The effects of b-FGF on ^{3}H-proline uptake by human cataract lens epithelial cells. *Indicates $p<0.05$; **indicates $p<0.01$.*

Discussion

Our results clearly show that IL-1 increased the mitosis of and synthesis of collagen by human cataract LECs, while IL-1ra decreased the mitosis significantly. We also reported previously that rabbit polyclonal anti-human IL-1 significantly inhibited the proliferation of human LECs.[7] Interleukin-1 is now regarded as a basic mediator of intercellular signals, both within the immune system as well as between the immune system and almost all other organ systems, and it possesses a wide spectrum of multifunctional activities.[8–10] It is known to initiate a cascade of inflammatory mediators.[11 12] It stimulates[13–16] or inhibits[17–19] the proliferation of various cells or cell lines. It also increases[20–23] or inhibits[24–28] the collagen synthesis in various cell lines. These apparently contradictory results appear to depend on the cell type, the culture conditions, and the presence of other modulating cytokines. IL-1ra[29] has been identified by cloning as the first described naturally occurring endogenous receptor antagonist of any cytokine. It has a molecular weight of 17 to 25 kD and inhibits IL-1 activities by competitive binding to a specific receptor.[29 30] The dose we used was determined from some reports which indicated that the IL-1β concentration in the anterior chamber at day 7 after intraocular lens implantation in rabbits[31] was 10·12 (SD 3·96) ng/ml aqueous humour; Rosenbaum *et al* injected 75 μg of human recombinant IL-1ra intravitreally in rabbit eyes to assess the potential activity of the IL-1ra in ocular inflammation by the intravitreal injection of IL-1.[32] Although IL-1ra significantly decreased the mitosis rate, it did not affect the collagen synthesis in this study. The collagen synthesis may have been compensated by the collagen synthesis induced by

cytokines other than IL-1, such as TGF-β or b-FGF, that are produced by the LECs themselves during culture.

TGF-β2 showed a significant suppressive effect on the mitosis, but at the same time increased significantly the collagen synthesis. We reported previously that LEC proliferation was induced by TGF-β, which, however, was based on the microscopic morphological observation. Both TGF-β and b-FGF are involved as pluripotent modulators in embryonic morphogenesis in a number of organs, including the eye, tissue repair, and fibrosis.[10 33 34] TGF-β was originally described as a factor that induced phenotypic transformation in fibroblast-like cells. In general, it increases collagen synthesis in a variety of cells or cell lines.[35–37] TGF-β[38] stimulates the proliferation of cells of mesenchymal origin, but has a suppressive effect on the mitosis of epithelial cells, which is consistent with the present results.

Basic FGF increased significantly the mitosis of and collagen synthesis by human LECs. It stimulates the proliferation and differentiation of a large variety of cells derived from the neuroectoderm, mesoderm, and neural crest.[39 40] It is also capable of angiogenesis.[40 41] Basic FGF appears to participate in the development of the lens.[34] Cultured corneal endothelial and LECs synthesise b-FGF,[40–44] which has a mitogenic effect on them. It was detected in the subcapsular epithelium of the lens.[45 46] Basic FGF upregulates[44 47] or downregulates[48 49] the collagen synthesis. These effects again appear to depend on the cell type and the culture conditions – that is, whether it is a primary culture or cell line. This property also may be significantly modified by the type of cytokine present.

Interleukin-1 was found in the aqueous humour[31] in rabbits and IL-6[50] in humans following intraocular lens implantation. IL-6 is known to be induced by IL-1.[51] Basic FGF[52] and TGF-β[53–55] were detected in the aqueous humour obtained from patients undergoing cataract surgery. Basic FGF was identified[56] immunohistochemically in the proliferating LECs around the lens capsule after cataract surgery. The above mentioned cytokines may be produced by LECs in vivo as well after cataract surgery, and LECs can be a source of these cytokines detected in the aqueous humour. The cytokines may act in an autocrine or paracrine manner to stimulate or inhibit the proliferation of LECs, enabling them to participate in the formation of a secondary cataract after cataract surgery.

In summary, human recombinant IL-1 increased the mitosis of and collagen synthesis by human cataract LECs in culture, while IL-1ra decreased the mitosis. Basic FGF increased both mitosis and collagen synthesis significantly. TGF-β suppressed the mitosis but increased the collagen synthesis significantly. These results suggest that the cytokines synthesised by LECs modulate the proliferation of LECs, enabling them to participate in the formation of secondary cataract after cataract surgery. Illuminating the role of these cytokines in LECs may lead potentially to the development of new therapies to inhibit their proliferation and, therefore, to prevent secondary cataract.

The authors have no proprietary interest in the methods and products mentioned in this paper.

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