Cytokine Modulation of Adhesion Molecule Expression on Human Retinal Pigment Epithelial Cells

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Purpose. The purpose of this study was to assess qualitatively the expression of adhesion molecules by human retinal pigment epithelium (RPE) and to study their regulation by inflammatory cytokines. These molecular events and the role played by inflammatory cytokines are important for selective lymphocyte trafficking into the eye during uveitis.

Methods. Expression of intracellular adhesion molecule-1 (ICAM-1), endothelial leukocyte adhesion molecule-1 (ELAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1), and vascular adhesion molecule (VCAM-1) by early passage human RPE cells was assessed by flow cytometry. In addition, the regulation of the expression of these molecules by the inflammatory cytokines interleukin-1 beta (IL-1β), IL-6, tumor necrosis factor alpha (TNFa), and interferon gamma (IFNγ) was determined. Reverse transcription–polymerase chain reaction (RT–PCR) was used to characterize further adhesion molecule expression.

Results. Flow cytometric analysis determined that ICAM-1 was constitutively expressed on RPE cell lines and that in the presence of TNFa, IFNγ, and IL-1β, there was a median fold increase in expression of 4.4, 5.4, and 4.4, respectively. In contrast, flow cytometric analysis of ELAM-1, PECAM-1, and VCAM-1 indicated that these adhesion molecules were not constitutively expressed at the cell surface; only the expression of VCAM-1 was upregulated by the presence of cytokines. The results of RT–PCR on RPE cells indicated that mRNA for all the adhesion molecules was present constitutively in some RPE cultures. After activation with IFNγ, TNFa, and IL-1β, RT–PCR analysis showed that the number of RPE cell lines expressing all the adhesion molecules increased.

Conclusions. ICAM-1 expression is markedly upregulated by inflammatory cytokines. Although mRNA for other adhesion molecules is expressed in RPE cells and is enhanced by inflammatory cytokines, this does not necessarily reflect the cell surface protein expression. Thus, the expression of adhesion molecules by RPE cells, and the subsequent recruitment of specific leukocytes, may be determined by the local cytokine environment. Invest Ophthalmol Vis Sci. 1995; 36:2262–2269.

The retina, in common with other neural tissue, requires a carefully controlled physiological environment, and to this end the eye possesses a blood–ocular barrier formed by the retinal pigment epithelium (RPE) and the endothelium of the retinal vasculature. The term “uveitis” describes the heterogeneous group of inflammatory disorders of the eye characterized by a breakdown of the blood–ocular barrier resulting in the passage of cells and proteins into the retina, vitreous, and aqueous humor. Although uveitis is a common cause of ocular disease and an important cause of blindness worldwide, the underlying pathologic mechanisms of this group of diseases remain unclear.

It has been shown that experimental autoimmune uveitis is predominantly a CD4⁺ (helper) T lymphocyte-mediated disease. In humans, there appears to be a selective recruitment of different leukocyte subsets during different stages of the disease. Studies of the cellular infiltrate present in the vitreous humor of
eyes with uveitis have shown that there is an early influx of T lymphocytes, particularly of the activated CD4+ subset. In the later stages, there is a preponderance of CD8+ (cytotoxic) T lymphocytes, whereas macrophages and B lymphocytes have been found in some patients with chronic posterior uveitis. Direct lymphocyte-mediated responses and the release of cytokines, growth factors, and enzymes at the inflammatory site contribute to the disease process. Hence, the recruitment of lymphocytes into the vitreous and aqueous humor is an important event.

Recruitment of immune cells at sites of inflammation is influenced by the local expression of cell adhesion molecules. Retinal pigment epithelial cells in vitro constitutively express ICAM-1, and retinal vascular endothelial cells may be induced to express this molecule by culture in the presence of IFNγ. Antibodies to ICAM-1 and its ligand LFA-1 markedly inhibit CD4+ lymphocyte–RPE interactions, but they only partially inhibit lymphocyte–endothelial cell interaction, indicating that binding of lymphocytes to the retinal endothelium occurs through other adhesion molecules. Several groups of cell–cell and cell–substratum adhesion molecules are now known, and those that are likely candidates for the recruitment of leukocytes during ocular inflammatory responses include members of the immunoglobulin superfamily: intercellular adhesion molecule-1 (ICAM-1); vascular cell adhesion molecule-1 (VCAM-1); platelet endothelial cell adhesion molecule-1 (PECAM-1), and the selectin–endothelial leukocyte adhesion molecule-1 (ELAM-1). The gene sequence codings for these molecules are known. Cytokines involved in inflammatory eye disease include interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and interleukin 6 (IL-6). Some are known to increase the expression of adhesion molecules on human umbilical vein endothelial in vitro, including IL-1β, TNFα, and IFNγ. These cytokines may be of lymphoid or nonlymphoid origin, and they are intimately involved in regulating immune responses. Thus, the induction and regulation of adhesion molecules by cytokines represent important aspects of inflammatory disease. Elucidation of the mechanism of upregulation of adhesion molecule expression by those cells that form the blood–ocular barrier may lead to the development of immunomodulatory strategies to prevent and treat intraocular inflammatory conditions. We have, therefore, undertaken a study to examine the cytokine modulation of adhesion molecular expression by RPE cells using flow cytometry and reverse transcription–polymerase chain reaction (RT–PCR) techniques. We conclude that mRNA encoding adhesion molecules may be expressed, but this does not necessarily result in cell surface protein expression.

The expression of RNA for ICAM-1, and to a lesser extent VCAM-1, was upregulated by inflammatory cytokines.

MATERIALS AND METHODS

Cell Lines

Methods for securing human tissue were humane, included proper consent and approval, and complied with the tenets of the Declaration of Helsinki. Human RPE cells were established from cadaveric eye using trypsin dissociation with seeding into tissue flasks in Eagle’s minimal essential medium (Life Technologies, Paisley, UK) containing 20% fetal calf serum (Life Technologies), penicillin (100,000 U/L) (Crystapen; Britannia Pharmaceuticals, Surrey, UK), streptomycin (100,000 U/L) (Life Technologies), and L-glutamine (2 mM) (Life Technologies) at 37°C in a 5% CO2/95% air atmosphere. All RPE cell lines showed characteristic epithelial morphology in culture, were pigmented in primary and early cultures, and stained positively with monoclonal antibody to cytokeratin 18. Cells were maintained in the above medium with 15% fetal calf serum and were used between passages 5 and 10.

Cytokines

IL-1β (specific activity of 1 × 10⁸ U/mg) was a generous gift from Immunex Research and Development Corporation (Seattle, WA) and was used at 20 ng/ml. TNFα (specific activity of 2.7 × 10⁶ U/mg) was a generous gift from Strangeways Research Laboratories (Cambridge, UK) and was used at 100 U/ml. IFNγ (specific activity 2 × 10⁶ IU/mg) was a generous gift from Boehringer Mannheim (Vienna, Germany) and was used at 100 U/ml. IL-6 (specific activity 4.1 × 10⁷ U/mg) was a generous gift from Zeneca (Macclesfield, UK) and was used at a concentration of 1000 U/ml.

Cell Culture

Cell populations were seeded into T75 tissue culture flasks at 1 × 10⁶ cells/cm² and were incubated for 24 hours at 37°C until subconfluent. The supernatant was removed, and the cells were washed once with serum-free medium. Fresh medium containing cytokine was added, and the cells were incubated for an additional 24 hours in the absence of serum. Cells were harvested using 1:1 mixture of 0.25% Trypsin (Life Technologies) and 0.02% ethylenediaminetetraacetic acid (BDH, Poole, UK) in phosphate-buffered saline (PBS) and were analyzed for the expression of adhesion molecules.

Flow Cytometric Analysis

After activation with cytokines, the RPE cells were harvested as described above and stained with the manu-
Retinal Pigment Epithelial Cells and Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted by a modification of the method of Chomczynski and Sacchi, using reagents from the RNeasy kit (Biogenesis, Bournemouth, UK). The RNA was dissolved in water containing 1000 U/ml RNaseasy pancreatic ribonuclease inhibitor (Pharmacia, St. Albans, UK) and stored at −20°C. Aliquots of the total RNA preparations were taken and adjusted to give 20 μg in 9.5 μl. One microliter of primer oligonucleotide pd(T)$_{12-18}$ (Pharmacia) at 0.5 μg/μl was added, and the mixture was heated to 70°C for 10 minutes and snap-cooled on ice. The reaction mixture was then made up to 20 μl containing 50 mM Tris HCl, pH 8.3; 75 mM KCl; 3 mM MgCl$_2$; 10 mM dithiothreitol; 15 U fresh RNAguard; 0.2 mM each dATP, dCTP, dGTP, dTTP (Pharmacia); and 200 U of Superscript RNase H$^-$MMTV reverse transcriptase (Life Technologies). The reaction was incubated at 37°C for 2 hours. The cDNA was then precipitated by the addition of 10 μl of 3 M sodium acetate, pH 5.5, and 80 μl of ethanol, followed by storage at −20°C for at least 2 hours.

The cDNA preparation was then pelleted for 5 minutes at 7000g, washed in 100 μl ice-cold 80% ethanol, and recentrifuged at 7000g for 2 minutes. The pellet was air dried and taken up in 20 μl water. Two microliter aliquots of this were added to the PCR reaction mixture to give a final volume of 100 μl containing the following: 10 mM Tris HCl, pH 8.3; 1.5 mM MgCl$_2$; 50 mM KCl; 0.01% (wt/vol) gelatin (Sigma, Poole, UK); 0.05% (wt/vol) Tween 20; 0.05% (wt/vol) Nonidet P40; 0.2 mM each dATP, dCTP, dGTP, and dTTP; 1 μM each oligonucleotide primer; and 2 U AmpliTaq DNA polymerase (Applied Biosystems, Warrington, UK). Negative control PCR reactions containing no cDNA were used to ensure there was no cDNA contamination while the PCR reaction mixtures were prepared. Fifty microliters of mineral oil were overlayed onto the PCR reaction mixtures, and amplification was carried out in a Perkin Elmer (Warrington, UK) thermal cycler as follows: 2 minutes at 94°C, followed by 35 cycles of 1 minute at 95°C, 1 minute at 50°C, and 3 minutes (increasing by 6 seconds per cycle) at 72°C. Final extension was for 15 minutes at 72°C. The reaction mixtures were then stored at 4°C until analysis.

Polymerase Chain Reaction Primers

Using published gene sequences for the adhesion molecules, exon specific primers were chosen that were specific for the adhesion molecule sequences. Oligonucleotide primers were synthesized on an Applied Biosystems 491 PCR-mate (Applied Biosystems) (Table 1).

Probes

Probe cocktails were purchased (R&D Systems Europe) and consisted of an equimolar mix of six single-stranded oligonucleotides labeled 5' with digoxigenin. Two of each set of six bound to the specific adhesion molecule PCR products. Stock solutions of the oligoprobes and primers were stored at −20°C, and working solutions were kept at 4°C. Repeated freezing and thawing, which is detrimental to oligonucleotides, was avoided.

Analysis of Polymerase Chain Reaction Products

One hundred microliters of chloroform and 25 μl of loading buffer (50% [vol/vol] glycerol, 50% 20% SSC, 0.01% bromophenol blue) were added to each reaction mixture; the tubes were shaken and centrifuged at 700g for 1 minute. The upper layer was decanted, and 25 μl was applied to a 1.5% NuSieve GTG (Flowgen Instruments, Sittingbourne, UK) gel containing 0.5 μg/ml ethidium bromide and TAE buffer (40 mM Tris acetate, pH 8.3; 1 mM ethylenediaminetetraacetic acid). Three microliters of unlabeled 123 bp DNA Mwt marker (Life Technologies) and digoxigenin-labeled DNA Mwt marker (Boehringer Mannheim, Lewes, UK) were used to determine the size of the products by ethidium bromide staining and by Southern blot analysis, respectively.

The gels were run at 5 V/cm in TAE containing 0.5 mg/ml ethidium bromide and photographed in ultraviolet light. They were then equilibrated in 0.4 M NaOH and blotted overnight to positively charged nylon membranes (Boehringer Mannheim, Lewes, UK). The blots were washed in 2 × 300 mM NaCl; 30 mM Tris HCl, pH 7.5 (TBS), air dried, and baked at 95°C for 30 minutes. Southern blots were prehybridized at 37°C for at least 1 hour in prehybridization solution (750 mM NaCl; 75 mM Tris HCl, pH 7.5;
TABLE 1. Oligonucleotides Used in Reverse Transcription-Polymerase Chain Reaction to Detect Adhesion Molecules

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ICAM-1</strong></td>
<td></td>
</tr>
<tr>
<td>Sense (exon 4)</td>
<td>245</td>
</tr>
<tr>
<td>Antisense (exon 5)</td>
<td></td>
</tr>
<tr>
<td><strong>VCAM-1</strong></td>
<td>500</td>
</tr>
<tr>
<td>Sense (exon 2)</td>
<td></td>
</tr>
<tr>
<td>Antisense (exon 4)</td>
<td></td>
</tr>
<tr>
<td><strong>PECAM-1</strong></td>
<td>787</td>
</tr>
<tr>
<td>Sense (exon 3)</td>
<td></td>
</tr>
<tr>
<td>Antisense (exon 6)</td>
<td></td>
</tr>
<tr>
<td><strong>ELAM-1</strong></td>
<td>943</td>
</tr>
<tr>
<td>Sense (exon 2)</td>
<td></td>
</tr>
<tr>
<td>Antisense (exon 7)</td>
<td></td>
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</tbody>
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RESULTS

Cell Surface Adhesion Molecule Expression—Flow Cytometric Analysis

Retinal pigment epithelial cells cultured for 24 hours under serum-free conditions were analyzed for adhesion molecule expression by flow cytometry. Initial studies confirmed that the adhesion molecule expression was not adversely affected when the RPE was enzymatically harvested. Using six different RPE cell cultures, it could be shown that only ICAM-1 was expressed at detectable levels at the cell surface, the intensity of staining ranging from a mean channel of fluorescence of 31 to 588 (Fig. 1), with variable expression in assays performed on the same cell line. Low-intensity staining or no significant elevation above background was shown for PECAM-1, VCAM-1, and ELAM-1 (data not shown). The variable expression of ICAM-1 by a given cell line could not be accounted for by changing culture conditions because ICAM-1 expression was assessed 24 hours after subculture when cells were growing exponentially as subconfluent cultures.

FIGURE 1. Expression of ICAM-1 on unstimulated retinal pigment epithelial cell lines, as assessed by flow cytometry. Median channel fluorescence is given after background subtraction (more than one symbol indicates replicate experiments).
Expression of ICAM-1, VCAM-1, PECAM-1, and ELAM-1 was assessed on five RPE cell cultures after a 24-hour culture in the presence of either IFNγ, IL-1β, IL-6, or TNFα at concentrations determined in this laboratory to activate RPE. No increased expression of ELAM-1 or PECAM-1 was observed in response to the presence of inflammatory cytokines; however, increased expression of ICAM-1 and VCAM-1 occurred upon co-culture with TNFα, IFNγ, and IL-1β (Fig. 2). Increases in expression of adhesion molecules are reported as fold increases above the unactivated levels of expression. Expression of ICAM-1 was increased by median fold increases of 4.4, 5.4, and 4.4 (ranges: 3.6 to 13.1; 2.5 to 24.2; 2.9 to 25.6) in the presence of TNFα, IFNγ, and IL-1β, respectively. IL-6 did not appear to influence greatly the expression of ICAM-1 in any of the RPE cell lines tested with a median fold increase of 1.1 (range: 0.0 to 1.4). Upregulation of expression of VCAM-1 also was detected in the presence of TNFα; IFNγ, and IL-1β, with median fold increases of 4.0, 3.4, and 15.5 (ranges: 1.0 to 71.3; 1.0 to 8.5; 0.0 to 40.7). The dramatic increases result from the low levels of constitutive fluorescence of this adhesion molecule. Again, IL-6 had no effect on VCAM-1 expression (data not shown).

**Adhesion Molecule mRNA Expression: Reverse Transcription–Polymerase Chain Reaction Analysis**

Six RPE cell lines were incubated for 24 hours in the presence or absence of exogenous cytokines, after which the cells were processed and assessed for the expression of messenger RNA for adhesion molecules, determined by RT–PCR. After RNA extraction, cDNA was prepared and amplified using synthetic oligonucleotide primers. Gel electrophoresis was performed...
on the samples, and the PCR products were visualized by ethidium bromide staining (Fig. 3). Although the PCR products of the expected size could be identified, there were numerous other bands that stained with ethidium bromide, and, despite optimization of experimental conditions using activated HUVEC as positive controls, these extra bands could not be eliminated when using the primers for PECAM-1 and ELAM-1. We therefore probed each of the PCR products to confirm the presence or absence of messenger RNA specific for the adhesion molecules. The results of probing the blots of the stained gels in Figure 3 are given in Figure 4. Although some nonspecific binding of the probes occurred, the bands of interest were demonstrated clearly. A PCR product of 245 base pairs was generated in PCR reactions primed with the ICAM-1 oligonucleotide primers, allowing us to conclude that the expression of messenger RNA for ICAM-1 occurs in both unstimulated and cytokine-stimulated RPE cells. This result was seen in 2 of 6 RPE cell lines; in three other cell lines, expression of ICAM-1 mRNA was seen only after cytokine activation. PCR products of 500 bp were generated in PCR reactions primed with the VCAM-1 oligonucleotide primers, indicating that the message for VCAM-1 was present. Four of the six RPE cell lines showed expression of mRNA for VCAM-1 in the absence or presence of exogenous cytokines; an additional cell line showed expression only after cytokine exposure. Fewer RPE cell lines expressed either PECAM-1 or ELAM-1 (PCR product sizes of 787 and 943 bp, respectively); however, two cell lines expressed mRNA for PECAM-1 in the absence or presence of exogenous cytokines, whereas two additional cell lines expressed mRNA only after culture in the presence of cytokines. Similarly, one of six cell lines expressed mRNA for ELAM-1 in the absence or presence of exogenous cytokines, and two other lines expressed mRNA only after exposure to cytokines.

**DISCUSSION**

Although the cause of uveitis remains unknown, it is thought that the disorder is precipitated by exogenous or endogenous antigens. The initial event probably involves antigen processing and presentation by antigen-presenting cells; whether this is mediated by endogenous ocular cells or infiltrating mononuclear cells has not been established. Retinal pigment epithelial cells have properties that allow them to act as accessory cells for immune responses in the eye: They show major histocompatibility complex antigen expression that can be enhanced by exposure to inflammatory cytokines; they are phagocytic, can process retinal antigens, and may, therefore, present antigen to macrophages and ultimately to T lymphocytes once recruited.

Cytokine production by activated cells mediate cell proliferation, migration, and differentiation, and a number of cytokines—including IL1, TNF, IL6, and IL8—have been implicated in the pathogenesis of inflammatory eye disease. The cellular source of these cells is unknown, though production by macrophages or other antigen-presenting cells is possible; RPE have been shown to produce IL-6, IL-8, and monocyte chemotactic protein. Alternatively, some form of systemic immune reaction may take place, activating immune cells, and the eye—because of its extremely high blood flow—is visited by these activated cells that release cytokines in the vicinity. This produces a slight upregulation of cell adhesion molecules, including those constitutively expressed. This enhanced expression can then begin recruiting immune cells to that area. Migration of inflammatory cells into the eye must involve adhesion to vessel walls, and, because RPE cells form the outer blood–ocular barrier, this may have important implications in the recruitment of leukocytic infiltrates into the vitreous humor.

In addition, we have shown that ICAM-1 expres-
sion at the level of both messenger RNA and protein is upregulated by IL-1β, TNFα, and IFNγ. Our findings for ICAM-1 are consistent with the hypothesis that the RPE cell acts as an accessory cell for inflammatory responses within the eye. The release of cytokines by RPE cells and by cells of the immune system may serve to enhance RPE-leukocyte interaction by increasing the expression of the ICAM-1 protein. This finding is supported by the work of others.2,8–10

It is unlikely that immunomodulatory treatment could be administered at an early stage in an inflammatory process to prevent release of these cytokines and the subsequent enhanced expression of adhesion molecules; therefore, treatment aimed at blocking or downregulating this expression would seem more appropriate. Studies in the rabbit have shown that monoclonal antibodies to ICAM-1 reduced the cellular infiltrate during inflammation.20 It must be emphasized that these in vitro findings do not necessarily reflect the situation in vivo, but they may provide clues to the likely sequence of molecular events occurring during the initiation and progression of posterior uveitis.

We have shown that, despite the lack of VCAM-1, ELAM-1, and PECAM-1 protein expression, as determined by flow cytometry, mRNA for these adhesion molecules is found in 4 of 6, 1 of 6, and 2 of 6 RPE cell lines, respectively. In addition, stimulation with TNFα, IFNγ, and IL-1β, at concentrations comparable to other studies,23–25,30 resulted in the expression of VCAM-1, ELAM-1, and PECAM-1 in 5 of 6, 3 of 6, and 4 of 6 RPE cell lines, respectively. The results show that inflammatory cytokines have the capacity to induce and/or enhance gene expression for adhesion molecules in RPE cells. Studies on other tissues within the eye have found constitutive expression of ICAM-1 with some expression of VCAM and ELAM in response to inflammatory cytokines.31–33

There were discrepancies between the detection of adhesion molecules by RT–PCR followed by ethidium bromide staining compared with Southern blot analysis. This is because of the differences in sensitivity of the two detection methods; Southern analysis using digoxigenin-labeled probes are far more sensitive and specific than ethidium bromide staining. Constitutive expression of ELAM-1 and PECAM-1 was detected by RT–PCR analysis in one and two RPE cultures, respectively, and not by flow cytometric analysis. This may be as a result of the fact that RT–PCR is exquisitely sensitive compared to flow cytometric analysis. The former detects mRNA from a few cells, but the latter requires extensive expression for detection; therefore, no biologic significance of mRNA expression for adhesion molecules can be ascribed. Clarification of the extent of mRNA expression could be achieved only by in situ RT–PCR, though lack of appropriate tissue would be limiting for these investigations. Alternat-
Cytokine Modulation of Adhesion Molecules on RPE


