Orbital fibroblast chemokine modulation: effects of dexamethasone and cyclosporin A

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Abstract

Aim—Orbital inflammation is common, but the mechanisms underlying leucocytic infiltration of orbital tissue are poorly understood. Human orbital fibroblasts (OF) express chemokines, interleukin 8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1), when exposed to proinflammatory cytokines. The effects of dexamethasone (DEX) and cyclosporin A (CSA) on OF IL-8 and MCP-1 were examined.

Methods—Cultured human OF were incubated with recombinant interleukin 1 β (rIL-1 β ; 0.2, 2.0, 20 ng/ml) alone or incubated with rIL-1 β and DEX (10^{-8} , 10^{-7} , 10^{-6} M) or CSA (3, 30, 300 ng/ml) for 24 hours. ELISA and northern blot analyses were performed to determine OF IL-8 and MCP-1 protein secretion and mRNA expression, respectively.

Results—OF lacked constitutive IL-8 or MCP-1 expression, but secreted significant amounts of these chemokines and expressed substantial steady state mRNA for both chemokines upon rIL-1 β stimulation. DEX caused dose dependent inhibition of IL-1 induced IL-8 ($p < 0.001$) and MCP-1 ($p < 0.05$) secretion and mRNA expression at all concentrations of rIL-1 β . CSA enhanced IL-1 induced OF IL-8 ($p < 0.001$) and suppressed rIL-1 β induced OF MCP-1 ($p < 0.05$) secretion when lower doses of rIL-1 β were used. These effects on secreted chemokines at different concentrations of rIL-1 β and immunomodulating agents were corroborated by steady state OF IL-8 and MCP-1 mRNA expression.

Conclusions—DEX is a potent inhibitor of OF IL-8 and MCP-1. In contrast, CSA enhances IL-1 induced OF IL-8 and suppresses OF MCP-1. These observations may explain the relative lack of CSA effectiveness in human orbital diseases that respond to corticosteroids.

(Br J Ophthalmol 1998;82:318-322)

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Accepted for publication
10 October 1997

Orbital inflammation accounts for approximately 57% of all orbital disorders.¹ These disorders, characterised by inflammatory cell infiltrates, include orbital cellulitis, orbital myositis, Graves' eye disease, idiopathic orbital inflammation (orbital pseudotumour), as well as inflammation associated with systemic diseases. The mechanisms mediating orbital inflammation, however, remain poorly understood.²⁻⁴

Infiltration of leucocytes into inflamed tissue is a complex phenomenon, probably orches-

trated by chemotactic gradients expressed via cytokine cascades. We believe that resident orbital cells may participate in the initiation and perpetuation of many orbital inflammatory disease processes. We have shown that orbital fibroblasts (OF) secrete mediators that may be crucial to orchestrating orbital immune and inflammatory responses.⁵ Specifically, stimulation with lipopolysaccharide (LPS) or recombinant (r) proinflammatory cytokines (interleukin 1 β (rIL-1 β), tumour necrosis factor α (rTNF- α), or interferon γ (rIFN- γ)) induces secretion of different OF chemokines, low molecular weight, proinflammatory cytokines that (1) chemoattract and activate distinct leucocyte subsets and (2) participate in the upregulation of inflammatory responses.⁶⁻⁸ Interleukin 8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) are the two most potent and best characterised members of the superfamily of low molecular weight CXC and CC leucocyte chemokines, respectively. Induced principally by IL-1, TNF, and LPS, IL-8 preferentially chemoattracts and activates neutrophils, while MCP-1 attracts and stimulates monocytes and lymphocytes.⁸ Thus, OF are anatomically well positioned and are functionally able to participate in the regulation of leucocyte movement and activation in diseased orbital tissue when exposed to ambient proinflammatory cytokines.

The production of novel chemotactic cytokines by OF is noteworthy because coordinating communication between the interstitium and vascular bed may dictate the initiation, maintenance, and resolution phases of orbital inflammation. Strategies aimed at modulating these potent leucocyte chemoattractant and activating agents may be helpful in the control of orbital inflammatory disease. To examine the effects of the immunosuppressive agents dexamethasone (DEX) and cyclosporin A (CSA), cultured human OF were co-incubated with these immunosuppressive agents and rIL-1 β , a known inducer of IL-8 and MCP-1. OF IL-8 and MCP-1 protein secretion was measured and steady state mRNA expression was analysed.

Methods

ORBITAL FIBROBLASTS AND STIMULATION WITH rIL-1 β AND IMMUNOMODULATING AGENTS

Human retro-ocular OF were obtained from the posterior surface of globes enucleated for uveal malignant melanoma by gently mincing orbital fat in Dulbecco's modified essential medium (DMEM) containing 15% fetal bovine serum. The OF were grown at 37°C and 5% carbon dioxide and were passaged by

trypsinisation and maintained in 25 cm² tissue flasks for up to three passages. Experimental incubations were begun by simultaneously overlying newly confluent OF cultures with serum free media alone or in the same media containing DEX alone, CSA alone, or rIL-1 β (Upjohn Company, Kalamazoo, MI, USA; 0.2, 2.0, and 20 ng/ml) either alone or with DEX (Sigma Chemical Company, St Louis, MO, USA; 10⁻⁸, 10⁻⁷, 10⁻⁶ M) or CSA (Sigma Chemical Company; 3, 30, 300 ng/ml) for 24 hours at 37°C and 5% carbon dioxide. The dosages of DEX and CSA were specifically used because they represent the drug concentrations at the cellular level: calculated dosage in total body water. The 24 hour time point was chosen based upon previous dose response and time course studies that indicated upregulation of IL-8 and MCP-1.⁵ The specific activity of rIL-1 β was 30 units/ng. After experimental incubations, the orbital fibroblast culture media were collected after centrifugation and stored at -70°C until ELISA assays for IL-8 or MCP-1 were performed. Cell monolayers were extracted for IL-8 and MCP-1 mRNA analyses.

IL-8 AND MCP-1 ELISA

Immunoreactive IL-8 or MCP was measured in OF supernatants using a modification of the double ligand ELISA method.^{9,10} Briefly, flat bottomed, 96 well microtitre plates (Nunc Immuno-Plate, Vanguard International, Neptune, NJ, USA) were coated with either rabbit anti-IL-8 or anti-MCP-1 antibodies, each at 1 ng/ μ l in 0.6 M NaCl, 0.26 M H₃BO₃, and 0.08 N NaOH (pH 9.6), at 4°C and then washed with PBS (pH 7.5) and 0.05% Tween 20 (wash buffer). These antibodies were raised in rabbits using rIL-8 and rMCP-1 as previously described.^{9,10} These antibodies were exhaustively tested against numerous other chemokines, including other chemokines within the CC and CXC families; they were found to be very specific with no cross reactivity. Microtitre plate non-specific binding sites were blocked with 2% bovine serum albumin in PBS and incubated for 90 minutes at 37°C. Plates were rinsed four times with wash buffer and diluted. Non-diluted (neat) and diluted (1:5 and 1:10) OF derived conditioned media (50 μ l) in duplicate were added and incubated for 1 hour at 37°C. Plates were washed four times with wash buffer, then 50 μ l/well of biotinylated rabbit anti-IL-8 (1:2000) or anti-MCP-1 (1:2000) were added for 30 minutes at 37°C. Plates were washed four times, streptavidin-peroxidase conjugate (Bio-Rad, Richmond, CA, USA) was added, and the plates were incubated for 30 minutes at 37°C. Plates were washed four times, chromogen substrate (Bio-Rad) was added, the plates incubated to the desired extinction at room temperature, and the reaction terminated with 50 μ l/well of 3 M H₂SO₄. Absorbance for each well at 490 nm was read in the ELISA reader. Standards were run with each ELISA and were calibrated using rIL-8 and rMCP-1 WHO standards: 1:2 log dilutions standards of rIL-8 or rMCP-1 concentrations ranging from 1 pg to 1000 ng

per well. This ELISA method consistently detected IL-8 or MCP-1 concentrations greater than 10 pg/ml in a linear fashion.

NORTHERN BLOT ANALYSIS OF IL-8 AND MCP-1 MRNA

Total OF cell RNA was extracted.^{11,12} Adherent OF were solubilised in 25 mM TRIS (pH 8.0) containing 4.2 M guanidine isothiocyanate, 9.5% Sarkosyl, and 0.1 M β -mercaptoethanol. An equal volume of 100 mM TRIS (pH 8.0) containing 1% SDS and 10 mM EDTA was added and the RNA extracted with chloroform phenol. The alcohol precipitated RNA was separated by formaldehyde/1% agarose gels and transblotted to nitrocellulose. The baked blots were prehybridised and then hybridised with either a γ ³²P-5' end labelled (ICN Biomedicals, Inc, Costa Mesa, CA, USA), 30-mer oligonucleotide probe complementary to either nucleotides 262–291 of the published cDNA sequence for IL-8 (5'-GTT-GGC-GCA-GTG-TGG-TCC-ACT-CTC-AAT-CAC-3')¹³ or to nucleotides 256–285 of the published cDNA sequence for MCP-1 (5'-TTG-GGT-TTG-CTT-GTC-CAG-GTG-GTC-CAT-GGA-3').¹⁴ The blots were stringency washed and autoradiographed. Equivalent amounts of total RNA loaded per gel lane were assessed by monitoring 28s and 18s rRNA, comparing the fluorescence of the northern blot lanes stained with ethidium bromide.

STATISTICAL ANALYSIS

Individual experiments were performed in triplicate on three different OF cell lines (nine times) and the results composited. The mean IL-8 or MCP-1 concentrations plus or minus standard error of the mean (SEM) were determined for each assay condition and quantitated. Various assay conditions were compared using Student's *t* test (two tailed) and probability values less than 0.05 were considered to be statistically significant.

Results

IL-8 AND MCP-1 ELISA ASSAYS OF HUMAN ORBITAL FIBROBLAST CELL SUPERNATANTS

OF lacked constitutive IL-8 and MCP-1 secretion in control media or culture with DEX alone or CSA alone. When exposed to rIL-1 β (20 ng/ml) for 24 hours, OF IL-8 and MCP-1 secretion increased as quantitated by ELISA (Figs 1 and 2; *p*<0.001). DEX produced dose dependent inhibition of both rIL-1 β induced OF IL-8 (*p*<0.001) and MCP-1 (*p*<0.05), the greatest effect noted with DEX 10⁻⁶ M. Similar effects of DEX on OF IL-8 and MCP-1 production were noted when lower rIL-1 β concentrations were used to induce these chemokines (Table 1).

Low (3 ng/ml) and moderate (30 ng/ml) doses of CSA augmented rIL-1 β induced IL-8 protein secretion at all doses of rIL-1 β stimulation (Fig 1; Table 1). High dose CSA (300 ng/ml) was toxic and resulted in OF lysis. OF MCP-1 secretion induced by high dose rIL-1 β (20 ng/ml) was not significantly altered by the presence of CSA (Fig 2). Low (0.2 ng/ml) and

Figure 1 Dose dependent effects of dexamethasone and cyclosporin A on rIL-1 β induced IL-8 secretion by orbital fibroblasts detected by ELISA. *Denotes $p < 0.001$ compared with rIL-1 β stimulated control.

Figure 2 Dose dependent effects of dexamethasone and cyclosporin A on rIL-1 β induced MCP-1 secretion by orbital fibroblasts detected by ELISA. *Denotes $p < 0.05$ compared with rIL-1 β stimulated control.

moderate (2.0 ng/ml) doses of rIL-1 β induced OF MCP-1, however, were significantly inhibited by CSA (30 ng/ml; $p < 0.05$; Table 1).

NORTHERN BLOT ANALYSIS OF OF IL-8 AND MCP-1 MRNA

OF lacked constitutive IL-8 and MCP-1 mRNA expression (Fig 3). Increased IL-8 and MCP-1 mRNA expression was present following stimulation with rIL-1 β (20 ng/ml). DEX reduced IL-1 induced IL-8 and MCP-1 mRNA expression in a dose dependent fashion with greatest reduction noted with DEX 10^{-6} M. Marked and slightly increased expression of IL-1 induced IL-8 and MCP-1 mRNA was noted with low doses of CSA (3 and 30 ng/ml),

respectively. At lower concentrations of rIL-1 β OF stimulation (0.2 and 2.0 ng/ml), DEX decreased IL-8 and MCP-1 steady state mRNA expression (Figs 3 and 4). In contrast, CSA potentiated rIL-1 induced OF IL-8 mRNA expression and suppressed IL-1 induced MCP-1 mRNA expression (Fig 4). Cell toxicity, as evidenced by cell lysis in culture and lack of extractable RNA, was seen with CSA 300 ng/ml.

Discussion

Orbital inflammation is poorly understood.¹⁻⁴ Few models of orbital inflammation exist to explain leucocyte infiltration within the orbit, a complex phenomenon orchestrated by proinflammatory cytokines, cell adhesion molecules, and chemotactic gradients that result in a cascading inflammatory response. By studying ways to attenuate these mechanisms of orbital inflammation, destructive orbital inflammatory processes may be controlled. We believe that resident orbital fibroblasts are critical to initiation and propagation of orbital inflammation. Previously, we have shown that OF differentially express intercellular adhesion molecule 1 (ICAM-1) and HLA-DR antigen¹⁵ and chemokines (IL-8 and MCP-1) in response to different proinflammatory stimulation (LPS, rIL-1 β , rTNF- α , or rIFN- γ).⁵ Similarly, other investigators have found variable expression of adhesion molecules (ICAM-1 and HLA-DR) and cytokine production (IL-1 α , TNF α , and IFN- γ) in orbital fibroblasts of patients with Graves' disease, a disorder characterised by chronic inflammatory cellular infiltrates and accumulation of excessive hydrophilic glycosaminoglycans (GAGs) within the orbit.¹⁶⁻²³ This accumulation of inflammatory cellular infiltrates and GAGs in Graves' orbitopathy leads to the clinical manifestations of proptosis, diplopia, periorbital swelling, and inflammation.^{4, 24}

Cytokines chemotactic for leucocytes, known as chemokines, are critical in propagating an inflammatory response. Their importance in ocular disease is supported by recent animal studies demonstrating IL-8 to be an important mediator of experimental intraocular inflammation,²⁵ and the detection of MCP-1 and IL-8 in proliferative vitreoretinopathy,^{26, 27} pseudophakic bullous keratopathy,²⁸ and herpetic keratouveitis (Wu *et al*, presented as a poster at the American Academy of Ophthalmology Annual meeting, Atlanta, 1995). We have previously shown that different proinflammatory stimulants activate different OF derived chemokines.⁵ Specifically,

Table 1 Effects of dexamethasone (DEX) and cyclosporin A (CSA) on orbital fibroblasts (OF) IL-8 and MCP-1 production

Stimulant	IL-8 (ng/ml) immunosuppressive			MCP-1 (ng/ml) immunosuppressive		
	None	DEX (10^{-6} M)	CSA (30 ng/ml)	None	DEX (10^{-6} M)	CSA (30 ng/ml)
None (control)	13.0 (1.8)	13.7 (0.6)	12.6 (0.7)	8.5 (0.1)	5.8 (0.4)*	6.3 (0.2)*
IL-1 0.2 ng/ml	23.3 (3.0)	18.4 (1.0)	54.0 (7.7)*	12.1 (0.8)	9.6 (0.8)	8.2 (0.7)*
IL-1 2.0 ng/ml	61.6 (0.8)	33.8 (4.0)*	112.3 (1.6)*	13.2 (1.1)	7.4 (0.8)*	6.3 (0.3)*
IL-1 20 ng/ml	78.4 (3.1)	38.9 (7.6)*	188.7 (31.5)*	22.6 (0.6)	13.9 (2.0)*	21.4 (3.4)

Numbers represent mean concentration (SEM) (ng/ml).

*Denotes $p < 0.05$ compared with control (unstimulated) or rIL-1 β stimulation.

Figure 3 Representative northern blots revealing the effects of dexamethasone (DEX) and cyclosporin A (CSA) on rIL-1 β induced (20 ng/ml) IL-8 and MCP-1 mRNA expression in human orbital fibroblasts at 24 hours. Equivalent total cellular RNA loading per lane is demonstrated by the electrophoretic profile (below) of 18s and 28s rRNA in all lanes, except the high dose CSA lane in which cell death occurred from cyclosporin toxicity.

Figure 4 Representative blots revealing the effects of dexamethasone (DEX) and cyclosporin A (CSA) on OF IL-8 and MCP-1 mRNA expression in human orbital fibroblasts at 24 hours in response to rIL-1 β OF stimulation (2.0 ng/ml). Equivalent total cellular RNA loading per lane is demonstrated by the electrophoretic profile (below) of 18s and 28s rRNA in all lanes. Similar results were found with rIL-1 β (0.2 ng/ml) stimulation (data not shown).

OF lack constitutive IL-8 or MCP-1 secretion or gene expression, but OF produce substantial dose dependent increases in IL-8 and MCP-1

secretion and mRNA expression in response to LPS or cytokine stimulation (rIL-1 β > rTNF- α > rIFN- γ), emphasising that immune "bystander" OF that reside in orbital tissue participate in orchestrating an orbital inflammatory response by secreting important leucocyte chemotaxins.

In this study, we confirm that OF produce and secrete IL-8 and MCP-1 in response to the proinflammatory cytokine IL-1 β , and we demonstrate that there are widely variable cellular responses to commonly used immunosuppressive agents (DEX and CSA). High rIL-1 β concentrations were used to stimulate IL-8 and MCP-1 so that the dramatic effects of DEX and CSA would be evident. Although IL-1 β is usually found at lower concentrations in fluids removed from inflamed tissue, very high local concentrations at the cellular level (microenvironment) may occur. Thus, 20 ng/ml may be biologically relevant at the sites where actual inflammation occurs. Our study demonstrates that DEX is an effective inhibitor of IL-8 and MCP-1 produced by OF at all rIL-1 β concentrations, whereas CSA enhances OF IL-8 production and inhibits OF MCP-1 production at lower rIL-1 β concentrations (Table 1). These ELISA results are corroborated by northern blot analyses (Figs 3 and 4) and may explain the lack of CSA effectiveness in a wide variety of orbital diseases responsive to corticosteroids. Interestingly, similar results have been reported with the CC chemokine, RANTES: downregulation of RANTES expression by glucocorticoids in T cells, supporting a putative mechanism for the anti-inflammatory properties of glucocorticoids.^{29 30}

Corticosteroids and CSA have numerous anti-inflammatory effects and act by different mechanisms to control inflammation. Corticosteroids cause vasoconstrictive inhibition of leucocyte trafficking, reduction of arachidonic acid derived inflammatory mediators, downregulation of cellular adhesion molecules and proinflammatory cytokine gene transcription (IL-1, IL-2, IFN- γ , TNF- α , IL-6), and destabilisation of cytokine mRNA.³¹ In addition, glucocorticoids inhibit chemokine gene transcription and destabilise chemokine mRNA.³² The known anti-inflammatory effects of CSA are less well understood. CSA binds to an intracellular protein receptor, resulting in inhibition of an early calcium dependent signal transduction event involved in lymphokine (IL-2, TNF, and IFN- γ) expression, apoptosis, and degranulation of mast cells, neutrophils, basophils, and cytotoxic T cells.³³ Since DEX and CSA may have toxic side effects which result in electrolyte, musculoskeletal, gastrointestinal, renal, and neurological abnormalities,³⁴ it is important to establish a physiological basis for their use in human disease. Indeed, the enhanced OF IL-8 gene expression and protein secretion in response to CSA may explain the low therapeutic index of this agent in orbital disease.

Recent studies are defining orbital fibroblasts as potential local immunomodulating cells in the orbit which are able to produce chemokines in response to proinflammatory

cytokine or LPS stimulation.⁵ Our study describes a mechanism of how DEX may work in orbital inflammatory disorders. Further in vitro and in vivo studies characterising the roles of inflammatory cytokines, cellular adhesion molecules, and chemokines in orbital inflammation may illuminate new approaches that modulate OF and better control orbital inflammatory disease while reducing treatment associated morbidity. Study of these chemokines in inflammatory disorders is indicated.

Supported by NIH grants EY-09441 (VME; SGE) and EY-07003.

The authors wish to thank Zo-Mei Bian and Mitch Gillet for their technical and intellectual contributions.

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