Retinal Pigment Epithelial Cells Secrete Interleukin-6 in Response to Interleukin-1

Stephen R. Planck,* Thao T. Dang,* David Graves,* David Tora,† John C. Ansel,† and James T. Rosenbaum*

Interleukin-6 (IL-6) is a peptide whose properties include the ability to activate T-lymphocytes, stimulate the secretion of immunoglobulin, induce neuronal differentiation, and trigger the release of acute phase proteins. We have detected IL-6-like activity in conditioned medium from cultured human retinal pigment epithelial (RPE) cells with a bioassay based on the ability of IL-6 to induce the proliferation of murine B-9 plasmacytoma cells. Biologic activity increased approximately 90-fold when the cells were cultured in the presence of IL-1α (30 units/ml). Western blot analysis confirmed that conditioned medium from IL-1α-stimulated RPE cells contained peptides with molecular weights ranging between 19,000 and 30,000 and reactive with antibody to IL-6. Finally, Northern blot analysis indicated that cells cultured in the presence of interleukin-1 contained a 1.2 kilobase transcript that hybridized to a cDNA probe specific for IL-6 messenger RNA. IL-6 peptide on Western blots and mRNA on Northern blots were undetectable unless cells were cultured in the presence of IL-1α. Although IL-6 is synthesized by a variety of cell types, this report is the first to detect its synthesis by an eye-specific cell type. Furthermore, these observations indicate that retinal pigment epithelial cells respond to IL-1, a cytokine that previously has been implicated in ocular inflammation. Invest Ophthalmol Vis Sci 33:78-82, 1992.

The retinal pigment epithelial (RPE) cell is believed to play a central role in ocular inflammation. In the presence of γ-interferon, the RPE cell will express class II HLA molecules and has the ability to present antigen to sensitized T cells in vitro. Furthermore, during phagocytosis, the RPE cell releases superoxide in much the same way as neutrophils. Because most clinical and experimental models of uveitis are clearly immune-mediated, cytokines produced by the RPE cell could contribute to the pathogenesis of these forms of inflammation as well as to the immunohistologic changes associated with retinitis pigmentosa.

Interleukin-6 (IL-6) is a recently recognized potential mediator of inflammation. This cytokine is synthesized by a variety of cells, including monocytes, synoviocytes, and fibroblasts. It has been known as interferon beta 2, hepatocyte stimulating factor, B cell stimulatory factor 2, and hybridoma/plasmacytoma growth factor. It possesses many biologic activities similar to those of interleukin-1 (IL-1). These properties include the ability to stimulate the synthesis of acute phase proteins, to act as a cofactor in T cell proliferation, to synergize with interleukin-3 in stimulating the growth of hematopoietic stem cells, and the ability to enhance immunoglobulin synthesis. Recently, the ability of IL-6 to induce neuronal differentiation was demonstrated. Normal, nontumor cells typically do not express IL-6 unless they are stimulated by another cytokine. In several cell types, the synthesis of IL-6 is induced by IL-1. IL-1 has been detected in ocular fluids and shown to possess inflammatory properties that make it a potential mediator of ocular inflammation. Glial cells from the retina and subretinal macrophages are among the sources of IL-1. We cultured human RPE cells and assessed their ability to respond to IL-1α with the synthesis of IL-6.

Methods

RPE Cell Cultures

Cultures of RPE cells were established from cadaveric human eyes as previously described. When initially established, these cultures had a homogeneous appearance of pigmented epithelioid cells (Fig. 1). Nearly all of the cells expressed the RPE-15 cell surface antigen specifically found on RPE cells. Cultures were tested on early passage numbers and in some cases on primary cultures.

From the Departments of Medicine, Ophthalmology, and Cell Biology & Anatomy; and the Department of Dermatology and Portland VA Medical Center, Oregon Health Sciences University, Portland, Oregon.
Supported by NIH grants EY06484 and EY07373.
Submitted for publication: March 5, 1991; accepted August 5, 1991.
Reprint requests: James T. Rosenbaum, MD, L329A, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Road, Portland, OR 97201-3098.
Phase contrast photomicrograph of RPE cells in culture. This picture was taken after the cells had been cultured for 1 week and immediately after the conditioned medium was removed for the experiment shown in Figure 2. The cells display a typical epithelial morphology, and some cells still contain pigment granules.

**B-9 Proliferation Assay**

The presence of IL-6-like activity in conditioned media from cultured cells was detected by the ability of conditioned media to induce the proliferation of murine B-9 plasmacytoma cells. Conditioned media were harvested 48 hours after incubation with cells in the presence or absence of human recombinant IL-1α (a gift of Ira Green, National Cancer Institute, Frederick, MD) and dialyzed against phosphate-buffered saline prior to testing. Serial two-fold dilutions of each sample were tested in triplicate. Five thousand B-9 cells were plated in 96-well tissue culture plates and cultured for 68 to 72 hours in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 5 × 10⁻⁵ M β-mercaptoethanol, 5% fetal bovine serum, penicillin, streptomycin, and dilutions of the RPE cell conditioned media. The cells were then pulse-labeled with 1 μCi of ³H-thymidine for 4 hours and washed onto glass fiber filters. The amount of incorporated radioactivity was determined by liquid scintillation counting. Maximal stimulation was determined by adding 5 units of human recombinant IL-6 (Genzyme, Cambridge, MA) instead of conditioned medium.

**Northern Blot Analysis**

Cultured cells were serum-deprived for 24 hours and then incubated with IL-1α or control medium for 18 hours prior to being harvested. Cells were lysed in 0.2% Nonidet P-40 (United States Biochemical Co., Cleveland, OH), and total cytoplasmic RNA was isolated. Twenty μg of RNA/lane was electrophoresed in 1.2% agarose gels containing 2.2 M formaldehyde, followed by capillary transfer of the RNA to Nytran membranes. Membranes were prehybridized with 50% formamide, and 5× saline standard-citrate (SSC) (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 250 μg/ml herring sperm DNA, 50 μg/ml polyadenylic acid, 0.1% sodium dodecyl sulfate, and 1× Denhardt’s solution (0.02% Ficoll [Pharmacia, Inc., Piscataway, NJ], 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin). Hybridization was performed with the same solution supplemented with ³²P-labeled IL-6 cDNA copied from a 0.9 kilobase Eco R1/Taq 1 cDNA fragment (a gift of T. Kishimoto, University of Osaka, Japan). Washing was done under stringent conditions (0.1× SSC at 62°C). Blots also were probed with a ³²P-labeled RNA-probe specific for cyclophilin (1B15 plasmid was a gift of Dr. James Douglass, Oregon Health Sciences University), a transcript that is not regulated by IL-1α. This technique was used to assure that lanes had been loaded with equal amounts of mRNA.

**Western Blot Analysis**

Conditioned media harvested after 48 hours incubation in the presence of 30 units/ml IL-1α or 10% calf serum were dialyzed against distilled water and concentrated 10-fold by lyophilization. The concentrated samples were electrophoresed using SDS-polyacrylamide (Pharmacia PHAST system, Piscataway, NJ) under reducing conditions. Proteins were passively transferred to Immobilon PVDF (Millipore, Bedford, MA) membrane at 70°C over 25 minutes. This blot was probed with a rabbit antiserum directed against recombinant human IL-6 (a gift of Lester May, Rockefeller University). The rabbit antiserum was then detected by a goat-anti-rabbit IgG antibody conjugated to alkaline phosphatase (Tago, Burlingame, CA). Prestained molecular weight markers (BioRad, Richmond, CA) were run as standards.

**Results**

To determine if RPE cells secrete IL-6, conditioned media from RPE cell cultures were tested for IL-6-like activity with the murine B9 cell proliferation assay. The bioassay is based on the ability of IL-6 to stimulate the proliferation and, hence, the ³H-thymidine incorporation into the DNA of the B9 cells. The graphs shown in Figure 2 represent the mean values of ³H-thymidine incorporation for different dilutions of conditioned media obtained from four primary cultures derived from one pair of eyes. Conditioned media from these RPE cell lines stimulated approximately half-maximal proliferation of the B9 cell line at a dilution of 1:270. When these RPE cells were incubated with human recombinant IL-1α (30 units/
Fold Dilution

Fig. 2. B9 cell proliferation bioassay for IL-6 in conditioned media obtained from RPE cell cultures with or without IL-1α. Each point represents the average value of 3H-thymidine incorporation obtained from the indicated dilution of conditioned medium from cultures of four RPE lines derived from one pair of eyes and grown in the presence (•) and absence (○) of 30 units/ml of IL-1α. In this assay, five units of recombinant human IL-6 induced 16,000 cpm of 3H-thymidine incorporation.

<table>
<thead>
<tr>
<th>IL-6</th>
<th>IL-1</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig. 3. Western blot detection of IL-6-like peptides in RPE cell-conditioned media. The left lane contains 20 units of recombinant human IL-6 peptide, which is expected to migrate faster than the glycosylated, secreted forms of IL-6. Lanes 1 contain conditioned media incubated with RPE cells for 48 hr with either 30 units/ml of IL-1α or 10% bovine calf serum, as indicated. These cells had been serum-deprived for 24 hr prior to incubation with the IL-1α or serum. Lanes 2 are identical to Lanes 1 except that the cells were not serum-deprived prior to IL-1α or serum stimulation. Lanes 3 show unconditioned medium plus IL-1α or serum. Peptides of molecular weights ranging from approximately 19,000 to 30,000 Daltons were detectable if, and only if, the RPE cells were cultured in the presence of IL-1α.

Although the B9 cell proliferation assay is believed to be specific for IL-6, the identity of this IL-6-like biological activity was further tested by Western blot (immunoblot) analysis. RPE cell cultures were first grown for 24 hours in the presence or absence of 10% bovine calf serum. Fresh culture medium was conditioned by incubation with these cells for 48 hours in the presence of 30 U/ml IL-1α and/or 10% bovine calf serum. As shown in Figure 3, conditioned medium from RPE cells grown in the presence of IL-1α contained at least 2 peptides reactive with antibody to IL-6 and showing apparent molecular weights within the known range for IL-6. The presence of several immunoreactive forms of human IL-6 also has been reported for other cell types. This results from variations in post-translational glycosylation and phosphorylation. Neither serum stimulation nor serum starvation had any apparent effect on IL-6 levels.

Finally, RPE cells were studied for the ability to express the mRNA for IL-6. As shown in Figure 4 (top panel), IL-6 mRNA was not detectable by Northern blot analysis when cells were grown in the presence of

<table>
<thead>
<tr>
<th>IL-6</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Bottom, The same blot as above reprobed with a 32P-RNA probe for cyclophilin, which is constitutively expressed. These results indicate that approximately equal quantities of RNA were loaded into each lane, and exclude the possibility that the variation in intensity of the IL-6 mRNA signals is due to loading artifacts.
10% fetal bovine serum. However, cells grown for 18 hours in the presence of IL-1α contained readily detectable mRNA. The addition of IL-1α (1–50 U/ml) markedly increased the expression IL-6 mRNA in a dose-dependent manner. This effect could be partially prevented by adding dexamethasone (10^{-6} M) to the culture medium with the IL-1α (10 U/ml). The bottom panel of Figure 4 shows the same blot probed for mRNA of the cyclosporin-binding protein, cyclophilin. It confirms that approximately the same amount of RNA was loaded onto each lane.

Discussion

These studies indicate that human RPE cells are capable of synthesizing IL-6 in response to IL-1α. The evidence for this is based on biologic activity, immunologic reactivity, and the presence of an appropriate mRNA transcript. The amount of IL-6 produced by IL-1α-stimulated RPE cells is comparable to what we have observed to be secreted by IL-1-stimulated human synoviocytes, and cells that are considered to be an excellent source of IL-6. This report demonstrates that RPE cells respond to IL-1 and provides evidence that they synthesize IL-6. Soluble or membrane-bound forms of IL-1 can be produced by several cell types within the eye, including Müller cells, macrophages, vascular endothelial cells, and ciliary body epithelium. Recently, the ability of cultured RPE cells to secrete a potent neuronal differentiation factor, pigment epithelium-derived factor, was reported. Because IL-6 also induces neuronal differentiation, it provides an additional mechanism whereby RPE cells might regulate retinal development.

Responses of RPE cells that result in pigmented scars are noted in various forms of retinal or choroidal inflammation, including sympathetic ophthalmia, Vogt-Koyanagi-Harada syndrome, toxoplasmosis, and birdshot choroidopathy. These and most other forms of posterior uveitis are suspected to be immunologically mediated. Therefore, a peptide, such as IL-6, which stimulates immunoglobulin secretion and acts as a cofactor for T lymphocytes, may be centrally involved in this form of inflammation.

In studies involving primary cell cultures, potential complications are contamination with other cell types and artifacts induced by the culture conditions. As a result, we examined separate cell lines and endeavored to test cells soon after establishment in culture so any alterations caused by the conditions in vitro were minimized. This study includes results based on RPE lines started from the eyes of 4 donors. Each line uniformly showed evidence for the synthesis of IL-6. Biologic activity was consistently detectable in freshly established cell lines, and IL-6 mRNA, which required more cells per assay, was consistently detected in IL-1α-stimulated cells studied after several passages. Because cells were not cloned, a subpopulation of contaminating cells, such as fibroblasts, could be responsible for the results observed. While the strength of the signal in the Western and Northern blots and the degree of biologic activity make it unlikely that a minor cell population is primarily responsible for the results, we cannot totally exclude this remote possibility. Even if such contamination is present, the cells clearly are eye derived, and the conclusion that IL-6 is synthesized by eye-derived cells would not be suspect.

The responsiveness of RPE cells to another cytokine, namely γ-interferon, has been previously reported. While the work in the present report was being completed, Elner et al reported that neutrophil chemotactic factor (IL-8) is produced by RPE cells in response to IL-1β. These data suggest that RPE cells express a functional receptor for IL-1. In light of our observations, attempts to detect and localize IL-6 in examples of ocular pathology would certainly be of interest. Indeed, Murray et al recently reported that 63% of the patients examined with Fuch’s heterochromic cyclitis or uveitis secondary to toxoplasmosis had elevated levels of IL-6 in their aqueous humor.

Key words: interleukin-6, interleukin-1, retinal pigment epithelial cells, inflammation, uveitis

References


