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Induction of interleukin-8 in human retinal pigment epithelial cells after denuding injury

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Abstract

***Aim*—To determine interleukin 8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) expression in response to mechanical injury in human retinal pigment epithelial (HRPE) cells.**

***Methods*—Enzyme linked immunosorbent assay (ELISA) was performed to determine IL-8 and MCP-1 secretion by HRPE cells after mechanical denudation. IL-8 and MCP-1 mRNA expression by HRPE cells was assessed using semiquantitative RT-PCR. The effects of immunosuppressive drugs, dexamethasone (DEX) and cyclosporin A (CSA), as well as immunosuppressive cytokines, interleukin 4 (IL-4), interleukin 10 (IL-10), and interleukin 13 (IL-13), on chemokine expression in HRPE cells after denuding injury were analysed.**

***Results*—Mechanical injury induced HRPE IL-8 mRNA and IL-8 secretion. Although MCP-1 mRNA was enhanced slightly after denuding injury, MCP-1 secretion was not increased. DEX and CSA inhibited HRPE chemokine expression after injury. IL-4 and IL-13 enhanced IL-8 and MCP-1 production by HRPE cells after injury while IL-10 had no effect.**

***Conclusions*—These results suggest that IL-8 may be involved in retinal inflammatory responses to injury and that DEX and/or CSA treatment may help control the inflammatory components of retinal diseases such as proliferative vitreoretinopathy.**

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Human retinal pigment epithelial (HRPE) cells, situated between the neurosensory retina and the choroid, form part of the blood-retinal barrier and are thought to be a key cell type in the pathogenesis of proliferative vitreoretinopathy (PVR).[1] PVR is a pathological intraocular wound healing response after retinal detachment, ocular perforation, or intraocular surgery.[1] Important factors for initiation of PVR are mechanical dispersal of HRPE cells, breakdown of the blood-retinal barrier, and infiltration of the vitreous and subretinal space with inflammatory cells.[2 3] HRPE cells migrate and proliferate to form contractile membranes, frequently containing inflammatory cells and often leading to tractional retinal detachment and visual loss, suggesting that HRPE cells might play a central part in the initiation and propagation of PVR as an aberrant, inflammation driven wound healing response. However, how HRPE cells may initially respond to mechanical injury remains largely unknown.

Inflammatory cells including monocytes/macrophages, neutrophils, and lymphocytes appear to be integral to the development of PVR.[1 4 5] We previously reported that interleukin 8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) were two chemokines responsible for the majority of HRPE derived leucocyte chemotactic activity that is induced by interleukin 1β (IL-1β), tumour necrosis factor-α (TNF-α), and interferon-γ.[6 7] Chemokines have been divided mainly into two subgroups based on the juxtaposition of the first two cysteine residues in their amino acid sequences. IL-8, a member of the C-X-C family, is primarily chemotactic for neutrophils and eosinophils while MCP-1, a C-C chemokine, attracts and stimulates monocytes and lymphocytes.[8] We have shown that IL-8 and MCP-1 are present in eyes from patients with PVR.[9] Therefore, chemokines produced by HRPE cells are likely to be involved in recruiting inflammatory cells in PVR. Mechanical stresses of stretching and detachment of pulmonary alveolar, bronchial, and intestinal epithelial cells induces IL-8 production.[10–12] In light of these facts, we hypothesise that HRPE cells may produce chemokines, particularly IL-8, in response to mechanical injury following retinal detachment, leading to leucocyte recruitment in the initiation and propagation of PVR.

In this study, we examined HRPE chemokine expression in response to mechanical denuding injury. In addition, we tested whether mechanical injury induced chemokine expression could be reduced by the immunosuppressive drugs, dexamethasone (DEX) and cyclosporin A (CSA), as well as potent anti-inflammatory cytokines, interleukin 4 (IL-4), interleukin 10 (IL-10), and interleukin 13 (IL-13). Strategies aimed at modulating retinal derived chemokines may be helpful in the control of clinical PVR.

Materials and methods

HRPE CELL CULTURE

HRPE cells were isolated from donor eyes within 24 hours of death as previously described in accordance with the Helsinki agreement.[6] In brief, the HRPE cells were trypsinised (0.25%) from Bruch's membrane into serum free Dulbecco's modified essential medium (DMEM) containing 0.02 mg/ml DNase I. Isolated HRPE cells were seeded into Falcon Primaria flasks in DMEM containing 15% fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 mg/ml), and amphotericin B (2.5 mg/ml) (Gibco Inc, Grand

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Island, NY, USA). The HRPE monolayers exhibited uniform immunohistochemical staining for fibronectin, laminin, and type IV collagen in a chicken wire distribution, characteristic for these epithelial cells. Cells were subcultured into six well plates and four chamber culture slides for enzyme linked immunosorbent assay (ELISA) and immunocytochemistry, respectively, grown to confluency, and used for experiments.

DAMAGING OF THE MONOLAYER OF HRPE CELLS

Before experiments, confluent monolayers of HRPE cells were incubated in serum free DMEM for 24 hours. Cells were preincubated with DEX (10^{-6} M) or CSA (30 ng/ml) for 1 hour or recombinant (r) IL-4, rIL-10, or rIL-13 (R&D Systems, Minneapolis, MN, USA) for 24 hours, damaged with a plastic comb as previously described,[13] and then washed with DMEM. HRPE cells migrated from the edge of the wound and repaired the denuded space by 72 hours. We selected the preincubation time above because 1 hour preincubation of DEX and CSA was enough to reduce chemokine production in experiments in previous reports[14 15] and because we previously found that 24 hours of preincubation was needed to inhibit HRPE HLA-DR expression by IL-10.[16] After experimental incubations, culture media were collected, and stored at −70°C until ELISA was performed.

ENZYME LINKED IMMUNOSORBENT ASSAY

ELISA was performed on serial dilution of HRPE supernatants. Antigenic IL-8 and MCP-1 were quantitated using a double ligand ELISA method as described previously.[9]

SEMIQUANTITATIVE REVERSE TRANSCRIPTION PCR

Synthetic oligonucleotide primers based on the cDNA sequences of human IL-8, MCP-1, and β-actin were prepared: IL-8, 5'-AAGCTGGC CGTGGCTCTCTTG-3' and 5'-AGCCCTC TTCAAAAACTTCTC-3'; MCP-1, 5'-GCTC ATAGCAGCCACCTTCATTC-3' and 5'-GTCTTCGGAGTTTGGGTTTGC-3'; and β-actin, 5'-GTGGGGCGCCCCAGGCA CCA-3' and 5'-CTCCTTAATGTCACGC ACGATTTC-3'. Polymerase chain reaction (PCR) was carried out in a semiquantitative manner, essentially as previously described.[17] First strand cDNA was synthesised using Moloney murine leukaemia virus reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA). RNA, 1 µg, was denatured at 65°C for 10 minutes and added to the reverse transcription mixture, as indicated by the manufacturer. After incubation at 37°C for 1 hour, cDNA was then subjected to PCR. Linearity range of the reaction was determined running 15–35 cycles. DNA was denatured for 5 minutes at 94°C, followed by 28, 26, and 20 PCR cycles for IL-8, MCP-1, and β-actin respectively. Each cycle included a 1 minute denaturation at 94°C, a 1 minute primer annealing at 65°C, and a 2 minute polymerisation at 72°C. Each reverse transcription-PCR reaction mixture was analysed by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The intensity of the ethidium bromide luminescence was measured by an image sensor with a computer controlled display.

IMMUNOCYTOCHEMISTRY

Immunocytochemical staining for HRPE cells IL-8 was performed as previously described.[7 8] HRPE cells were fixed with 4% paraformaldehyde. HRPE cells were overlaid with blocking serum (Vector Laboratories, Inc, Burlingame CA, USA) for 20 minutes and incubated with goat polyclonal anti-human IL-8 antibody (Santa Cruz, CA, USA) at 4°C overnight, followed by incubation with biotinylated anti-goat antibody (Vector Laboratories, Inc) for 50 minutes, and streptavidin-biotinylated horseradish peroxidase complexes (Vector Laboratories, Inc) for 30 minutes. Reaction product was developed in buffer containing 3-amino-9-ethylcarbazole (5 mg/ml) and 0.01% H_2O_2 yielding granular, red-brown, reaction product. Negative controls using goat serum as primary antibody did not yield observable immunocytochemical reaction product.

STATISTICAL ANALYSIS

Individual experiments were performed in triplicate three times on three different HRPE cells from different donors who were 37, 51, and 60 years old. Values for protein of supernatant are expressed as a final concentration per 5×10^5 cells. Data are expressed as mean (SEM)). The data in the graphs are the combined results of the three experiments. Analysis of variance (ANOVA) with a post hoc analysis (Scheff multiple comparison test) and Student's *t* test were used to determine differences between multiple and two group, respectively; p values < 0.05 were considered to be statistically significant.

Results

Denuding injury induced IL-8 secretion, but not MCP-1 secretion by the HRPE cultures (Fig 1). Immunocytochemical analysis revealed that the expression of IL-8 protein was observed in HRPE cells at the edge of the scrape and motile HRPE cells (Fig 2). Slight staining for immunoreactive IL-8 was detected in some HRPE cells untouched by the comb. DEX and CSA inhibited IL-8 secretion induced by denuding injury (Fig 3). MCP-1 secretion by injured and uninjured HRPE cultures was inhibited by DEX and CSA (Fig 3). Consistent with ELISA data, denuding injury induced steady state IL-8 mRNA (Fig 4A, 4B).

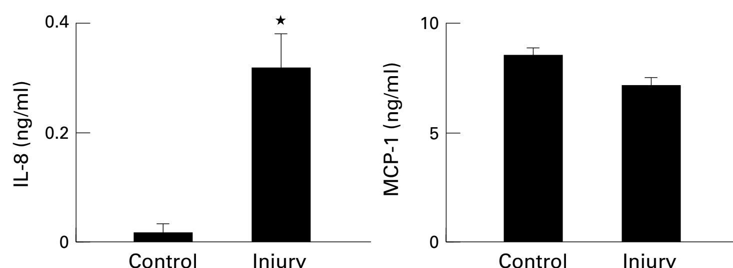

*Figure 1 IL-8 and MCP-1 secretion by HRPE cells after denuding injury. HRPE cells were damaged with a plastic comb. After experimental incubations for 24 hours, IL-8 and MCP-1 in culture media were measured using ELISA. *p<0.05, compared with control.*

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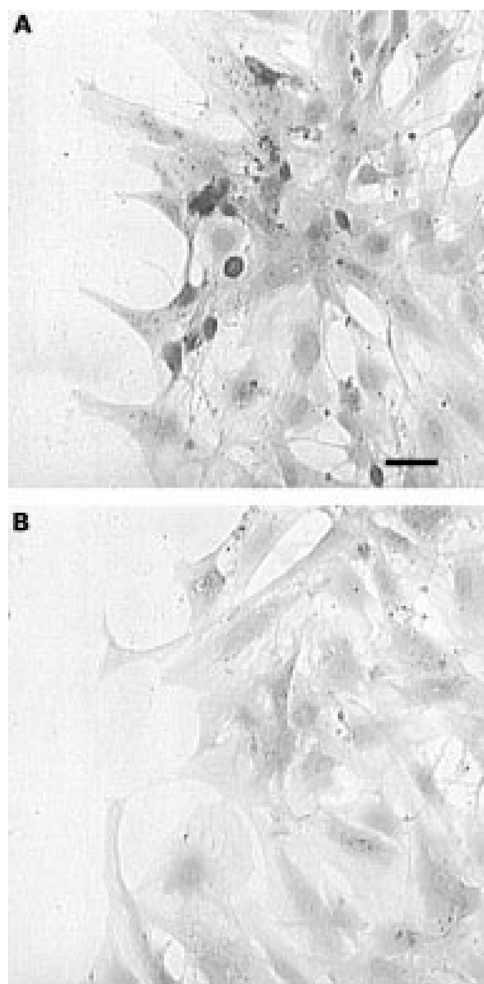

Figure 2 Immunocytochemical staining for IL-8 of HRPE cells 12 hours after denuding injury. HRPE cells were damaged and incubated for 12 hours. Cells were fixed and incubated with anti-IL-8 antibody (A) or goat serum (B). Bound antibodies were detected by a conventional avidin-biotin-peroxidase. (A) IL-8 is prominently detected mainly in HRPE cells at the edge of the scrape and motile HRPE cells. Slight staining for immunoreactive IL-8 is detected in some cells untouched by the comb. (B) No immunoreactivity is observed. Scale bar = 100 µm.

MCP-1 mRNA was slightly enhanced by denuding injury, even though MCP-1 secretion was not increased. DEX and CSA reduced denuding injury induced steady state IL-8 mRNA expression and MCP-1 mRNA expression of injured and uninjured HRPE cultures.

We next investigated the effects of immunosuppressive cytokines, IL-4, IL-10, and IL-13, on injured HRPE chemokine expression. Denuding injury induced HRPE IL-8 production was enhanced by IL-4 and IL-13, which also enhanced MCP-1 secretion by injured HRPE cultures (Fig 5). IL-10 had no significant effects on chemokine secretion. IL-8 and MCP-1 mRNA expression in injured HRPE cultures was enhanced by IL-4 and IL-13 (Fig 6A, 6B). IL-10 had no effects on injured HRPE chemokine mRNA expression.

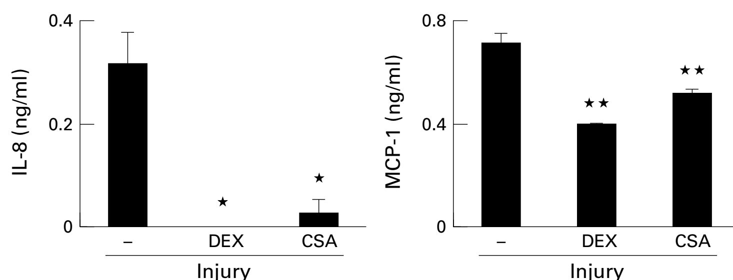

*Figure 3 The inhibition of dexamethasone (DEX) and cyclosporin A (CSA) on HRPE IL-8 and MCP-1 secretion after denuding injury. HRPE cells were preincubated with DEX or CSA for 1 hour and damaged. Culture media were assayed using ELISA. *$p < 0.05$; **$p < 0.01$, compared with supernatant from injured HRPE cells without agents (–).*

Discussion

In PVR, mechanical injury from non-surgical and surgical trauma damages and disperses HRPE cells. This may lead membrane formation and contraction characteristic of PVR. Inflammatory cells attracted into the vitreous and subretinal space may appear to be essential to evolving PVR.[4 5] Therefore, mechanisms recruiting and activating selective inflammatory cells, such as the regulated secretion of chemokines by resident, injured retinal cells, may be important in the pathogenesis of PVR. Among retinal cells, HRPE cells are anatomically well positioned and functionally able to participate in chemokine secretion.

In general wound healing, chemokines have the unique potential to activate and selectively guide various leucocyte subtypes to specific microanatomical sites of wound during different phases of tissue repair. Initially, neutrophils accumulate in the damaged tissue before monocyte/macrophage and lymphocyte infiltration. Accumulating leucocytes produce a battery of proinflammatory cytokines and growth factors which are thought to be involved in development of PVR.[9 18] In our study, we found that mechanical injury triggers HRPE IL-8 production that may lead to neutrophil recruitment in the retinal wound healing response. Accordingly, IL-8 has been detected in subretinal fluid from the eyes with retinal detachment and in the vitreous of eyes with PVR[9 19] and neutrophils were found in the subretinal space and retina in the experimental retinal detachment.[20] Although IL-8 could be produced by many cells including retinal glial cells, endothelial cells, and leucocytes, HRPE cells may be a main source of IL-8. IL-8 was detected in the HRPE cells at the edge of the scrape and in motile cells by immunocytochemistry, indicating these cells are the main sources of IL-8 in our culture model. Activated NF-κB, an important transcription factor for IL-8, has been demonstrated in vascular endothelial cells at the wound edge and cells replicating after denuding injury,[21] supporting the notion that cells adjacent to injury may produce IL-8. There is increasing evidence concerning the intracellular responses to mechanical stress, and including IL-8 secretion in responses to mechanical stress. Deformation of cytoskeletal structures has been reported to be important for IL-8 induction in bronchial epithelial cells,[12] suggesting that cellular deformation caused by injury might induce HRPE IL-8 production. We found slight immunoreactive IL-8 in HRPE cells untouched by the comb, suggesting such cells may also produce low levels of IL-8 in response to juxtacrine or paracrine signals from cells at the edge of the injury.

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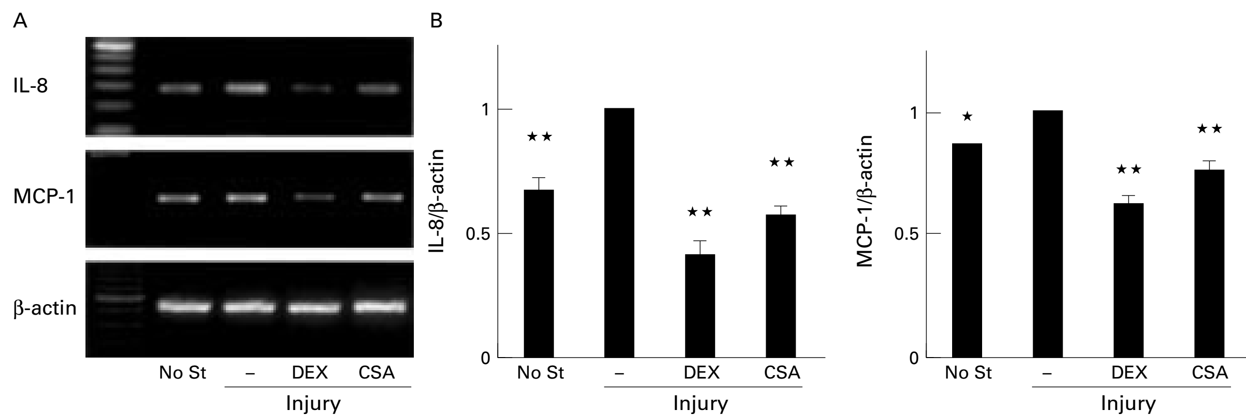

*Figure 4 Expression of IL-8, MCP-1, and β-actin mRNA by HRPE cells. (A) HRPE cells were preincubated with DEX or CSA for 1 hour, and then damaged. After 4 hours, total RNA was extracted from the HRPE cells, and semiquantitative RT-PCR was performed. These representative data are from one of three independent experiments. No St = no stimulant. (B) Results are expressed as a ratio of each PCR product/β-actin band density. Values represent means (SEM) (n=3). *p <0.05; **p <0.01, compared with mRNA from injured HRPE cells without agents (−). No St = no stimulation.*

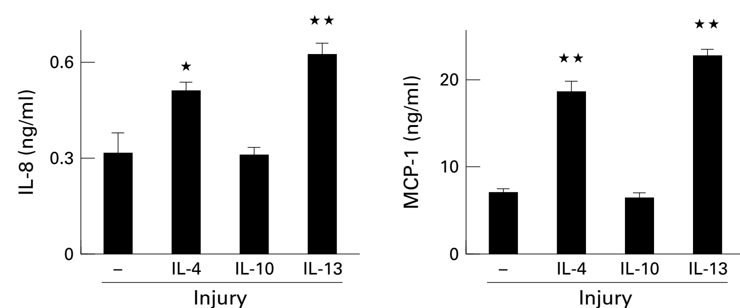

*Figure 5 The effects of IL-4, IL-10, and IL-13 on damaged HRPE IL-8 and MCP-1 secretion. HRPE cells were preincubated in serum free DMEM, or the same medium containing rIL-4, rIL-10, or rIL-13 for 24 hours, damaged, and incubated for 24 hours. IL-8 and MCP-1 were measured by ELISA. *p <0.05; **p <0.01, compared with supernatant from HRPE cells without cytokines.*

Unstimulated HRPE cell cultures consistently demonstrated basal MCP-1 production whereas no detectable IL-8 levels were observed as we previously reported.[7 22 23] However, very few monocytes are found in normal retinal tissue.[24] MCP-1 may cause desensitisation of the monocyte chemotactic response,[25 26] suggesting that low constitutive levels of MCP released by normal HRPE cells in normal eyes may serve to temper MCP-1 chemotactic responses and not recruit monocytes. Following injury, we could not detect increased HRPE MCP-1 secretion, but MCP-1 mRNA was slightly enhanced, suggesting that there might be suppressive regulation at the post-transcriptional level of MCP-1 production. Although IL-8 is not necessarily the only chemokine induced by injury of HRPE cells, mechanical injury seems to selectively induce chemokines.

In this study, DEX and CSA inhibited injury induced IL-8 production as well as MCP-1 production in control and injured HRPE cultures. Corticosteroids and CSA both appear to impair wound healing.[27 28] Corticosteroids have also been shown to inhibit experimental PVR.[29] Corticosteroids have numerous anti-inflammatory effects including downregulation of prostaglandin, cell adhesion molecules, proinflammatory cytokines, and chemokines.[30] In particular, corticosteroids inhibit chemokine gene transcription and destabilise chemokine mRNA.[31] Although CSA is also known to suppress proinflammatory cytokines and chemokines, the mechanisms of mediating

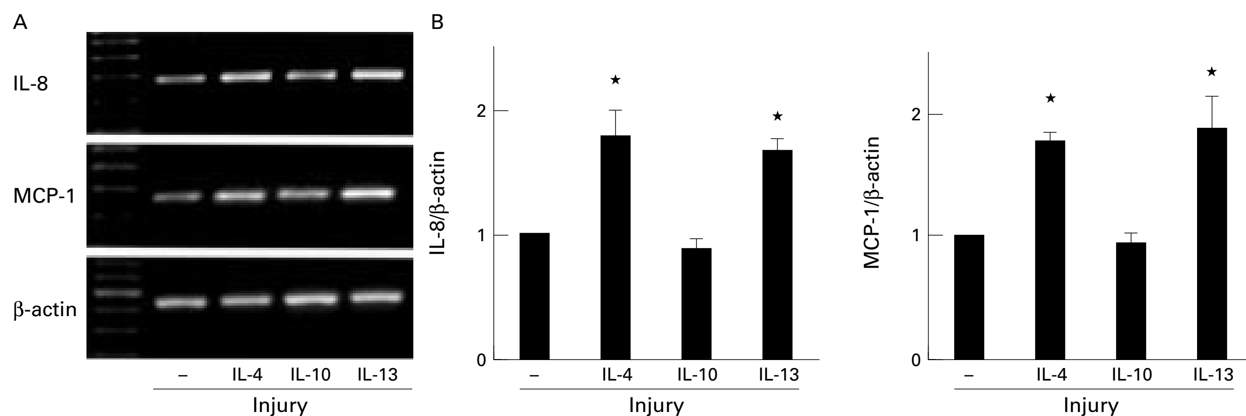

*Figure 6 The effects of IL-4, IL-10, and IL-13 on damaged HRPE IL-8 and MCP-1 mRNA expression. (A) HRPE cells were preincubated in serum free DMEM, or the same medium containing rIL-4, rIL-10, or rIL-13 for 24 hours, damaged, and incubated for 4 hours. Total RNA was extracted from the HRPE cell, and semiquantitative RT-PCR was performed. These representative data are from one of three independent experiments. (B) Results are expressed as a ratio of each PCR product/β-actin band density. Values represent means (SEM) (n=3). *p <0.05, compared with mRNA from HRPE cells without cytokines (–).*

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CSA chemokine inhibition are less well understood. We previously reported that IL-1β induced HRPE IL-8 and MCP-1 secretion is inhibited by DEX, but not by CSA, while TNF-α induced chemokine secretion was sensitive to CSA.[32] Our present data suggest that inhibition of retinal derived chemokines by DEX and/or CSA treatment before surgery may reduce surgically induced inflammation that promotes PVR recurrence by helping to control pathological inflammatory responses of PVR.

IL-4, IL-10, and IL-13 are Th2 cytokines that are considered to be anti-inflammatory since they downregulate proinflammatory cytokine release by activated monocytes in various diseases.[33] IL-10 and IL-13 have been detected in subretinal fluid from eyes with retinal detachment, but the roles of these cytokines in this setting remain unknown.[34] We previously reported that IL-4 induces HRPE IL-8 and MCP-1, but that IL-10 has no significant effects on constitutive or induced IL-8 and MCP-1.[16 35] In this study, IL-4 and IL-13 enhanced IL-8 and MCP-1 secretion by injured HRPE cells, while IL-10 had no such effects. Therefore, these cytokines seem to have potentiate the chemokine inducing effects of injury on HRPE chemokine secretion, in contrast with their effects on monocytes.

In summary, mechanical injury induces HRPE IL-8 production which may trigger recruitment of leucocytes. The IL-8 expression is inhibited by DEX and CSA, but not by Th2 cytokines. These findings may help the prevention and treatment of ocular inflammation by administration of DEX and CSA before surgery. Further in vitro and in vivo studies characterising the roles of chemokines and their regulation may lead to better control of inflammatory components of retinal diseases such as PVR.

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