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TGF-β and IL-1β act in synergy to enhance IL-6 and IL-8 mRNA levels and IL-6 production by human retinal pigment epithelial cells

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SUMMARY

Cytokines produced by human retinal pigment epithelial (RPE) cells may function as important regulators of intraocular inflammation. We investigated the effect of transforming growth factor-β (TGF-β) on interleukin-1β (IL-1β) induction of IL-6 and IL-8 mRNA levels and protein production by human RPE cells. Both TGF-β and IL-1β alone induce IL-6 mRNA and IL-6 production in human RPE cells and synergize to enhance IL-6 mRNA levels and IL-6 production over a range of TGF-β (0·1–10 ng/ml) and IL-1β concentrations (5–500 U/ml). TGF-β was also found to enhance IL-1β induction of IL-8 mRNA levels at lower IL-1β concentrations (50 U/ml) but had no effect at higher IL-1β concentrations (500 U/ml). However, TGF-β had no synergistic effect on IL-1β induction of IL-8 secretion. These results suggest that expression of IL-6 and IL-8 in human RPE cells is regulated by different transcriptional and translational mechanisms, and that RPE cells are important regulators of cytokine production within the ocular microenvironment.

INTRODUCTION

The retinal pigment epithelium which forms part of the blood–retinal barrier is believed to be an important regulator of posterior segment ocular inflammatory responses. Retinal pigment epithelial (RPE) cells have been shown to secrete cytokines *in vitro* after stimulation with interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α).[1,2] During an inflammatory response, in addition to infiltrating cells, resident ocular cells such as Muller cells[3] are among the possible sources of IL-1 and TNF within the eye.

Several of the cytokines produced by stimulated RPE cells including IL-6 and IL-8 are known to be involved in lymphocyte activation and chemotaxis,[4,5] and induce an inflammatory response when injected intravitreally.[6,7] Transforming growth factor-β (TGF-β) is also present within the eye[8] and is one of the cytokines responsible for the immunosuppressive properties of the aqueous humor.[9] TGF-β however is also capable of enhancing or down-regulating an inflammatory response depending on the cell type.[10] It can thus inhibit IL-1-induced IL-6 production by monocytes[11] but enhances IL-6 production by other cell types such as fibroblasts[12] and intestinal epithelial cells.[13] TGF-β appears to have no effect on IL-8 production by colonic epithelial cells.[14] The aim of the present study was to determine the influence of TGF-β on IL-1β induction of IL-6 and IL-8 by human RPE cells. The results demonstrate that TGF-β has a synergistic effect on IL-1 induction of IL-6 mRNA levels and protein production in human RPE cells over a range of IL-1β and TGF-β concentrations.

TGF-β also has a modest synergistic effect on IL-1 induction of IL-8 mRNA levels when combined with low IL-1 concentrations but has no effect on IL-8 mRNA levels at higher IL-1 concentrations. TGF-β has no synergistic effect on IL-1-induced IL-8 secretion suggesting that in RPE cells different transcriptional and translational mechanisms are involved in the regulation of IL-6 and IL-8 production.

RPE cells would therefore appear to play an important role in the regulation of an ocular inflammatory response by regulating cytokine production within the eye.

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Abbreviations: RPE, retinal pigment epithelium; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α.

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MATERIALS AND METHODS

Cell isolation and culture

Human eyes were obtained from the Amsterdam eye bank after the corneas had been removed for corneal transplants. The donor age ranged from 20 to 80 years. Eyes were received within 48–72 hr of death. Human RPE cells were isolated from donor eyes and cultured as previously described.[15]

Briefly, a 5-mm band of sclera was removed. The vitreous was then aspirated with a syringe and the retina was removed by cutting the optic nerve. The eye cup (posterior segment) was then washed with Hanks' balanced salt solution (HBSS), Ca^{2+} and Mg^{2+} free (Gibco, Paisley, UK) and filled with 0·25%

trypsin (ICN Flow, Irvine, UK) for 1–2 hr at 37°. The cells were then released from the basement membrane by gentle aspiration. Cells were washed twice in Glasgow minimal essential medium (GMEM) (Gibco) and suspended at 5×10^5 cells/ml for donors aged less than 30 years and 1×10^6/ml for older donors.

Cells were then plated in 24-well tissue culture plates in GMEM + 10% fetal calf serum (FCS) (Gibco). On the following day, cells were gently washed with warm HBSS and the medium was replaced. When cells reached confluency they were passaged by trypsinization and gradually expanded by plating into 6-well plates, then into 25-cm^2 and 75-cm^2 flasks (Nunc, Roskilde, Denmark). Rapidly replicating cells (fibroblasts) were discarded. The resultant cultures consisted of monolayers of polygonal cells which were vimentin (Dako, High Wycombe, UK) and cytokeratin positive (Becton Dickinson, San Jose, CA).

Treatment of cells

In all experiments, simultaneous parallel assays were performed on fourth to fifth passaged RPE cells in sister flasks containing near confluent cells seeded at the same time and density from the parent cultures.

Assayed RPE cells were either unstimulated or treated with human recombinant TGF-β1 (R&D Systems, Abingdon, UK), recombinant IL-1β alone (Genzyme, West Malling, UK) or a combination of these cytokines. Specific activities were: TGF-β1, 1·67–5×10^7 U/mg and IL-1β 5×10^8 U/mg.

RPE mRNA analysis

Tissue culture flasks (75 cm^2) were overlaid with 15 ml of medium alone or medium containing varying concentrations of IL-1β (5–500 U/ml), TGF-β alone (0·1–10 mg/ml) or a combination of both cytokines for 4 hr. Cells were then washed and total RPE cell RNA was extracted as previously described.[16] Briefly the adherent RPE cells were solubilized in 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7·0) 0·5% Sarkosyl and 0·1 M 2-mercaptoethanol (all from Sigma, Poole, UK).

RNA was extracted using phenol-chloroform isoamyl alcohol and the aqueous layer removed. Isopropanol-precipitated RNA was then resolved by electrophoresis in 1·2% agarose gels containing formaldehyde and transferred to a nylon membrane (Hybond N, Amersham International, Amersham, UK). RNA was also transferred to membranes using a standard dot blot procedure. An aliquot of 10 μg of the appropriate RNA sample was applied in triplicate to a nylon membrane using a dot blot manifold (Gibco) with vacuum pressure.

The filters were prehybridized and then hybridized with a single-stranded oligonucleotide probe cocktail specific for IL-8 or β-actin (British Biotechnology, Oxford, UK). The oligonucleotides were 5′ end labelled (5′ labelling kit, Amersham International), with γ-adenosine triphosphatase (γ-ATP) to a specific activity of $> 10^6$ c.p.m./ng DNA. The filters were subsequently hybridized with a 1·0 kilobase (kb) *Eco*RI fragment for IL-6 cDNA.[17] The cDNA probe was labelled by random priming with a α-cytidine triphosphate (α-CTP), using the megaprime labelling kit (Amersham International) to a specific activity of $> 10^6$ c.p.m./ng DNA. Non-specific labelling was then removed by repeated washing in decreased concentrations of SSC (0·3 M NaCl/30 mM sodium citrate, pH 7·0) at 42° and at 65° for the oligonucleotides and the cDNA respectively. The filters were exposed to autoradiography film (Hyperfilm, MC Amersham, UK) for 24 hr.

Quantification of mRNA

Each of the dot blot membranes of the test RNA were hybridized sequentially with IL-8, β-actin and IL-6 probes. After each hybridization had been carried out, the level of the radioactivity on the filters was determined using matrix 96 (Canberra, Packard, Pangbourne, UK). The IL-8/β-actin, IL-6/β-actin ratios were determined for each sample. The level of the respective mRNA in serum-free medium was compared to the level of mRNA obtained in cells which had been incubated in medium containing IL-1β or TGF-β alone or a combination of both these cytokines.

Cytokine bioassays

Prior to treatment with human cytokines, RPE cell cultures were rinsed and placed in fresh medium plus 0·2% bovine serum albumin (BSA). Assays were begun by overlaying RPE cultures with either 2 ml of medium alone (unstimulated control) or 2 ml of medium containing varying concentrations of TGF-β, IL-1β alone or a combination of these cytokines for 48 hr. Following experimental incubations, conditioned media were collected, centrifuged to remove particulates and stored at −70° until bioassay production was performed. Specific enzyme-linked immunosorbent assay (ELISA) kits were used for each cytokine: IL-6 (Eurogenetics, Teddington, UK); IL-8 (R&D Systems).

RESULTS

TGF-β and IL-1β enhance IL-6 and IL-8 mRNA levels in human RPE cells

Human unstimulated RPE cells express minimal amounts of IL-6 and IL-8 mRNA. When RPE cells were cultured for 4 hr in the presence of varying concentrations of TGF-β alone (0·1–50 ng/ml) or varying concentrations of IL-1β (5–500 U/ml) alone they exhibit a dose-dependent increase in IL-6 and IL-8 mRNA levels as determined by Northern blot analysis (Fig. 1). A 1·3 kb message and a 1·8 kb message was observed for IL-6 and IL-8 respectively. These messages were identical to those found in other cell types for IL-6 and IL-8.[18]

TGF-β and IL-1β act synergistically to induce IL-6 mRNA levels in human RPE cells

When both TGF-β and IL-1β were added to RPE cell cultures IL-6 mRNA levels were greater than those obtained with either TGF-β or IL-1 alone (Fig. 2).

The synergistic effect of TGF-β on IL-1β-induced IL-6 mRNA levels was observed over a range of TGF-β concentrations (0·1–10 ng/ml) and a range of IL-1β concentrations (5–500 U/ml). When human RPE cells were stimulated with 5, 50 and 500 U/ml of IL-1 alone for 4 hr, a three-, 10- and 14-fold increase in IL-6 mRNA respectively was observed compared to control values. TGF-β alone at most concentrations increased IL-6 mRNA levels by twofold (Fig. 2a).

In contrast the combination of 10 ng/ml of TGF-β to

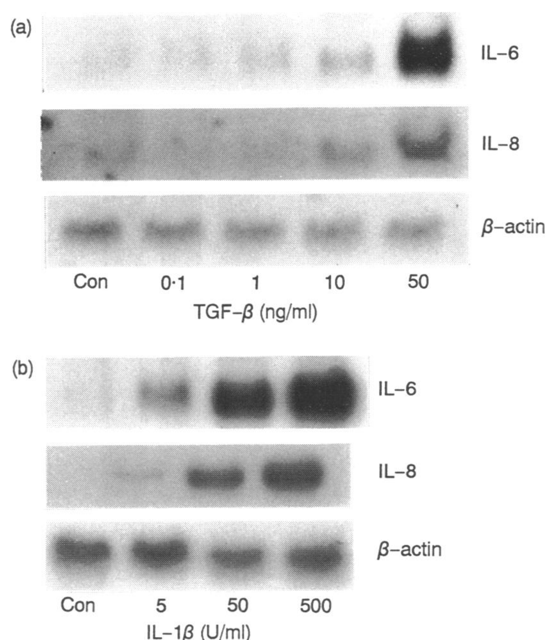

Figure 1. (a) Northern blot analysis of the effect of increasing concentrations of TGF-β on IL-6 and IL-8 mRNA levels in human RPE cells. (b) Northern blot analysis of the effect of increasing concentrations of IL-1β on IL-6 and IL-8 mRNA levels in human RPE cells. Cells were incubated in medium alone or in medium plus cytokines for 4 hr. The results are representative of two separate experiments.

cultures containing 5, 50, or 500 U/ml of IL-1 resulted on average in a 15-, 30- and 45-fold increase in IL-6 mRNA above control values which represents respectively a five- and threefold increase in the level of IL-6 mRNA when compared to cultures treated with IL-1 alone (Fig. 2a).

A synergistic effect was also found when TGF-β was added at 1 ng/ml to cultures containing 5, 50 or 500 U/ml of IL-1 with 4·4-, 14- and 15-fold increases in IL-6 mRNA being observed when compared to control values, which represent a 1·6-, 2·1- and 1·2-fold increase in the level of IL-6 mRNA when compared to cultures containing IL-1 alone (Fig. 2b).

Similarly when TGF-β was added at 0·1 ng/ml to cultures containing 5, 50 or 500 U/ml of IL-1β, the level of IL-6 mRNA increased from 4·6- to 14-fold above control values which represents a 3·5-, 2- and 2·3-fold increase in IL-6 mRNA levels when compared to cultures treated with IL-1 alone (Fig. 2c).

Effect of TGF-β and IL-1β on IL-8 mRNA levels in human RPE cells

TGF-β over a range of concentrations (0·1–10 ng/ml) was also found to have a modest synergistic effect on IL-1β induction of IL-8 mRNA levels in human RPE cells (Fig. 3). However unlike IL-6 mRNA this synergistic effect was noted at only one IL-1β concentration (50 U/ml) whereas TGF-β had no synergistic effect on IL-1β induction of IL-8 mRNA at higher (500 U/ml) IL-1β concentrations and little effect at lower (5 U/ml) IL-1β concentrations (Fig. 3).

Table 1. Effect of IL-1β and TGF-β on IL-6 production by human RPE cells

	TGF-β (ng/ml)			
	0	0·1	1	10
IL-1β (U/ml)	IL-6 (pg/ml)			
0	320	1200	1800	2000
5	5600	24 000	32 000	32 000
50	40 000	44 000	48 000	42 000
500	48 000	54 000	52 000	54 000

Human RPE cells 4 × 10^5/flask were cultured for 2 days in medium containing IL-1β or TGF-β alone, or a combination of both cytokines. The supernatants were then harvested and IL-6 content was determined by ELISA as described in the Materials and Methods. Results are expressed as mean values of triplicate samples. SD < 10% of the mean. The results are representative of three separate experiments.

The level of IL-8 mRNA was on average 20 times higher in cells stimulated with 50 U/ml of IL-1β when compared to control values (Fig. 3). The addition of TGF-β at 10 ng/ml and 0·1 ng/ml resulted in a further 1·5-fold increase in the level of IL-8 mRNA when compared to cultures containing IL-1β alone (Fig. 3a and c).

TGF-β and IL-1β act synergistically to enhance IL-6 production by human RPE cells

Both TGF-β and IL-1β alone were found to enhance IL-6 production by human RPE cells. IL-6 production increased in parallel with increasing concentrations of IL-1 (5–500 U/ml) or increasing concentrations of TGF-β (0·1–10 ng/ml) although IL-1 was a significantly more potent inducer of IL-6 than TGF-β (Table 1).

TGF-β synergized with IL-1 to enhance IL-6 secretion. This synergy was most apparent at low IL-1 concentrations (5 U/ml) with approximately four- to fivefold increases in IL-6 secretion being observed over a range of TGF-β concentrations (0·1–10 ng/ml) (Table 1).

However at higher IL-1 concentrations (50–500 U/ml) significantly more IL-6 was produced by IL-1 alone although a synergistic effect on increased IL-6 secretion was still observed when TGF-β (0·1–10 ng/ml) was added (Table 1).

Effect of TGF-β on IL-1 induction of IL-8 production by human RPE cells

TGF-β alone appeared to suppress low levels of constitutive IL-8 production observed in human RPE cells. IL-1 produced a dose-dependent increase in IL-8 production. However the high levels of IL-8 observed after IL-1 stimulation were not increased further after the addition of TGF-β (Table 2).

Low levels of TGF-β (0·1 ng/ml) were found to suppress IL-8 production when low IL-1 (5 U/ml) levels were used. This suppression was not apparent at higher IL-1 or TGF-β concentrations (Table 2).

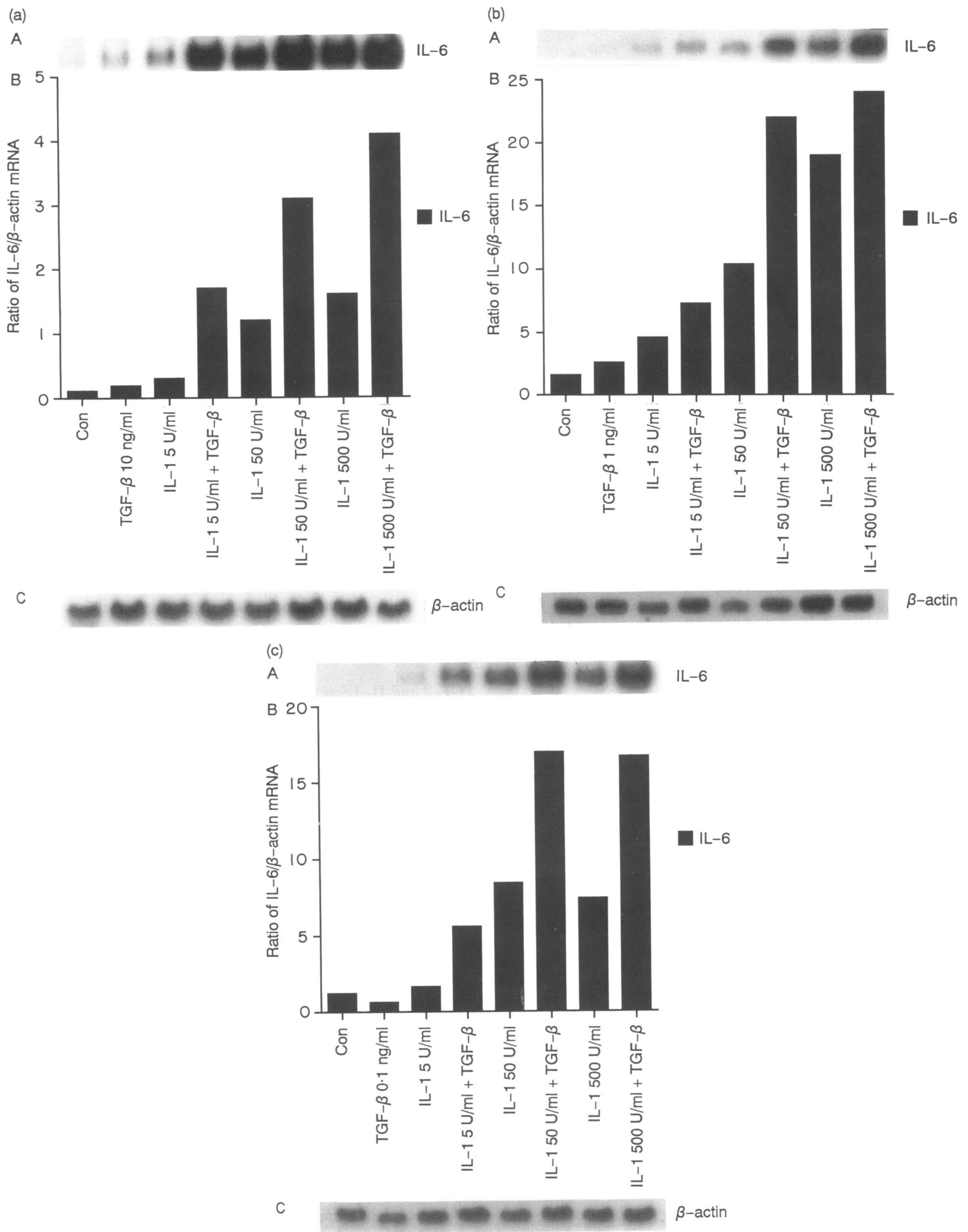

Figure 2. Northern blot analysis of the synergistic effect of TGF-β on IL-1β induction of IL-6 mRNA in human RPE cells. Cells were incubated for 4 hr in medium containing (a) TGF-β (10 ng/ml) or (b) TGF-β (1 ng/ml) or (c) TGF-β (0·1 ng/ml) with or without IL-1β (5–500 U/ml) Cells were also incubated in medium alone or medium plus IL-1β alone (5–500 U/ml). (A) Northern blot analysis showing IL-6-specific transcripts. (B) Quantitative analysis of IL-6 mRNA levels. Results are expressed as mean values of triplicate samples. SD $<$ 15%. (C) β-actin-specific transcripts that corresponded to the Northern blot in (A). Results are representative of three separate experiments.

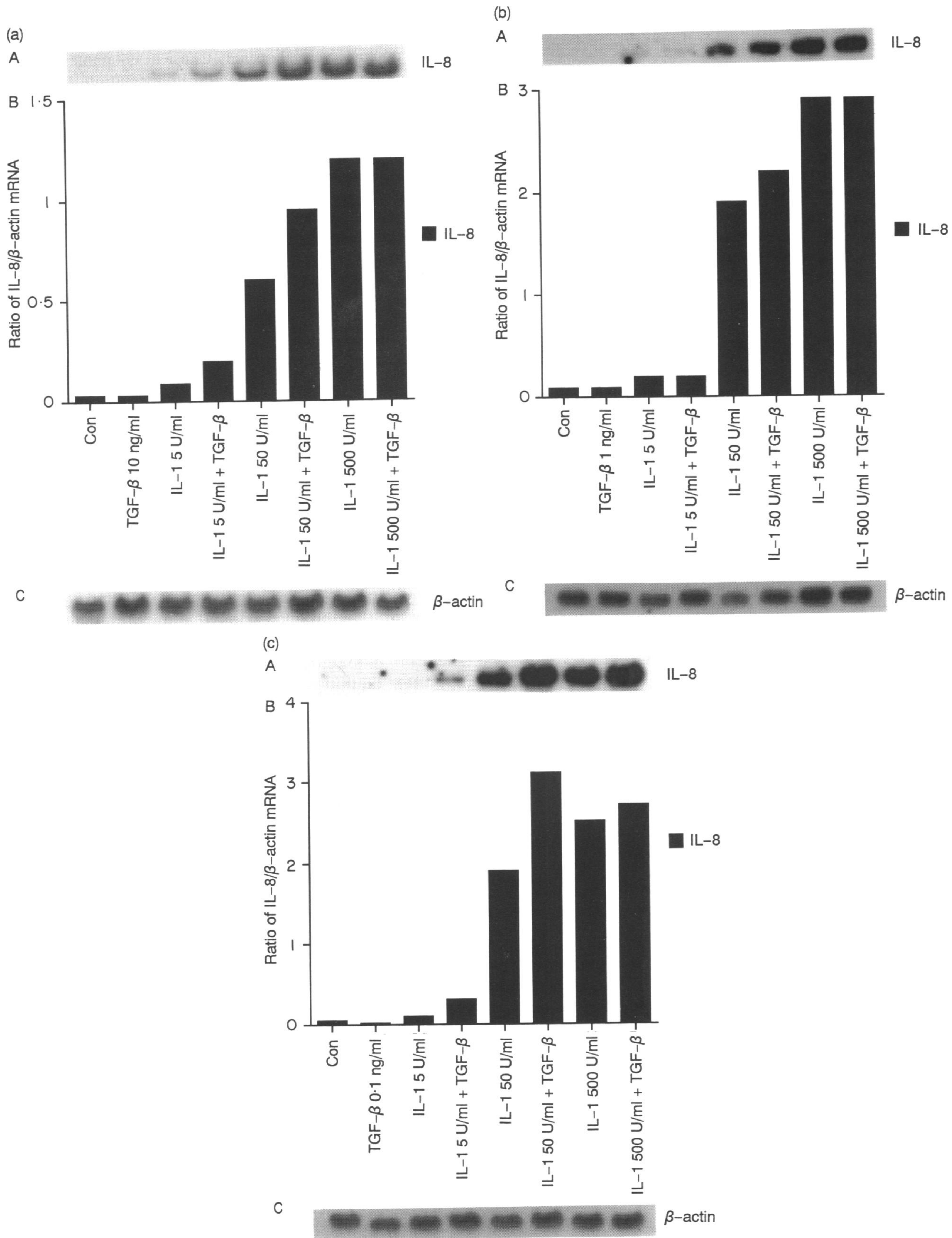

Figure 3. Northern blot analysis of the synergistic effect of TGF-β on IL-1β induction of IL-8 mRNA in human RPE cells. Cells were incubated for 4 hr in medium containing (a) TGF-β (10 ng/ml) or (b) TGF-β (1 ng/ml) or (c) TGF-β (0·1 ng/ml) with or without IL-1β (5–500 U/ml). Cells were also incubated in medium alone or medium plus IL-1β alone (5–500 U/ml). (A) Northern blot analysis showing IL-8-specific transcripts. (B) Quantitative analysis of IL-8 mRNA levels. Results are expressed as mean values of triplicate samples. SD < 15%. (C) β-actin-specific transcripts that correspond to the Northern blot in (A). Results are representative of three separate experiments.

Table 2. Effect of IL-1β and TGF-β on IL-8 production by human RPE cells

IL-1β (U/ml)	TGF-β (ng/ml)			
	0	0·1	1	10
	IL-8 (pg/ml)			
0	950	180	146	100
5	20 000	7200	20 000	18 000
50	128 000	108 000	140 000	120 000
500	200 000	200 000	200 000	200 000

Human RPE cells 4×10^5/flask were cultured for 2 days in medium containing IL-1β or TGF-β alone, or a combination of both cytokines. The supernatants were then harvested and IL-8 content determined by ELISA as described in the Materials and Methods. Results are expressed as mean values of triplicate samples. SD < 10% of the mean. The results are representative of three separate experiments.

DISCUSSION

Several studies have suggested that cytokines play an important role in the pathogenesis of intraocular inflammation.[19] The source of cytokine production is clearly important to the response. Thus an ocular inflammatory response may be influenced by cytokines released by resident ocular cells in addition to infiltrating inflammatory cells.

IL-1 plays a central role in the induction of an inflammatory response and is produced by many cell types including macrophages, fibroblasts endothelial cells[20] and specific eye-derived cells such as Muller cells.[3] IL-1 is also known to invoke an inflammatory response when injected intravitreally.[7] RPE cells produce IL-6 and IL-8 after *in vitro* stimulation with IL-1β.[1,2]

IL-6 is a multifunctional cytokine involved in the regulation of inflammatory and immunological responses.[21] Its functions include the enhancement of T-lymphocyte responses and the induction of T- and B-cell differentiation.[21] It is also thought to function as an overall activation signal that recruits diverse host defence mechanisms that serve to limit tissue injury.[22] Elevated levels of IL-6 have been detected in the vitreous of patients with immunoinflammatory disorders.[23] IL-8 is a well-defined chemotactic factor for neutrophils and lymphocytes.[5]

Both IL-6[6] and IL-8[7] also cause an inflammatory response when injected intravitreally. However in the case of IL-6 it has yet to be determined whether it exacerbates ocular inflammation or has a protective role. Several studies support the suggestion that it may have a protective role as IL-6 has been shown to inhibit T-cell responses via induction (through macrophages) of TGF-β[24] and repeated intraocular injections of IL-6 are known to result in ocular unresponsiveness to LPS in rats.[25]

TGF-β is a homo-dimeric peptide that is expressed in various parts of the eye[8] and has been found to play an important role in the maintenance of the immunosuppressive ocular environment.[9] We have also observed that cultured RPE cells secrete low levels of active TGF-β (our unpublished observations).

However in addition to its immunosuppressive effects TGF-β has potent, although usually transient inflammatory properties.[10] This cytokine promotes inflammation through the recruitment of monocytes and the induction of IL-1 and other cytokines.[10] It can inhibit IL-1 induction of IL-6 in monocytes[11] but synergizes with IL-1 to enhance IL-6 production in fibroblasts[12] and intestinal epithelial cells.[13]

However the extent of the synergy between IL-1 and TGF-β depends on the cell type as in fibroblasts. TGF-β enhances IL-1 induction of IL-6 expression at low IL-1 concentrations but inhibits IL-6 production when higher concentrations of IL-1 are used.[12] Human RPE cells appear to behave in a similar manner to epithelial cells from other sources such as the intestine[13] in that TGF-β enhances IL-1 induction of IL-6 at high or low IL-1 concentrations. The present study has shown in more detail that this synergy is directly reflected at the level of IL-6 mRNA.

Although TGF-β appears to have no effect on IL-1 induction of IL-8 production by colonic epithelial cells[14] the influence of TGF-β on IL-1 induction of IL-8 mRNA was not reported in this study.

Using human RPE cells we have found that TGF-β has a synergistic effect on IL-1 induction of IL-8 mRNA levels but this effect is less pronounced than that observed with the level of IL-6 mRNA and occurs within a narrower IL-1 concentration range with no synergistic effect being observed at high IL-1 concentrations. This synergistic effect is not reflected at the levels of IL-8 production as IL-1 induces significant IL-8 production from human RPE cells, which is unaffected by the addition of TGF-β, suggesting that TGF-β does not affect IL-1 receptor expression.

Recent studies have shown that interferon-γ (IFN-γ) can synergistically enhance TNF-α-induced IL-8 production in a human gastric cancer cell line[26] through synergistic activation of transcription factors without up-regulating the TNF-α receptor. This is in contrast to reports on the synergistic action of TGF-β and IL-1 on IL-6 production which have suggested that TGF-β makes the cells more responsive to IL-1 by up-regulating the expression of the IL-1 receptor.[13]

Studies with colonic epithelial cells also revealed a contrast between the stimulus dose-dependent IL-8 release and a non-dose-related appearance of IL-8 mRNA[14] suggesting that IL-8 expression is controlled by both transcriptional and translational factors.

The results of the present study suggest that the RPE cells play an important role in ocular inflammatory response by regulating the expression of cytokines such as IL-6 and IL-8 at both transcriptional and translational levels. Recent studies have indicated that the activity of IL-1 is regulated by an endogenous peptide that competes with IL-1 for binding to a specific receptor.[27] This IL-1 receptor antagonist (IL-1RA) is synthesized by a variety of cells including macrophages.[28]

Although RPE cells do not actively secrete IL-1 they have been shown to express an intracellular (i.c.) IL-1RA.[29] In addition intravitreal injection of the IL-1RA is very effective in reducing IL-1-induced inflammation in the rabbit eye.[30] TGF-β has been shown to sequentially induce IL-1 and an IL-1RA in monocytes[28] thus down-regulating the initial inflammatory response. Within the ocular environment TGF-β may also be able to amplify an inflammatory response initially by synergizing with cytokines such as IL-1 and then subsequently

down-regulate the expression of IL-1-induced cytokines such as IL-6 and IL-8 by stimulating the production of an IL-1RA. During the initial response however the increased production of IL-6, stimulated by IL-1 and TGF-β, may itself serve to limit the damage caused by the host response. Therefore RPE cells may make a significant contribution to redress the balance of normal cytokine homeostasis after an initial inflammatory response within the eye.

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