Dexamethasone and Cyclosporin A Modulation of Human Retinal Pigment Epithelial Cell Monocyte Chemotactic Protein-1 and Interleukin-8

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Purpose. To examine the modulation of interleukin-1 beta (IL-1β)- and tumor necrosis factor-alpha (TNF-α)-stimulated monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8) secretion and transcription in human retinal pigment epithelial (HRPE) cells by dexamethasone (DEX) and cyclosporin A (CSA).

Methods. Cultured HRPE cells were stimulated with IL-1β (0.2 to 20 ng/ml) or TNF-α (0.2 to 20 ng/ml) for 8 or 24 hours without (control) and with DEX (10⁻⁸ to 10⁻⁶ M) or with CSA (0.3 to 30 ng/ml). Secreted levels of HRPE MCP-1 and IL-8 were measured in the media using enzyme-linked immunosorbent assay (ELISA). Both MCP-1 and IL-8 mRNA were analyzed by Northern blot.

Results. Although DEX (10⁻⁸ to 10⁻⁶ M) inhibited IL-1β-stimulated MCP-1 and IL-8 production, it did not inhibit TNF-α-stimulated chemokine secretion. In contrast, CSA significantly inhibited TNF-α-stimulated, but not IL-1β-stimulated, HRPE MCP-1 and IL-8 secretion. Both DEX and CSA inhibitions showed dose dependence. Northern blot analysis of HRPE steady state MCP-1 and IL-8 mRNA corroborated the ELISA measurements of secreted MCP-1 and IL-8.

Conclusions. Although DEX and CSA inhibit HRPE MCP-1 and IL-8 secretion, this is dependent on whether the inducing inflammatory mediator is IL-1β or TNF-α. IL-1β-induced chemokine secretion is sensitive to DEX, whereas MCP-1 and IL-8 induced by TNF-α are inhibited by CSA. This information may be useful in explaining in vivo observations and in suggesting targeted clinical treatments and combinations of immunosuppressive agents.

The production of cytokines is important for propagating, sustaining, and resolving inflammatory responses in all tissues, including the eye. Although many of these proteins were first identified in leukocyte secretions, many human cell types are now known to secrete a class of chemotactic cytokines, termed chemokines, that preferentially attract and activate specific leukocyte populations. Chemokine secretion occurs in response to soluble, inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α),² that are present at sites of inflammation but that are not themselves chemotactic.³ Uveitic models confirm the in vitro inflammatory nature of IL-1β and TNF-α,⁴ suggesting that inhibition of these inflammatory mediators and their effects may curtail ocular inflammatory responses.

Monocyte chemotactic protein (MCP-1) and interleukin-8 (IL-8) are the best-characterized members of two superfamilies, the C-C and the C-X-C chemokines, respectively. These chemokines display different in vivo and in vitro effects in various cell types and experimental conditions. Emphasis has been placed on MCP-1 chemotaxis and activation of monocytes and lymphocytes, as well as on similar IL-8 effects on neutrophils, eosinophils, and lymphocytes.⁵ Cellular responses include conformational changes, receptor expression, respiratory burst, and the formation of other bioactive proteins and lipids.⁶ In the eye, the strategic position and functional properties of the retinal pigment epithelium (RPE) suggest this cell type plays a major role in regulating ocular inflammatory responses. Retinal pigment epithelial cells

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Proprietary interest category: N.

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FIGURE 1. Baseline TNF-α and IL-1β-stimulated MCP-1 and IL-8 secretion into HRPE cell culture media. Values represent mean ± SEM. *Significant (P < 0.05) increase in HRPE MCP-1 or IL-8 secretion when compared to unstimulated cells. TNF = tumor necrosis factor; IL = interleukin; MCP = monocyte chemotactic protein; HRPE = human retinal pigment epithelium.

form a key part of the blood-retina barrier and, thus, are in position to interact with infiltrating leukocytes from the choroidal circulation.10,11 Retinal pigment epithelial cells respond to IL-1β and TNF-α by secreting chemokines, including IL-8 and MCP-1. Interleukin-8 accounts for the majority of RPE-derived in vitro neutrophil chemotactic activity, whereas MCP-1 secretion appears to be responsible for a significant component of RPE cell-derived in vitro monocyte chemotactic activity.12,13

Dexamethasone (DEX) and cyclosporin A (CSA) each have numerous anti-inflammatory effects, which include suppression of cytokine-mediated responses.14,15 Although some of these effects may be caused by systemic immunosuppression, intraocular drug concentrations are achieved with systemic and topical DEX or CSA administration, ranging from 10^-8 to 10^-6 M for DEX and 10 to 100 ng/ml for CSA.16,17 Using enzyme-linked immunosorbent assay (ELISA), we measured the inhibitory effects of clinically relevant concentrations of these two agents on IL-1β and TNF-α-stimulated human RPE (HRPE) MCP-1 and IL-8 secretion. Northern blot analysis was used to assess steady state levels of HRPE MCP-1 and IL-8 mRNA. Our in vitro observations may help explain and predict the effects of these drugs in vivo.

METHODS

Human Retinal Pigment Epithelial Cell Culture and Experimental Incubations

Human retinal pigment epithelial cells were isolated and grown from donor eyes (after informed consent and institutional review board approval were obtained in accordance with the tenets of the Declaration of Helsinki) as previously described.18 Fourth- to sixth-passaged cells from four cell lines were used for all experiments. Then HRPE cells were placed in either serum-free Dulbecco’s minimum essential medium or serum-free Dulbecco’s minimum essential medium containing dexamethasone (DEX; 10^-6 to 10^-8 M; Sigma, St. Louis, MO) or cyclosporin A (CSA; 0.3 to 30 ng/ml; Sigma). The HRPE cells from each of these conditions either were left unstimulated or were stimulated with human recombinant IL-1β (0.2 to 20 ng/ml; activity 30 U/ng protein; Upjohn, Kalamazoo, MI) or with TNF-α (0.2 to 20 ng/ml; activity 20 U/ng protein; Cetus, Emeryville, CA) for 8 or 24 hours. After experimental incubations, culture media were collected and stored at −70°C until ELISA assays were performed. Cell lysates were collected for mRNA isolation as previously described.19 Assays for 8- and 24-hour points showed similar trends. All data represent 8-hour incubations.

Interleukin-8 and Monocyte Chemotactic Protein-1 Enzyme-Linked Immunosorbent Assay

Antigenic MCP-1 or IL-8 were quantitated using a double-ligand ELISA method, as previously described.20 Sequential dilutions of HRPE supernatants were incubated with biotinylated rabbit anti-MCP-1 (1:2000) or anti-IL-8 (1:2000) in these assays. The ELISA were calibrated using standard concentrations of rMCP-1 or rIL-8 (Pepro Tech, Rocky Hill, NJ) ranging from 1 pg to 1000 ng/well. This ELISA method consistently detected MCP-1 or IL-8 concentrations > 0.5 ng/ml. All assays were performed in triplicate.

Human Retinal Pigment Epithelium Interleukin-8 and Monocyte Chemotactic Protein-1 Northern Blot Analysis

Total cellular RNA was extracted and probed as previously described.21 The blots were hybridized with 32P-
FIGURE 2. Modulation of IL-1\(\beta\)-induced MCP-1 (A) and IL-8 (B) secretion by DEX (10\(^{-6}\) M) and CSA (30 ng/ml) at three concentrations of IL-1\(\beta\) (0.2, 2, 20 ng/ml). Values represent mean ± SEM. *Significant (P < 0.05) decrease in HRPE MCP-1 or IL-8 secretion compared to HRPE stimulated with an equal concentration of IL-1\(\beta\) but without inhibitor. Absolute basal MCP-1 secretion was 3.64 ng/ml, whereas positive control levels of MCP-1 were 11.7 ng/ml for 0.2 ng/ml IL-1\(\beta\), 12.7 ng/ml for 2 ng/ml IL-1\(\beta\), and 14 ng/ml for 20 ng/ml IL-1\(\beta\). Baseline IL-8 was below detectable levels for the assay, whereas positive control levels of IL-8 were 40.3 ng/ml for 0.2 ng/ml IL-1\(\beta\), 80.5 ng/ml for 2 ng/ml IL-1\(\beta\), and 103.4 ng/ml for 20 ng/ml IL-1\(\beta\). TNF = tumor necrosis factor; IL = interleukin; MCP = monocyte chemotactic protein; DEX = dexamethasone; HRPE = human retinal pigment epithelium.

5end-labeled 30-mer oligonucleotide probes complementary to nucleotides of the published cDNA sequences for human IL-8 or human MCP-1.\(^{22,23}\) The probe sequences were 5'GTT-GGC-GCA-GTG-TGG-TCG-ACT-CTC-AAT-CAG3' for IL-8 and 5'-AGT-GAA-TGA-GTA-GCA-GCA-GGT-GAG-TGG-GGO3' for MCP-1. Blots were autoradiographed and quantitated by laser densitometric measurements. Equivalent amounts of total RNA loaded per gel lane were assessed by monitoring 28s and 18s rRNA. To take into account lane loading...
variation, MCP-1 and IL-8 bands were normalized. Total mRNA did not vary more than 20% between lanes and was generally within 10%. To quantify differences in mRNA expression with inhibitory drugs, the densitometric measurements in autoradiographs from unstimulated, control cultures were subtracted from raw measurements in autoradiographs from experimental conditions. To account for lane loading variation, this number was divided by the rRNA densitometry counts in that lane.

**Statistical Analysis**

All ELISA assays were carried out in triplicate, and the data were expressed as the mean ± SEM. Groups of data were evaluated by analysis of variance. Data that appeared to be statistically different were compared to each other by the Student’s t-test using the Bonferroni method for comparing the means of several groups. Differences in data, expressed as mean ± SEM, were considered significant if \( P < 0.05 \).

**RESULTS**

**Effects of Interleukin-1β, Tumor Necrosis Factor-α, Dexamethasone, and Cyclosporin A on Basal Human Retinal Pigment Epithelial Cell Monocyte Chemotactic Protein-1 and Interleukin-8 Secretion**

Unstimulated HRPE cells consistently demonstrated basal MCP-1 production of <5 ng/ml, whereas basal IL-8 production was below the levels detectable by our assay. Eight-hour incubations with IL-1β (0.2 to 20 ng/ml) and TNF-α (0.2 to 2 ng/ml) produced significant dose-dependent increases in HRPE cell chemokine secretion (Fig. 1). Data for 24-hour collections showed similar trends.

**Inhibition of Interleukin-1β-Stimulated Human Retinal Pigment Epithelial Cell Chemokine Secretion**

Dexamethasone (10^{-6} M) significantly reduced IL-1β-induced MCP-1 and IL-8 HRPE secretion at 2 or 20 ng/ml IL-1β, but not at 0.2 ng/ml IL-1β. Dexamethasone was equally effective in inhibiting IL-1β-stimulated MCP-1 and IL-8 secretion. In contrast, CSA (30 ng/ml) had no significant inhibitory effect on the IL-1β induction of MCP-1 or IL-8 at any dose of IL-1β (Fig. 2). Dexamethasone inhibition of IL-1β-induced MCP-1 and IL-8 secretion was dose dependent (Fig. 3). Inhibition of MCP-1 and IL-8 secretion was significant \( (P < 0.05) \) for 10^{-7} M and 10^{-6} M DEX, whereas 10^{-8} M DEX significantly reduced IL-1β-induced MCP-1 but not IL-8.

In additional experiments, DEX and CSA significantly inhibited basal MCP-1. Unstimulated MCP-1 measured 2.6 ± 0.2 ng/ml, whereas incubation with 10^{-6} M DEX alone produced 2.2 ± 0.2 ng/ml MCP-1. Incubation with 30 ng/ml CSA alone resulted in 1.9 ± 0.5 ng/ml MCP-1 (difference between control significant at \( P < 0.05 \)). Basal IL-8 secretion, as well as values for 10^{-6} M DEX or 30 ng/ml CSA alone, were below detectable levels of the assay.

Different cell lines yielded approximately the same degree of inhibition of IL-1β induction of RPE cell chemokines; 10^{-6} M DEX reduced IL-1β induction of MCP-1 by an average of 36% ± 5%, whereas it reduced the IL-1β induction of RPE cell IL-8 by an average of 31% ± 6% in the four cell lines tested. Differences in the relative potencies of 10^{-6} M DEX
Inhibition of Interleukin-1β-Stimulated Human Retinal Pigment Epithelial Cell Steady State Chemokine mRNA

Cytokine-induced MCP-1 and IL-8 steady state mRNA expression was reduced by DEX but not by CSA, paralleling the observations of chemokine secretion. In a
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**FIGURE 6.** Modulation of TNF-α-induced MCP-1 (A) and IL-8 (B) by CSA (30 ng/ml) and DEX (10^{-6} M) at three concentrations of TNF-α (0.2, 2, 20 ng/ml). Values represent mean ± SEM. *Significant (P < 0.05) decrease in HRPE MCP-1 or IL-8 secretion compared to HRPE stimulated with an equal concentration of TNF-α but without inhibitor. Absolute basal MCP-1 secretion was 3.3 ng/ml, whereas positive control levels of MCP-1 were 14.1 ng/ml for 0.2 ng/ml TNF-α, 30.7 ng/ml for 2 ng/ml TNF-α, and 43 ng/ml for 20 ng/ml TNF-α. Baseline IL-8 was below detectable levels for the assay, whereas positive control levels of IL-8 were 9.9 ng/ml for 0.2 ng/ml TNF-α, 17.2 ng/ml for 2 ng/ml TNF-α, and 32.7 ng/ml for 20 ng/ml TNF-α. TNF = tumor necrosis factor; CSA = cyclosporin A; DEX = dexamethasone; IL = interleukin; MCP = monocyte chemotactic protein; HRPE = human retinal pigment epithelium.

representative experiment at 2 ng/ml IL-1β, normalized densitometry revealed a decrease in MCP-1 mRNA with DEX, whereas an increase was noted with CSA (Fig. 4A). For IL-8, a decrease in steady state mRNA also was seen with 10^{-6} M DEX, whereas an increase in expression was noted with 30 ng/ml CSA (Fig. 4B). Basal, unstimulated MCP-1 and IL-8 steady state mRNA were reduced by DEX and CSA (Fig. 4). Dexamethasone inhibition of the IL-1β induction of MCP-1 and IL-8 mRNA expression was dose depen-
dent, with 10^{-7} \text{ M} and 10^{-8} \text{ M} DEX displaying effects that were less inhibitory (Fig. 5).

**Inhibition of Tumor Necrosis Factor-\(\alpha\)-Stimulated Human Retinal Pigment Epithelial Cell Chemokine Secretion**

In contrast to the findings for IL-1\(\beta\)-induced HRPE chemokines, TNF-\(\alpha\)-induced MCP-1 and IL-8 secretion were significantly inhibited by CSA but not by DEX (Fig. 6). In fact, DEX significantly increased TNF-\(\alpha\)-induced IL-8 secretion. Cyclosporin A significantly \((P < 0.05)\) inhibited TNF-\(\alpha\)-stimulated MCP-1 secretion at higher concentrations of TNF-\(\alpha\) (2 and 20 ng/ml), but not at low-dose TNF-\(\alpha\) (0.2 ng/ml; Fig. 6A). Tumor necrosis factor-\(\alpha\)-stimulated IL-8 was also sensitive to CSA inhibition at the lower doses of TNF-\(\alpha\) (0.2 and 2 ng/ml), but not at high-dose TNF-\(\alpha\) (20 ng/ml; Fig. 6B). Cyclosporin A inhibited TNF-\(\alpha\)-induced MCP-1 and IL-8 in a dose-dependent fashion, with higher doses (3 and 30 ng/ml), but not low-dose (0.3 ng/ml) CSA producing significant inhibition (Fig. 7).

Different cell lines yielded approximately the same degree of inhibition of TNF-\(\alpha\) induction of RPE cell chemokines. Cyclosporin A (30 ng/ml) reduced TNF-\(\alpha\) induction of MCP-1 by an average of 47\% \pm 5\%, whereas it reduced TNF-\(\alpha\) induction of RPE cell IL-8 by an average of 42\% \pm 2\% in the four cell lines tested. Differences in the relative potencies of CSA inhibition were even smaller within a cell line in replicate assays.

**Inhibition of Tumor Necrosis Factor-\(\alpha\)-Stimulated Human Retinal Pigment Epithelial Cell Steady State Chemokine mRNA**

Cyclosporin A reduced TNF-\(\alpha\) stimulated MCP-1 and IL-8 gene expression. Dexamethasone also reduced TNF-\(\alpha\)-stimulated MCP-1 gene expression, whereas it had a minimal effect on TNF-\(\alpha\)-stimulated IL-8 gene expression (Fig. 8). Cyclosporin reduction of TNF-\(\alpha\)-stimulated MCP-1 and IL-8 expression showed dose dependence, with significant inhibitions at 3 and 30 ng/ml CSA (Fig. 9).

**DISCUSSION**

Human retinal pigment epithelial cells produce and secrete the chemokines MCP-1 and IL-8 in response to the cytokines IL-1\(\beta\) and TNF-\(\alpha\), suggesting a role for these cells in regulating ocular inflammatory responses.\(^{12,13}\) In this study, we found that IL-1\(\beta\) inductions of HRPE MCP-1 and IL-8 secretion were inhibited by DEX but not by CSA. In contrast, TNF-\(\alpha\)-induced MCP-1 and IL-8 secretion was sensitive to CSA but not to DEX. Inhibitions by DEX and CSA were dependent on the inhibitory drug concentration as well as on the dose of the stimulating cytokine. Measurements of secreted antigenic MCP-1 and IL-8 were corroborated by Northern blot analyses of steady state HRPE MCP-1 and IL-8 mRNA.

These observations should be considered in the context of other known effects of these two drugs on cytokine-stimulated chemokines that appear to be cell-type specific. Dexamethasone has been shown to inhibit IL-1\(\beta\) and TNF-\(\alpha\)-induced MCP-1 and IL-8 in human fibroblast and fibrosarcoma cell lines,\(^{14,24,25}\) whereas DEX does not inhibit TNF-\(\alpha\)-induced IL-8 secretion in human bronchial epithelial cells.\(^{26}\) This suggests that TNF-\(\alpha\)-induced epithelial chemokines may not be as sensitive to inhibition by DEX. Indeed, our results also show that DEX is less effective in inhibiting TNF-\(\alpha\)-induced MCP-1 and IL-8 secretion compared to its effect on IL-1\(\beta\) stimulations.
finding that CSA preferentially inhibits TNF-α-induced MCP-1 and IL-8 suggests that DEX and CSA may be complementary inhibitors for the IL-1β-

and TNF-α-mediated components of inflammatory lesions.

The proposition that the inflammation induced by these two cytokines is distinct and responds differently to immunomodulating agents is supported by recent in vivo models of inflammation using IL-1β and TNF-α. For example, the intravitreal injection of IL-1β pro-

**FIGURE 8.** Northern blot of TNF-α-stimulated MCP-1 (A) and IL-8 (B) steady state mRNA expression showing inhibition by CSA (30 ng/ml). Equivalent total cellular RNA loading per lane is demonstrated by the electrophoretic profile of 18s and 28s rRNA (second row). Densitometric measurements (normalized for lane loading variation, as indicated by rRNA levels) revealed a 96% decrease in TNF-α-stimulated MCP-1 mRNA expression and a 51% decrease in IL-8 mRNA with CSA in this experiment. DEX reduced TNF-α-stimulated MCP-1 and IL-8 mRNA by 20% and 0%, respectively. TNF = tumor necrosis factor; IL = interleukin; CSA = cyclosporin A; DEX = dexamethasone; MCP = monocyte chemotactic protein; HRPE = human retinal pigment epithelium.

**FIGURE 9.** Northern blot of TNF-α-stimulated MCP-1 (A) and IL-8 (B) steady state mRNA expression showing inhibition by 0.3, 3, and 30 ng/ml CSA. Equivalent total cellular RNA loading per lane is demonstrated by the electrophoretic profile of 18s and 28s rRNA (second row). Densitometric measurements (normalized for lane loading variation, as indicated by rRNA levels) revealed 2%, 27%, and 96% decreases in TNF-α-stimulated MCP-1 mRNA expression by 0.3, 3, and 30 ng/ml CSA, respectively, in this experiment. TNF-α-stimulated IL-8 mRNA expression was reduced 6%, 22%, and 31% by 0.3, 3, and 30 ng/ml CSA, respectively. TNF = tumor necrosis factor; IL = interleukin; CSA = cyclosporin A; DEX = dexamethasone; MCP = monocyte chemotactic protein; HRPE = human retinal pigment epithelium.
vokes acute neutrophilic inflammation\(^7\) that is poorly responsive to CSA,\(^7\) whereas endotoxin-induced uveitis, which involves IL-1\(\beta\)-mediated responses, is sensitive to DEX.\(^9\) In contrast, intravitreal TNF-\(\alpha\) elicits a delayed, prolonged, chronic inflammatory response consisting of lymphocytes and mononuclear phagocytes,\(^8\) similar to that observed in experimental autoimmune uveitis, which is suppressed by CSA.\(^9\)\(^,\)\(^10\)

Mechanisms for the inhibition of cytokine-stimulated MCP-1 and IL-8 by DEX and CSA are being studied. Glucocorticoids inhibit chemokine gene transcription and destabilize chemokine mRNA.\(^3\) Transcriptional repression may be mediated by glucocorticoid response elements contained in the chemokine gene.\(^2\)\(^,\)\(^3\)\(^,\)\(^5\) The DEX downregulation of IL-1\(\beta\)-induced MCP-1 and IL-8 steady state mRNA expression that we observed are likely to involve both mechanisms. The inability of DEX to suppress MCP-1 and IL-8 induced by low-dose (0.2 ng/ml) IL-1\(\beta\) suggests that the different mechanisms by which IL-1\(\beta\) stimulates HRPE chemokines may have variable glucocorticoid sensitivities, depending on the cytokine dose. The mechanisms mediating CSA chemokine inhibition are less well understood, but they appear to involve intracellular second messengers such as cyclophilin and calmodulin.\(^4\)\(^,\)\(^3\)\(^,\)\(^4\) As with corticosteroids, we think the inhibition of MCP-1 or IL-8 by CSA is not mediated by the suppression of TNF-\(\alpha\), \(\gamma\)-IFN, or IL-1\(\beta\) because these could not be detected by sensitive ELISA in HRPE cell homogenates and supernatants.\(^12\)\(^,\)\(^13\)

The importance of inhibiting chemokines in ocular diseases is supported by recent animal studies demonstrating IL-8 to be a mediator of experimental intraocular inflammation and by the detection of MCP-1 and IL-8 in proliferative vitreoretinopathy, proliferative diabetic retinopathy, and pseudophakic bullous keratopathy.\(^5\)\(^,\)\(^35\)\(^,\)\(^38\) Selective inhibition of inflammatory mechanisms by DEX, CSA, and other agents provides for targeted or multidrug treatments that are likely to be more effective in controlling intraocular inflammation while reducing treatment-associated morbidity.

**Key Words**

chemokine, cyclosporin, cytokine, dexamethasone, glucocorticoids, interleukin-1\(\beta\), interleukin-8, monocyte chemotactic factor-1, retinal pigment epithelium, tumor necrosis factor-\(\alpha\)

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