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The effect of interleukin-1 on cytokine gene expression by human corneal epithelial cells

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Abstract:

The purpose of this study was to characterize the pattern of cytokine gene expression by human corneal epithelial cells (HCEC) in response to interleukin-1 (IL-1). Primary cultured HCEC (P-HCEC) or SV40 transformed HCEC (SV40-HCEC) were treated for 6 hr with serum-free growth-media alone or with recombinant human IL-1β or IL-1α (10 ng ml^{-1}). ^{33}P labeled cDNA was generated from total RNA, then hybridized to a human cytokine expression array. An autoradiograph was generated for each experimental condition and results analysed semi-quantitatively. Reverse transcription polymerase chain reaction (RT-PCR) was performed to detect mRNA for IL-8, growth related oncogene-β (GRO-β), intercellular adhesion molecule (ICAM)-1 and Ephrin A5. P-HCEC and SV40-HCEC demonstrated comparable cytokine profiles. For P-HCEC ($n=2$) the expression of 35 genes was upregulated or only detectable following IL-1β treatment whereas the expression of nine genes was downregulated or undetectable after IL-1β treatment. In SV40-HCEC ($n=3$), the expression of 48 genes was upregulated or only detectable following IL-1β treatment and the expression of 10 genes was downregulated or undetectable after IL-1β treatment. Some genes that demonstrated increased expression included cadherin-5, ICAM-1, GRO-α, GRO-β, GRO-γ, Activin A (bA subunit), tumor necrosis factor-α, IL-6, and IL-8. Genes that showed decreased expression included the chemokine receptor—CXCR-4, ciliary neurotrophic factor (CNTF), c-kit ligand, Ephrin A5, G-protein coupled receptor RDC-1 and FGF family FGFR2. Bayesian analysis of the SV40-HCEC data ($n=3$) revealed the expression of 15 genes that were significantly ($p<0{\cdot}05$) differentially regulated. Within these 15 genes, the expression of chemokines (GRO-α, GRO-β, IL-8), fibroblast growth factor 13 and the cytokine IL-6 were the most upregulated, while ephrin A5 and chemokine receptor-4 were the most downregulated. IL-1α treatment ($n=1$ P-HCEC; $n=1$ SV40-HCEC) produced results very similar to IL-1β treatment. RT-PCR revealed differential regulation of IL-8, GRO-β, ICAM-1 and ephrin A5 in accordance with gene array data. In conclusion, the data demonstrate that IL-1 treatment of HCEC differentially regulates the expression of other cytokine and related genes, thus adding to the body of evidence that IL-1 is a major mediator of ocular surface inflammatory reactions. Since the expression of a large number of genes can be studied simultaneously, gene array studies such as these offers the advantage of understanding global changes in response to a specific stimulus. Thus our study provides insight in to the ocular surface response in conditions of inflammation and corneal wound healing where the levels of IL-1 are known to be increased.

Keywords: cornea; epithelial cells; gene expression; interleukin-1; cytokines; inflammation; ocular surface; cell culture

1. Introduction

The interleukin-1 (IL-1) family of genes comprises two proinflammatory forms (IL-1α and IL-1β) and one naturally occurring anti-inflammatory form, IL-1 receptor antagonist (IL-1Ra) (Dinarello, 1998). IL-1 is produced by a variety of cell types including macrophages, monocytes, neutrophils, lymphocytes, and epithelial cells and can exert an effect on almost every cell type of the body through binding to the type 1 IL-1-receptor (IL-1R1) (Dinarello, 1996, 1998).

The proinflammatory forms of IL-1 play significant roles in ocular surface immune and inflammatory responses and wound healing. IL-1 and its receptor are constitutively expressed by the corneal epithelium (Wilson et al., 2001). Keratocytes also constitutively express IL-1R, whereas

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production of IL-1 by these cells is stimulated by an autocrine loop after injury (West-Mays et al., 1995; Wilson et al., 2001). IL-1β regulates the secretion of chemotactic cytokines such as IL-6 and IL-8 by human corneal epithelial cells (HCEC) in the event of a microbial challenge (Xue et al., 2001) and thus promotes the initial response of HCEC to microbial invasion. IL-1β may further enhance ocular surface antimicrobial protection by inducing the expression of antimicrobial peptides such as human β-defensin-2 and cationic antimicrobial peptide-37 by corneal (McDermott et al., 2003; Ruan et al., 2002) and conjunctival (Narayanan et al., 2003) epithelial cells. IL-1 has been described as the 'master regulator' of corneal wound healing (Wilson et al., 1999). This cytokine modulates keratocyte apoptosis, has a negative chemotactic effect on keratocytes (Wilson et al., 1996) and stimulates corneal fibroblast production of hepatocyte growth factor and keratinocyte growth factor (Weng et al., 1997). These growth factors in turn regulate corneal epithelial proliferation, migration and differentiation (Wilson et al., 1994). IL-1 also stimulates keratocyte expression of enzymes such as matrix metalloproteinases suggesting a role for the cytokine in remodeling during wound healing (Girard et al., 1991). The cytokine also stimulates keratocyte expression of a variety of chemokines, proteases and transcription factors, several of which are important during wound healing (Mahajan et al., 2002). Though IL-1 plays important roles in acute corneal inflammation and wound healing, increased levels of this cytokine at the ocular surface are thought to contribute to the epithelial damage observed in patients with aqueous-deficient or evaporative dry eye (Solomon et al., 2001), ultraviolet-light induced inflammation (Kennedy et al., 1997), bullous keratopathy (Rosenbaum et al., 1995), alkali injuries (Planck et al., 1997; Sotozono et al., 1997) and keratoconus (Becker et al., 1995).

Discovering the effect of IL-1 on corneal epithelial cell gene expression will help clarify specific mechanisms associated with inflammatory processes and diseases of the ocular surface in which this cytokine plays a major role. The current study used gene array technology to investigate the simultaneous expression of cytokines and related genes in human corneal epithelial cells in response to IL-1β or IL-1α. Some of these results have been presented in preliminary form (Narayanan S and McDermott AM, ARVO Abstract #3201, 2002).

2. Materials and methods

2.1. Cell culture

Cell culture reagents were from Invitrogen (Carlsbad, CA), unless otherwise stated. Two human corneas unsuitable for transplantation were obtained from eye banks. The tissues were obtained in accordance with the guidelines of the Declaration of Helsinki regarding research involving human tissue. The donors were 71 and 67 years of age. Primary cultured corneal epithelial cells (P-HCEC) were grown in EpiLife™ media with Human Corneal Growth Supplement (HCGS)™ (Cascade Biologics, Portland, OR) on FNC-coated (Athena ES, Baltimore, MD) plates (McDermott et al., 2003). The cells grew to confluency by one week and were then passed using trypsin–EDTA. Cells of passage 1–2 were used. P-HCEC stained positively for the corneal epithelial-specific 64 kDa cytokeratin-3 (data not shown).

SV40-transformed HCEC (SV40-HCEC; passages 19, 22 and 26) (Araki-Sasaki et al., 1995) were cultured with Ham's F12 and Dulbecco's minimum essential medium (1:1 v/v) containing 0·5% dimethylsulphoxide, 10% fetal bovine serum and gentamicin (30 μg ml^{-1}).

2.2. Cytokine treatment of corneal epithelial cells

P-HCEC and SV40-HCEC were grown to approximately 80% confluency in 25 cm^2 tissue culture flasks, then placed in HCGS-free (P-HCEC) or serum-free (SV40-HCEC) media overnight. The cells were then treated with HCGS/serum-free growth media alone or with 10 ng ml^{-1} recombinant human IL-1α or IL-1β (R&D Systems, Minneapolis, MN) for 6 hr. Cells were trypsinized and pelleted by centrifugation. Cell pellets were stored in 350 μl Qiagen lysis buffer at −80°C. The short treatment period (6 hr) was chosen to avoid secondary effects such as de novo secretion of other molecules that may alter cytokine expression. Six hours was adequate for IL-1 to induce the expression of certain genes of interest in earlier studies (Cubitt et al., 1995; Li de et al., 2003; McDermott et al., 2003; Narayanan et al., 2003). The expression of genes that peaked significantly before or after the treatment period would not have been detected in our experiments.

2.3. Human cytokine expression array

A human cytokine expression array (R&D Systems) was used to detect cytokine gene expression. The array contained cloned cDNA's (10 ng per spot) printed as PCR products onto a nylon membrane. The experiments were initially done on arrays containing 390 genes. Repeat experiments were done on arrays with 862 genes since the manufacturer increased the number of genes tested on the array. Both versions contained nine positive and five negative controls. The genes on both versions of the arrays could be grouped into 27 families of cytokine genes. The procedure is briefly described below; full details are available from the manufacturer.

RNA extraction was performed using an RNeasy mini-kit (Qiagen, Valencia, CA). During the extraction, RNase-free DNase (Qiagen) was used to eliminate genomic DNA. Radiolabeled cDNA was generated by reverse transcription using 4 μg of total RNA, human cytokine gene specific primers (R&D Systems) and 40 μCi of ^{33}P-α-deoxycytidine

5′ triphosphate (2000 Ci mmol^{-1}, Perkin Elmer Life Sciences, Boston, MA). The use of a ^{33}P isotope helped to avoid the 'bloom' effect which occurs in gene arrays hybridized with ^{32}P radio-labeled cDNA. Unincorporated radioactive nucleotides were separated from incorporated nucleotides by spin column purification. The percentage of incorporation, which varied between 19 and 24%, was assessed by liquid scintillation counting. The arrays were pre-hybridized at 65°C in hybridization solution containing salmon testes DNA (100 μg ml^{-1}; Sigma, St. Louis, MO). The arrays were then hybridized overnight with the radiolabeled cDNA at an average final concentration of 6×10^6 cpm ml^{-1}. The arrays were washed in solutions containing differing concentrations of saline–sodium phosphate–EDTA buffer with sodium dodecyl sulfate, wrapped in clear plastic film and subjected to autoradiography (Kodak BioMax MS X-ray film; Kodak, Rochester,NY) for 3 days. Films were developed using a Kodak RP X-OMAT processor.

2.4. Image and data analysis

Autoradiographs were scanned on a desktop scanner (HP4470c, Hewlett Packard, Palo Alto, CA) at identical settings to obtain digital images. A Gene Array Macro (GAM) was developed for Optimas image analysis software (Version 6.5; Media Cybernetics, Silver Spring, MD), to convert the digital images into a matrix of grayscale intensities. GAM calculated the grayscale values ranging from 0 (for white) to 255 (for black) of each spot, where a product may be expected on the gene array. Additionally, to calculate the average background intensity across the whole array, the program also determined grayscale values of 230 empty spots, where no gene product was present.

Since GAM was a novel program, we initially established repeatability and detection limits with respect to the images analysed. We tested the ability of GAM to provide repeatable data for autoradiographs that were scanned on more than one occasion, the effect of rotating the same autoradiograph prior to scanning and the effect of rotating the same image post-scanning. All of the above testing showed very high correlations ($r^2=0{\cdot}99$), which verified that GAM provides excellent repeatability for the conditions mentioned. To establish a detection limit, we determined the difference in background-subtracted intensities of each gene following repeated measurement of an image with GAM. The mean difference of the subtracted intensities was 0·02–0·17 (95% confidence intervals from 0·07 to 0·14). Based on the intensities of a small number of outliers (data not shown) in the above measures we established a conservative detection limit of five grayscale values for the GAM program.

The GAM output was in the form of a Microsoft Excel-based spreadsheet. Mean background intensity sampled from the 230 empty spots on the scanned image was subtracted from the grayscale values of each gene on the scanned image. A further 10 grayscale values were subtracted from the intensities of each gene. These additional 10 grayscale units represent twice the detection limit as well as at least two standard deviations of the mean background of all experiments. Any gene which had a grayscale intensity value greater than zero following these corrections was used for further analysis. The intensity of expression of these genes on the arrays of the IL-1 treated cells was multiplied by a factor equal to the ratio of the mean background between the two (media and IL-1) arrays to equalize for any background variation. The data were expressed as the ratio of the background-corrected grayscale value of a gene in the IL-1 treated array to that of the same gene in the media-treated array. For upregulated genes, this ratio was a positive number greater than 1 and was used as the upregulation value for the gene concerned. For downregulated genes, which had ratios between 0 and 1, the negative inverse of the ratio was used to determine the downregulation value. The expression of certain genes was only detectable or became undetectable following IL-1β treatment. In such cases, the absolute grayscale value was used to describe the data (a positive number for genes expressed only after IL-1β treatment and a negative number for genes whose expression was undetectable following IL-1β treatment). In all conditions, only data from genes that demonstrated consistent changes in the same direction for all SV40-HCEC experiments are shown.

A global normalization of the SV40-HCEC data was performed prior to further statistical analysis. For the global normalization, we first corrected the data for mean background and detection limit, and then scaled the intensities of each gene by dividing by the corresponding median intensity of all genes on each array. A natural logarithm transformation of the intensity data was then applied to study the log fold-change in gene expression (Minitab Software Version 12, Minitab Inc., State College, PA). The log fold-change data demonstrated moderate to excellent repeatability (r^2 range 0·60–0·90) between SV40-HCEC experiments. The combined log fold-change data (not shown) was uni-modal, symmetric, and centered at 0 ($p=0{\cdot}89$, t-test). This demonstrated that the expression of many genes remained relatively unchanged following IL-1 treatment. However, the log fold-change data were not normally distributed due to higher kurtosis ($p=0$, normality tests). Based on simulation, a $t4$-distribution (t-distribution with four degrees of freedom) fitting was applied to the combined data. Genes with log fold-changes outside the $t_{4,0{\cdot}025}$ or $t_{4,0{\cdot}975}$ limits (which was equivalent to 0·0623 or 16·056 in fold-change) and which consistently responded across experiments, were chosen for significance analysis. The expression of fifteen genes was found to have log fold-changes equal to or more extreme than these limits and these genes were selected for significance analysis using Bayesian probability intervals (Casella and Berger, 2002).

2.5. Reverse transcription polymerase chain reaction:

250 ng of RNA was used in each reaction in one-step RT-PCR (Superscript I kit; Invitrogen). Reverse transcription was performed at 50°C for 60 min. Following denaturation of the enzyme (94°C, 5 min), amplification of cDNA was performed for 25 cycles (ephrin A5) or 28 cycles (IL-8, intercellular adhesion molecule-1 [ICAM-1] and growth related oncogene-β [GRO-β]) as follows: denaturation 94°C for 1 min, annealing 60°C for 1 min, and extension 72°C for 1 min. β-Actin was used as an internal control to ensure equal amounts of starting RNA were added to each reaction. Primer sequences used and expected product sizes were as follows: β-actin—Forward 5′ CCTCGCCTTTGCCGATCC 3′; Reverse 5′GGATCTTCATGAGGTAGTCAGTC 3′ 626 bp; IL-8 (Xue et al., 2001)—Forward: 5′ CTTGGCAGCCTTC-CTGATTT 3′; Reverse: 5′ CAGCCCTCTTCAAAAACTTC 3′; 229 bp; ICAM-1 (Takizawa et al., 2000)—Forward: 5′ TATGGCAACGACTCCTTCT 3′; Reverse: 5′ CATT-CAGCGTCACCTTGG 3′; 202 bp; GRO-β—Forward: 5′ CTCAAGAATGGGCAGAAAGC 3′; Reverse: 5′ TCAAA-CACATTAGGCGCAAG 3′; 214 bp; Ephrin A-5 (Varelias et al., 2002)—Forward: 5′ CTACGCTGTCTACTGGAA-CAGC 3′; Reverse: 5′ GACTCATGTACGGTGTCATCTGC 3′ 458 bp. Products were analysed on ethidium-bromide stained 1·3% agarose gels. Digital images were captured on an Alpha-imager (Alpha Innotech, San Leandro, CA) gel-documentation system. The PCR products were sequenced (Seqwright, Houston, TX) in order to confirm their identity. Controls in which the nucleic acid or Reverse Transcriptase was omitted were also performed, in which case no PCR product was obtained (data not shown).

3. Results

3.1. Human cytokine expression arrays—IL-1β treatment of HCEC

Autoradiographs from representative arrays are shown in Fig. 1. Areas in which changes in gene expression are readily visible between the media-treated cells (Fig. 1) control and the IL-1β treated cells control have been marked.

Each array included nine constitutively expressed positive control genes, which were not expected to change with treatment or between experiments. Fig. 2 demonstrates the mean (±S.E.M.) ratios of IL-1β: media treatment of these nine genes from experiments performed on SV40-HCEC. The ratios were very close to the expected value of 1, with the exception of Transferrin-R (1·42±0·4). Similar results were obtained with P-HCEC (not shown).

Table 1 shows various genes whose expression was only detectable or was no longer detectable following IL-1β treatment. The data are the grayscale intensity of gene expression. Positive numbers indicate the expression of genes that were detectable following IL-1β treatment; negative numbers indicate the expression of genes that were undetectable following treatment. It is evident from the table that some genes such as the adhesion molecule cadherin-5 (CAD-5), the chemokines GRO-α, GRO-β, GRO-γ, IL-8 and monocyte chemotactic protein (MCP)-1, the cytokines IL-1α, IL-1β and IL-10 were expressed at a greater intensity than other genes in SV40 and P-HCEC. Interestingly, some genes such as ICAM-1, MCP-2, MCP-3, IL-6, integrin α-4 and tumor necrosis factor-α (TNF-α) were expressed in SV40-HCEC after IL-1β treatment, but not in P-HCEC. Genes that were expressed only in the media-treated control cells (i.e. undetectable following IL-1β treatment) included: the chemokine receptor—CXCR-4, ciliary neurotrophic factor (CNTF) and the G-protein coupled receptor RDC-1.

Table 2 shows the genes whose expression was modulated (upregulated or downregulated) by IL-1β. The data are expressed as the ratio of the intensities of IL-1β: media treatment of various genes expressed by P-HCEC and SV40-HCEC. In general, SV40-HCEC demonstrated greater changes in the intensities of expression of various genes compared to P-HCEC. Notably the expression of integrin β-6 and Activin A (βA subunit) was increased considerably, especially in SV40-HCEC following treatment with IL-1β. The expression of platelet-derived growth factor-alpha chain (PDGF A), Ephrin A5 and FGF family

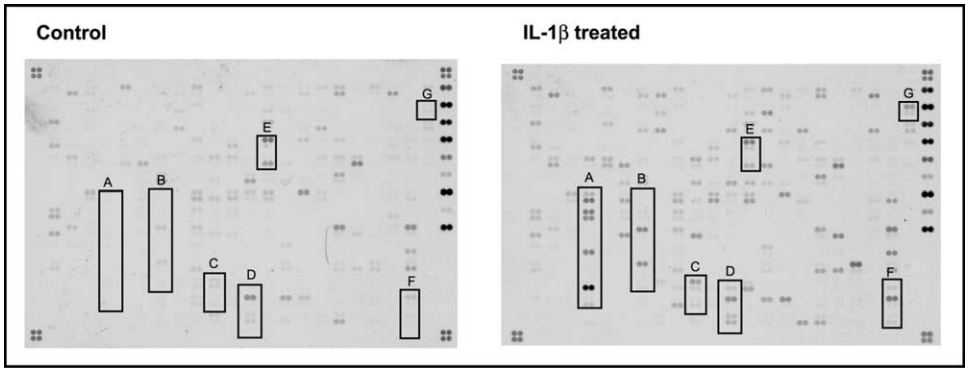

Fig. 1. Cytokine gene expression by SV40-HCEC. IL-1β treatment of SV40-HCEC differentially regulated the expression of several cytokine and related genes. The areas marked on the images are: A—chemokines; B—chemokine receptors; C—Ephrins; D and E—integrins; F and G—TNF superfamily. The left panel demonstrates the array of the media-treated control cells while the right panel shows the array of the IL-1β treated cells.

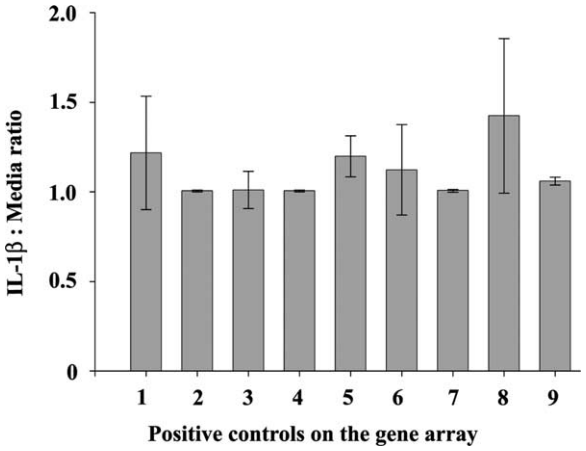

Fig. 2. Expression of positive control genes on the gene array. Nine genes that are constitutively expressed by human cells were denoted as positive controls on the gene array. The data are the mean ± SEM of the IL-1β: media treatment ratios for the nine positive control genes from three SV40-HCEC experiments. 1 = β-2 microglobulin, 2 = β-actin, 3 = Cylophilin A, 4 = GAPDH, 5 = HLA-A0201, 6 = HPRT, 7 = L-19, 8 = Transferrin R, 9 = α-Tubulin.

FGFR2 were downregulated in SV40 and P-HCEC following treatment with IL-1β. The expression of certain genes such as Ephrin A1, CD40, TNF-related apoptosis inducing ligand (TRAIL) and insulin-like growth factor-II (IGF-II) was not detected in P-HCEC but was present in basal conditions and upregulated by IL-1β treatment of SV40-HCEC.

Differences in gene expression between the two primary cultured corneas were also noted. Increased expression of certain genes such as MCP-1, macrophage inflammatory protein (MIP)-3α, RANTES, growth-arrest specific-6 (GAS-6), transforming growth factor-α (TGF-α), and matrix metalloproteinase-13 (MMP-13) was noted in the P-HCEC cultured from donor no. 1 but not detected in cells from donor no. 2. Other genes such as CAD-5, IL-12, IL-10, FGF-13 and inducible nitric oxide synthase (iNOS) were increased in expression in P-HCEC from donor no. 2 but not detected in donor no. 1. In some experiments, SV40 ($n=1$) and P-HCEC ($n=1$) were treated with IL-1α instead of IL-1β. The results (not presented) showed that IL-1α treatment induced a pattern of cytokine gene expression comparable to that of IL-1β treatment.

Following logarithmic transformation of the SV40-HCEC data and Bayesian probability analysis, the expression of 15 genes was found to be significantly modulated ($p<0{\cdot}05$) in IL-1β treated cells. The genes with statistically significant increased expression in IL-1β treated cells were: adhesion molecule—CAD-5; chemokines—GRO-α, GRO-β, GRO-γ, IL-8; cytokines—IL-1α, IL-1β, IL-6; FGF-13; TNF-family—TRAIL. The genes with statistically significant decreased expression were: chemokine receptor—CXCR4; cytokines—c-kit ligand, CNTF; Ephrin A5; G-protein coupled receptor—RDC-1.

Table 1
The expression of cytokine genes that were only detectable or were no longer detectable following IL-1β treatment of HCEC

Gene families and genes	P-HCEC no. 1	P-HCEC no. 2	SV40 HCEC (mean ± SE)
Adhesion molecules			
CAD-5	0·00	4·04	$27{\cdot}2\pm1{\cdot}7^{++}$
ICAM-1 (CD54)	0·00	0·00	24·9 ± 11·3
Chemokines and receptors			
GRO-α(CXCL-1)	31·45	7·12	$114{\cdot}8\pm12{\cdot}9^{++}$
GRO-β(CXCL-2)	34·09	8·81	$111{\cdot}2\pm14{\cdot}5^{++}$
GRO-γ(CXCL-3)	32·32	7·73	$107{\cdot}8\pm18{\cdot}5^{++}$
ENA-78 (CXCL-5)	3·49	3·73	1·05 ± 0·7
MCP-1 (CCL-2)	9·58	0·00	69·7 ± 29·3
MCP-2 (CCL-8)	0·00	0·00	87·4 ± 47·9
MCP-3 (CCL-7)	0·00	0·00	47·8 ± 12·9
MIP-3α(CCL-3)	4·30	0·00	22·6 ± 7·9
IL-8 (CXCL-8)	12·66	4·21	$111{\cdot}4\pm15{\cdot}6^{++}$
Rantes (CCL-5)	4·73	0·00	15·3 ± 2·16
CXCR-4	0·77	0·00	$-1{\cdot}8\pm0{\cdot}8^{--}$
c-Kit ligand	**	**	$-4{\cdot}0\pm2{\cdot}6^{--}$
Cytokines and receptors			
IL-1 α	21·51	11·57	$40{\cdot}83\pm16{\cdot}7^{++}$
IL-12 p35	0·00	20·29	7·59 ± 5·3
IL-1β	23·91	1·69	$46{\cdot}94\pm19{\cdot}2^{++}$
IL-10	0·00	4·77	18·93 ± 13·4
IL-6	0·00	0·00	$32{\cdot}97\pm7{\cdot}8^{++}$
IL-16	0·00	5·70	12·82 ± 9·0
IL-4 Rα	2·86	0·00	0·51 ± 0·8
GAS6	2·1	0·00	16·1 ± 0·88
EGF family			
TGF-α	2·53	3·09	17·29 ± 9·7
FGF family			
FGF-13	0·00	24·69	$9{\cdot}42\pm6{\cdot}7^{++}$
G-protein coupled receptors			
RDC-1	**	**	$-1{\cdot}7\pm1{\cdot}4^{--}$
Integrins			
Integrin α-4	0·00	0·00	128 ± 15·0
Neurotrophic group			
Neuropilin-1	1·26	3·13	10·02 ± 3·6
CNTF	−1·50	0·00	$-3{\cdot}8\pm2{\cdot}4^{--}$
Nitric oxide metabolism			
INOS	0·00	59·92	12·45 ± 8·8
TNF superfamily			
CD40	1·1	5·00	3·1 ± 1·6
TNF-α	0·00	0·00	9·67 ± 2·5

P-HCEC ($n=2$) or SV40-HCEC ($n=3$) were treated with serum-free growth media or with 10 ng/ml IL-1β for 6 hr. The table identifies the genes that were either only detectable or were no longer detectable following IL-1β treatment. The table provides data of the grayscale intensity of gene expression following correction for the background and the detection limit (positive values when genes were detectable and negative values when genes were no longer detectable after IL-1β treatment). The value 0·00 indicates a gene that was not detected in the experiment concerned. **c-Kit ligand and RDC-1 were no longer detectable following IL-1β treatment of SV40-HCEC but were downregulated in P-HCEC and the data for their expression in P-HCEC is shown in Table 2. ++Represents statistically significant ($p<0{\cdot}05$) increase, whereas −−represents a statistically significant decrease in gene expression following a Bayesian analysis of the data.

Table 2
IL-1β treatment upregulates or downregulates the expression of a variety of cytokines by HCEC

Gene families and genes	P-HCEC no. 1	P-HCEC no. 2	SV40-HCEC (mean ± S.E.)
Adhesion molecules			
P-Cadherin	1·86	1·23	1·2 ± 0·02
BCAM (CD 239)	1·09	34·81	−1·1 ± 0·06
ALCAM (CD 166)	1·57	−1·44	1·5 ± 0·22
CAD-8	2·33	7·52	1·4 ± 0·58
Binding proteins			
Follistatin	1·02	1·09	5·2 ± 1·8
Cytokines and receptors			
IGF-II	0·00	0·00	2·3 ± 0·2
IFN-γ R1	2·17	0·00	4·4 ± 1·6
PBEF	1·75	1·16	9·7 ± 4
c-Kit ligand	−1·44	−2·1	see Table 1
PDGF-A	−2·3	−2·9	−17·8 ± 12
Ephrins			
EphB2	0·00	−1·16	2·81 ± 0·5
Ephrin-A1	0·00	0·00	88·67 ± 35·4
EphA2	2·25	1·17	1·32 ± 0·1
Ephrin-A5	−1·3	−1·42	−2·8 ± 0·95 $^{--}$
FGF family			
FGF-R2	−1·2	−1·03	−3·3 ± 1·2
G-protein coupled receptors			
RDC-1	−1·4	−1·3	see Table 1
Integrins			
Integrin-β6	1·30	0·00	111·22 ± 64·3
Integrin-β8	1·00	−1·27	3·19 ± 1·1
Integrin-α2	−1·19	0·00	3·61 ± 1·2
Integrin-α5	−1·17	−1·31	7·16 ± 3·4
Integrin-α6	1·22	−1·38	4·42 ± 0·8
Proteases and related factors			
EMMPRIN	1·34	−1·37	−1·30 ± 0·1
Urokinase R	14·55	1·10	7·58 ± 4·1
MMP-13	4·00	0·00	2·36 ± 0·03
TIMP-2	0·00	3·17	−0·36 ± 0·5
TGF-β family			
Activin A (bA subunit)	3·39	2·50	127·97 ± 51·23
TNF superfamily			
TNFRSF5/ CD40	0·00	0·00	27·03 ± 8·1
TNFRSF6/Fas	1·35	1·49	2·23 ± 1·0
TNFSF10/ TRAIL	0·00	0·00	55·26 ± 22·0 $^{++}$

P-HCEC ($n=2$) or SV40-HCEC ($n=3$) were treated with serum-free growth media or 10 ng ml^{-1} IL-1β for 6 hr. The IL-1β: media ratio of the grayscale values following corrections for background and detection limits are shown. The value 0·00 illustrates a gene that was not detected in the experiment concerned. $^{++}$Represents statistically significant ($p<0·05$) increase, whereas $^{--}$ represents a statistically significant decrease in gene expression following a Bayesian analysis of the data.

3.2. RT-PCR detection of cytokine genes in HCEC

To confirm some of the results obtained with gene array experiments, semi-quantitative RT-PCR was performed to detect IL-8, GRO-β, ICAM-1 and Ephrin A5 mRNA. Representative results are shown in Fig. 3. Relative to β-actin, expression of IL-8 was upregulated 1·3 ± 0·2 (mean ± S.E.M.) fold in SV40-HCEC ($n=3$) and 1·3-fold in P-HCEC ($n=2$); ICAM-1 was upregulated 1·2 ± 0·01 fold in SV40-HCEC ($n=3$) and did not demonstrate any notable change in expression in P-HCEC ($n=2$); GRO-β was upregulated 1·3 ± 0·07 fold in SV40-HCEC ($n=3$) and 1·2 in P-HCEC ($n=2$); Ephrin A5 was downregulated 0·83 ± 0·02 fold in SV40-HCEC ($n=3$) but remained unchanged in P-HCEC ($n=2$).

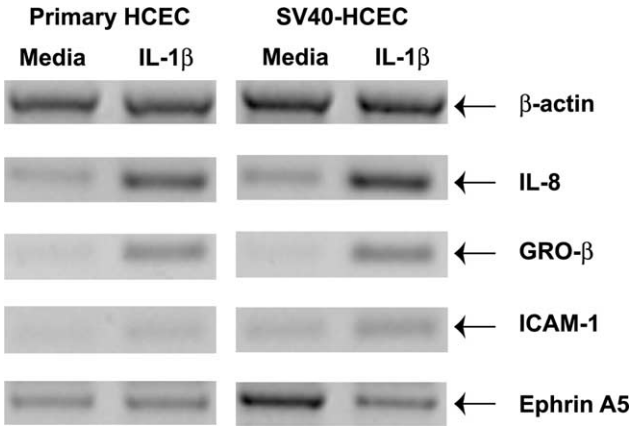

Fig. 3. Cytokine expression by HCEC treated with IL-1β. P-HCEC ($n=2$) or SV40-HCEC ($n=3$) were treated with IL-1β for 6-hr. The figure shows representative RT-PCR products for β-actin, IL-8, ICAM-1, GRO-β and Ephrin A5 from one experiment.

4. Discussion

We have shown that exposure of HCEC to IL-1 differentially regulated the expression of several chemokines, cytokines, cytokine receptors, proteases, growth factors and members of the TNF superfamily. Overall cytokine gene expression (with or without IL-1 stimulation) between primary cultured HCEC and SV40-transformed HCEC was very similar. In keeping with evidence that IL-1α and IL-1β have similar physiological effects (Dinarello, 1998), we observed that stimulation of HCEC with these cytokines induced a comparable pattern of change in gene expression.

Data analysis performed in a manner comparable to previous studies (Cao et al., 2002; Lee et al., 2001) revealed that in P-HCEC there were 35 genes whose expression was either upregulated or only detectable following IL-1β treatment and nine genes whose expression was either downregulated or undetectable after IL-1β treatment. In SV40-HCEC, the expression of 48 genes was upregulated or only detectable only following IL-1β treatment, whereas the expression of 10 genes was downregulated or no longer detectable after exposure to IL-1β. Performing the gene array experiment in three passages of SV40-HCEC not only

increased the validity of our data but also allowed further statistical analysis. A natural logarithm transformation of the intensity data was applied to the SV40-HCEC data to study the log-fold change in gene expression. The log-fold change in the expression of various cytokine genes in SV40-HCEC demonstrated good repeatability between experiments. Further analysis of the data using stringent criteria revealed statistically significant differential regulation of only 15 genes. However, this does not necessarily indicate that the changes seen with the other genes were inconsequential. Inherent variability between gene array experiments done on similar samples between and within arrays probably contribute to the differences observed (Jenssen et al., 2002). It is also conceivable that the changes observed, especially in genes with small ratios of change, would achieve statistical significance with further repetitions of the same experiment. Furthermore, since we only investigated the effect of IL-1β exposure for a 6-hr duration, peak expression that would have reached statistical significance before or after the 6-hr period is likely to have been missed in some cases. It must also be noted that small changes in the expression of some genes could cause dramatic changes in cell function and therefore, small changes in gene expression should not be ignored.

We confirmed our gene array results by performing RT-PCR to detect the change in expression of IL-8, ICAM-1, GRO-β and ephrin A5. The RT-PCR results were consistent with the gene array experiments. Other studies have shown similar effects of IL-1, with IL-8 being upregulated in HCEC (Cubitt et al., 1993) and GRO-β in hepatocytes (Rowell et al., 1997). One study (Yannariello-brown et al., 1998) demonstrated that unstimulated primary corneal epithelial cells expressed only low levels of ICAM-1 expression and IL-1 treatment of these cells did not induce any change in ICAM-1 expression. Thus the RT-PCR results corroborated with earlier studies and confirmed our own gene array findings.

We have identified for the first time in HCEC that IL-1 treatment can differentially alter the expression of members of the ephrin family and their receptors. The ephrins are thought to regulate cell shape and size during development (Oates et al., 1999). Interestingly, we found that certain integrin genes and adhesion molecules were also differentially regulated following IL-1 exposure. Other studies have shown that changes in ephrin regulation can affect cell adhesion and cell morphology. Both these effects are dependent on the activation of certain integrins such as integrin-β1 (Davy and Robbins, 2000). Based on our results and other studies (Boyd and Lackmann, 2001; Davy and Robbins, 2000), we hypothesize that the ephrin family of receptors and ligands may act in conjunction with integrins and other adhesion molecules to regulate epithelial cell morphology during corneal epithelial wound healing.

Several chemokines were significantly upregulated in our study. Notably, a pattern of chemokine expression similar to that observed here was seen in inflamed human corneas (Spandau et al., 2003). IL-1 also induces a comparable pattern of chemokine expression in corneal fibroblasts (Mahajan et al., 2002) and corneal endothelial cells (Yamagami et al., 2003). In our study, the neutrophil attractants IL-8, GRO-α, GRO-β and GRO-γ (Wuyts et al., 1998) were significantly upregulated in HCEC. One previous study also demonstrated that IL-1 could induce GRO-α expression in HCEC (Cubitt et al., 1997). Early recruitment of neutrophils to a site of injury is a primary innate defense mechanism and is especially important in the cornea for preventing infections which otherwise may compromise vision. Moreover, preventing leukocytes from entering the injured cornea has been shown to decrease epithelial healing rates (Gan et al., 1999). Thus, in addition to known activities, IL-1 could play a role in corneal wound healing by indirectly promoting leukocyte chemotaxis.

Here we have shown upregulation of TNF-α and TRAIL, another member of the TNF-family of cytokines, in HCEC stimulated by IL-1β. TNF-α induces corneal fibroblast apoptosis in corneal wound healing (Mohan et al., 2000), is increased in healing alkali-burned and post-traumatic corneas (Planck et al., 1997) and is also thought to play a role in the pathogenesis of ocular surface epithelial damage in Sjögren's syndrome (Jones et al., 1998). TRAIL has recently been shown to be involved in the lymphocytic destruction of the salivary gland of patients with Sjögren's syndrome (Matsumura et al., 2002). Thus our results suggest a possible secondary role for IL-1 in corneal wound healing and ocular surface inflammation by induction of the TNF family of cytokines.

In summary our study has shown that IL-1 treatment of HCEC alters the expression of several cytokines and related genes that are important in corneal inflammation. Our results not only confirm findings in previous studies but also provide novel information on changes in gene expression in HCEC exposed to IL-1. The study provides further evidence for the role of IL-1 in corneal inflammation and repair and will significantly improve our understanding of the pathways involved in corneal disease.

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