Role and Expression of CD40 on Human Retinal Pigment Epithelial Cells

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PURPOSE. To examine the CD40 costimulatory molecule expression on normal resting or activated adult human retinal pigment epithelium (hRPE) cells and to evaluate its role as an activation molecule considering the potential antigen presentation functions of hRPE cells.

METHODS. Expression of HLA-DR and costimulatory (CD40, B7.1, B7.2, CD54, and CD58) molecules on hRPE cells was analyzed by flow cytometry. CD40 triggering was performed using soluble CD40L or cocultures with CD40L transfected fibroblasts. Interleukin (IL)-6, -8, -10, and -12 secretions were measured by enzyme-linked immunosorbent assay. Antigen presentation function of hRPE cells was assessed by coculturing hRPE cells with allogeneic T cells. T-cell proliferation was measured by [3H]-thymidine incorporation, and T-cell apoptosis by measurement of caspase-3 activity.

RESULTS. Interferon (IFN)γ–activated hRPE cells expressed CD40, but not B7.1 or B7.2. Although interferonγ enhanced IL-6 and IL-8 production, CD40 triggering of IFNγ–activated hRPE cells did not induce IL-12 secretion. hRPE cells did not stimulate allogeneic resting T cells and downregulated phytohemagglutinin-activated allogeneic T cells via a cell-to-cell contact-dependent mechanism. Some induction of apoptosis was detected.

CONCLUSIONS. CD40 is expressed on IFNγ-activated hRPE cells. Its ligation leads to an increased production of IL-6 and IL-8 but fails to induce B7.1 or B7.2 expression, or to induce IL-12 secretion. Accordingly, hRPE cells do not activate allogenic T cells but inhibit T-cell proliferation, partly through induction of apoptosis. These results suggest that hRPE cells could be implicated more in a deviant antigen presentation. If the exact molecular mechanisms are unclear, it is likely that CD40–CD40L interaction could play a role in this process. (Invest Ophthalmol Vis Sci. 2000;41: 3485–3491)

Together with the retinal blood vessels, the retinal pigment epithelium (RPE) forms the blood-retina barrier (BRB), isolating the posterior segment of the eye from the rest of the body, in particular from inflammatory cells. Some pathologic conditions (e.g., uveitis1 or proliferative vitreoretinopathy2,3) are associated with a breakdown of the BRB. Some pathologic conditions (e.g., uveitis1 or proliferative vitreoretinopathy2,3) are associated with a breakdown of the BRB isolating the posterior segment of the eye from the rest of the body, in particular from inflammatory cells.

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CD40, a cell surface receptor that belongs to the TNF-receptor family, is expressed on a wide variety of cells other than bone-marrow-derived cells, such as endothelial cells, fibroblasts, keratinocytes, or epithelial cells. These adherent cells express a functional CD40 whose ligation induces phenotypic modifications and cytokine secretion, as well as stimulation or inhibition of proliferation. Rezai et al. recently reported that normal resting or activated fetal hRPE cells did not induce allogeneic responses probably because they did not express the costimulatory molecules B7.1 and B7.2, but they did not investigate CD40 expression. The purposes of our study were to examine CD40 expression on normal resting or activated adult hRPE cells and to evaluate its role as an activation molecule by studying three known consequences of CD40-CD40L interactions: induction of costimulatory molecules, upregulation of adhesion molecules, and induction of cytokine secretion. Finally, we evaluated the potential consequences of CD40-CD40L interactions on the antigen presentation function of hRPE cells.

**METHODS**

**Cells**

hRPE cells were isolated from human healthy donor eyes as previously described. In brief, after removal of the cornea and the anterior segment, the vitreous and the sensory retina were dissected and discarded. The eyecup was then incubated four times for 15 minutes at 37°C in trypsin to allow dissociation of the hRPE cells. Cells recovered from the last three incubations were plated in 3-cm petri dishes at 100,000 cells/dish in Dulbecco’s modified Eagle’s medium (GIBCO–BRL, Life Technology, Merelbeke, Belgium) supplemented with 10% fetal calf serum (FCS; GIBCO), penicillin 100 U/ml, and streptomycin 100 μg/ml (GIBCO). At confluence, cells were trypsinized and resuspended in larger culture flasks at 500,000 cells/flask. hRPE cells were used between passages 3 and 5. The purity of the hRPE cultures was evaluated by morphologic criteria and by immunohistochemistry for positive staining with antibodies against cytokeratins 7, 8a/b, AE-1, AE-3, and CAM-52.

Mouse 3T6 fibroblasts transfected with the CD40L cDNA (CD40L/3T6 cells) and control 3T6 nontransfected fibroblasts were cultured in RPMI 1640 medium (GIBCO), supplemented with 10% FCS, 1% glutamine, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. G418 (Geneticin; Sigma Chemical, Bornel, Belgium) at 200 μg/ml was added to the culture medium of CD40L/3T6 cells.

Human peripheral blood mononuclear cells (PBMCs) of healthy donors were isolated from buffy coats by density gradient centrifugation on Lymphoprep solution (Nycomed, Oslo, Norway). T lymphocytes were then purified by Lymphokwik T (One Lambda, Los Angeles, CA) according to the manufacturer’s instructions. Their purity was greater than 98%.

DCs were generated from adherent PBMCs by culture in the presence of GM-CSF and IL-4, as previously described.

**hRPE Activation**

Experiments were performed on nonactivated or activated hRPE cells. hRPE cells were activated by 72 hours’ incubation with 500 U/ml IFNγ (but also scaled from 100 to 1000 U/ml in some experiments). Activation was also done in presence of various other stimuli: lipopolysaccharide (LPS; 100 ng/ml), IFNγ plus LPS, GM-CSF (10,000 U/ml), IL-1 (30 U/ml), or TNFα (50 U/ml), all from Biosource Europe SA (Nivelles, Belgium).

**Flow Cytometry**

The expression of MHC class II (HLA-DR), B7.1 (CD80), B7.2 (CD86), CD40, CD54 (ICAM-1), and CD58 (leucocyte function-associated antigen [LFA]-3) molecules on hRPE cells was quantified by flow cytometry analysis with mouse anti-human fluorescein isothiocyanate (FITC)– or phycoerythrin (PE)-conjugated specific antibodies and control isotypes (all from Becton–Dickinson, Mountain View, CA). Cells were prepared according to standard procedures. Briefly, 500,000 cells were washed and incubated for 10 minutes at 4°C in phosphate-buffered saline containing 0.1% NaN3, 1% bovine serum albumin, and 10% human serum, to inhibit subsequent nonspecific labeling after antibody binding to fragment crystalline receptors (FcR). The cells were then incubated with saturating amounts (1 μg/10⁶ cells) of FITC- or PE-conjugated antibodies directed against specific surface antigens, for 30 minutes at 4°C, in the dark. Cells were then washed and resuspended in staining buffer before being analyzed using a FACScan flow cytometer and the LYSIS II software (Becton–Dickinson).

**CD40 Ligation**

For CD40 stimulation experiments, 200,000 hRPE cells were first pretreated during 72 hours at 37°C with 500 U/ml IFNγ or medium (as control). The CD40 expression was evaluated by fluorescence-activated cell sorter analysis. CD40 ligation assays were performed in two different ways: either by incubating 200,000 hRPE cells with 1 μg/ml soluble CD40L (Immunex, Seattle, WA), or by coculturing 200,000 hRPE cells with 50,000 transfected CD40L/3T6 or control nontransfected 3T6-irradiated mouse fibroblasts. All of the CD40 stimulation assays were done during 48 hours at 37°C, in the presence or absence of 500 U/ml IFNγ. Culture supernatants were then harvested and tested by enzyme-linked immunosorbent assay (ELISA) for their content of various cytokines. hRPE cells were trypsinized and prepared for flow cytometry analysis as previously described.

**Cytokine Secretion**

Human IL-6, -8, -10, and -12 were quantified in serial dilutions of hRPE culture supernatants by using Cytoscreen cytokine ELISA kits (all from Biosource Europe SA), according to the manufacturer’s instructions.

**T-Lymphocyte Activation**

Allogeneic purified T lymphocytes (500,000 cells/ml) were cocultured, in the absence or presence of phytohemagglutinin (PHA; at 5 μg/ml), with irradiated (30 Gy) hRPE cells (500,000 cells/ml) previously activated during 72 hours at 37°C with 500 U/ml IFNγ (or medium as control), or supernatants from hRPE cultures, either activated or not. The cells were cultured in 96-well flat-bottomed tissue-culture plates, in a total volume of 200 μl RPMI 1640 supplemented with 10% FCS, 1% glutamine, 1% nonessential amino acid, penicillin 100 U/ml, and 100 μg/ml streptomycin, and 5 × 10⁻⁵ M of 2-mercaptoethanol. T-cell proliferation was measured on day 8, by thymidine incorporation, after an 18-hour pulse with [³H]-thymidine (1 μCi/well).
Measurement of Caspase-3 Activity in RPE/T-Cell Cocultures

Allogeneic purified T lymphocytes (1,500,000 cells) were cocultured for 24 hours, in the presence of PHA (at 5 μg/ml), with irradiated (30 Gy) hRPE cells (150,000 cells) in 24-well flat-bottomed tissue-culture plates, in a total volume of 2 ml RPMI 1640 supplemented with 10% FCS, 1% glutamine, 1% nonessential amino acid, 100 U/ml penicillin, and 100 μg/ml streptomycin, and 5 x 10^-5 M of 2-mercaptoethanol. RPE and T cells were then collected by pipetting and gentle trypsinization. The level of caspase-3 activity was determined using the Ac-DEVD-AMC, caspase 3 (CPP 32) fluorogenic substrate (Pharmingen, San Diego, CA), according to the manufacturer’s instructions. Briefly, cells were washed in Hanks’ balanced salt solution and suspended in 200 μl of cell lysis buffer. Cells were incubated on ice for 8 minutes; then 100 μl of the lysate was added to 100 μl of protease buffer and 5 μl of Ac-DEVD-AMC, followed by 2 hours of incubation at 37°C. AMC (7-amino-4-methylcoumarin) cleaved was measured using a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength range of 430 to 460 nm.

RESULTS

CD40 but No B7.1 or B7.2 Expression on IFNγ-Activated hRPE Cells

The expression of MHC class II (HLA-DR), CD40, B7.1 (CD80), B7.2 (CD86), CD54 (ICAM-1), and CD58 (LFA-3) molecules was quantified by flow cytometry analysis on resting and activated cultured hRPE cells. Figure 1 shows that HLA-DR expression, which was undetectable at resting state, was strongly induced, as described previously, by treating hRPE cells with IFNγ.7,8 Similarly, there was no constitutive expression of CD40 molecules at the surface of cultured resting hRPE cells; CD40 expression was markedly induced by 72 hours’ stimulation of hRPE cells with IFNγ, in a dose-dependent manner (see Fig. 2). However, neither B7.1 (CD80) nor B7.2 (CD86) was expressed on hRPE cells, even after their stimulation with IFNγ. None of the other cytokines that we have used (LPS, IL-1, TNFa, or GM-CSF) alone or in combination to activate hRPE were able to induce CD40, B7.1, or B7.2 expression (data not shown). The adhesion molecule CD54 was spontaneously expressed on hRPE cells and upregulated by IFNγ treatment. In contrast, hRPE cells never expressed the CD58 adhesion molecule. hRPE cells did not express CD40L molecules in any of the experimental conditions tested (data not shown).

CD40 Ligation on hRPE Cells: Failure of Induction of Expression of B7.1 and B7.2

To test whether CD40 engagement was able to induce B7.1 or B7.2 expression, or to upregulate HLA-DR, CD40, or CD54 expression at the surface of hRPE cells, we triggered activated hRPE cells expressing the CD40 molecule by incubation with soluble CD40L or by coculture with CD40L/3T6-transfected mouse fibroblasts. First, incubation of activated hRPE cells with soluble CD40L (Fig. 3) showed no induction of B7.1 and B7.2 expression. Moreover, HLA-DR, CD40, and CD54 were not upregulated by CD40 engagement. In some experiments, however, incubation with soluble CD40L induced a slight decrease of HLA-DR expression on cultured hRPE cells. Similar data were obtained from experiments performed with CD40L/3T6-transfected fibroblasts (data not shown). Control experiments, performed using the same reagents on human DCs, showed upregulation of HLA-DR, CD40, B7.1, and B7.2 molecules as illustrated by typical values of mean fluorescence intensity: 641, 166, 19, and 210, respectively, before CD40L stimulation and 1465, 310, 79, and 1189, respectively, after CD40L stimulation.
CD40 ligation on hRPE Cells: Absence of IL-12 Secretion but IL-6 and IL-8 Enhanced Production

One of the major consequences of CD40 ligation on APCs is to induce IL-12 secretion.26 The CD40 signaling pathway is also known to play a role in other cytokine regulation.19,20 Therefore, we tested IL-6, -8, -10, and -12 production by hRPE cells either resting nonactivated, or activated by IFNγ, or triggered by CD40L. As shown in Figure 4, cultured resting hRPE cells were found to produce IL-6 and IL-8 spontaneously, but not IL-12 or IL-10 (or beyond detectable level). IFNγ activation of cultured hRPE cells did not induce IL-12 secretion but had opposite effects on IL-6 (upregulation) and IL-8 (downregulation) production. On CD40 ligation, activated hRPE cells still did not secrete IL-12 but drastically upregulated IL-6 and IL-8 production. The same results were obtained using either soluble CD40L or CD40L/3T6 transfected fibroblasts. As positive controls for IL-12 secretion, we used supernatants from LPS-stimulated human immature DCs.25 IL-10 secretion was never observed, whereas it was clearly positive in hRPE cells transduced with a retroviral vector carrying the human IL-10 cDNA, used as positive control.

Absence of Allogeneic Resting T Cells Stimulation by hRPE Cells

Although expressing CD40 molecules, hRPE cells neither expressed B7.1 or B7.2 nor secreted IL-12 on CD40 triggering. These characteristics let us suspect that hRPE cells were non-professional APCs unable to activate resting T cells. As shown in Figure 5, results from mixed lymphocytes allo-reactions confirmed this hypothesis: purified normal T lymphocytes cocultured 8 days with resting or IFNγ-activated allogeneic hRPE cells were not induced to proliferate, as evaluated by [3H]-thymidine incorporation assays. In contrast, PHA stimulation of responder T lymphocytes induced cell proliferation, showing their intrinsic capability to respond.

Inhibition of Allogeneic Activated T Cells Proliferation by hRPE Cells

Figure 5 shows also that when T cells were activated with PHA at the same time they were cocultured with allogeneic resting hRPE cells, their proliferation rate was strongly decreased compared with incubation with PHA alone. Coculture in the presence of hRPE cells pretreated with IFNγ was associated with a somewhat more profound inhibition. Moreover, when acti-
Our study demonstrates that CD40 was expressed on hRPE cells when stimulated by IFNγ. Despite CD40 and MHC class II expression, our results show that adult stimulated hRPE cells expressed neither B7.1 or B7.2. Moreover, even under CD40 triggering, one of the powerful way to induce B7 in different systems, hRPE cells still did not express B7 molecules. The absence of B7.1 and B7.2 expression on adult hRPE cells is in agreement with the work of Rezai et al., in which they found neither B7.1 nor B7.2 expression on fetal hRPE cells, although no information was provided on CD40 expression on those cells. We, thus, found on adult IFNγ-activated hRPE cells the coexpression of MHC class II and CD40, but B7.1 and B7.2 expression was not seen. This phenotype is not limited to hRPE cells and is also shared by Langerhans cells from the hair follicle, Grave’s thyroid epithelial cells, and gingival fibroblasts. The inability of hRPE to express either B7.1 or B7.2 suggests that they would not be able to act as conventional APCs. Antigen presentation in the absence of B7 costimulation may result in a deviant antigen presentation, leading in fact to ignorance, anergy, or apoptosis. However, there is growing evidence that T-cell activation may also occur through an alternate pathway involving CD40–CD40L stimulation. For example, stimulation of CD40 may induce BB-1 or CD44H surface expression on fibroblasts and Chinese hamster ovary cells, respectively. Both are able to costimulate T-cell proliferation and are distinct from B7.1 and B7.2. One other alternative pathway, which could be implicated in a B7-independent T-cell activation by hRPE cells, is the CD2–CD58 pathway. Nonetheless, our finding of an absence of CD58 expression makes this unlikely, although Liversidge et al. found that rat RPE cells may stimulate CD2 on T cells through CD48 and CD59. Actually, controversy exists over these molecules as ligands for CD2.

Induction of T-Cell Apoptosis by hRPE Cells

We finally tested the induction of apoptosis in T lymphocytes after their interaction with hRPE cells. To address this question we measured the activity of caspase-3, a sensitive method that has been previously used by others to detect apoptosis in keloid fibroblasts. Table 1 shows that caspase-3 activity was detected after 24 hours, only in cocultures of activated T cells and RPE cells, suggesting some T-cell apoptosis.

**Table 1.** Allogeneic hRPE Cells Induced Some T-Cell Apoptosis

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Caspase Activity, μM</th>
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</thead>
<tbody>
<tr>
<td>RPE− or RPE+</td>
<td>&lt;1</td>
</tr>
<tr>
<td>T Ly PHA</td>
<td>1.3</td>
</tr>
<tr>
<td>T Ly PHA and RPE−</td>
<td>8.5</td>
</tr>
<tr>
<td>T Ly PHA and RPE+</td>
<td>5.3</td>
</tr>
<tr>
<td>T Ly PHA and Sup RPE−</td>
<td>1.1</td>
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<tr>
<td>T Ly PHA and Sup RPE+</td>
<td>1.1</td>
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</table>

T lymphocyte (T Ly; 1,500,000 cells/well) activated by PHA were cocultured during 24 hours with hRPE cells (150,000 cells/well), IFNγ-activated (RPE+), or not (RPE−) and with supernatant from RPE culture (sup RPE−, sup RPE+). Apoptosis is reflected by the induction of caspase-3 activity. Caspase-3 activity is measured by cleavage of the fluorogenic substrate Ac-DEVD-AMC. Data are expressed in moles of AMC cleaved by caspase-3. Results are from one representative experiment of two.
cells could activate T cells, it is unlikely that they would induce a Th1 response, which is usually associated with autoimmune diseases, like experimental autoimmune uveitis.\(^{57}\)

In contrast to IL-12, IL-6 and IL-8 secretions were upregulated after CD40 ligation. IL-6 and IL-8 are not implicated as is IL-12 in antigen presentation and Th1 cell pathway but play a role in inflammatory cell activation and chemoattraction. IL-6 stimulates B and T cell differentiation. IL-8, a C-X-C family chemokine, is chemotactic for neutrophils, lymphocytes, eosinophils, and stimulates diapedesis of T cells. Secretion of these cytokines after stimulation of CD40 by CD40L has been described in fibroblasts and monocytes.\(^{20,38}\) In hRPE cells, IL-8 secretion is polarized toward the basal side and induced in response to lymphocyte products.\(^{39,40}\) Our results show that soluble molecules are not the only way to increase the secretion of IL-6 and IL-8 by hRPE cells and suggest that direct cellular interactions are also playing a role. This result may have implications in uveitis or in proliferative vitreoretinopathy in which an infiltration of the retina by lymphocytes has been demonstrated.\(^2\) Similarly, it could also be implicated in proliferative diabetic retinopathy. IL-8 has, indeed, been found in the vitreous of patients with this disorder and has been identified as an angiogenic agent.\(^{41}\)

Aldogether, the absence of B7.1 and B7.2 expression and IL-12 secretion suggests that hRPE cells would not be able to activate resting T cells. This is supported by our T-lymphocyte activation assays, which clearly showed that nonactivated T cells did not proliferate in hRPE cell coculture. Rezai et al.\(^{23}\) also found that fetal hRPE cells did not induce a significant alloimmune response. These authors could stimulate T cells only with the potent superantigen SEA, which is known to bypass the costimulatory pathway.\(^{42}\)

Hence, the absence of B7.1 and B7.2 expression and IL-12 secretion could implicate hRPE cells in a deviant antigen presentation, leading to T-lymphocyte anergy or apoptosis.\(^{43}\) This hypothesis is supported by our results showing that activated lymphocytes were strongly inhibited by hRPE cells. Similarly, Liverisgide et al.\(^{51}\) demonstrated that in addition to being poor presenters of antigen hRPE cells could suppress lymphocyte proliferation, even in the presence of professional APCs, through the action of transforming growth factor-\(\beta\), prostaglandin E\(_2\), and an unidentified membrane bound component. CD40 could be a good candidate for the latter. Moreover, the increase of caspase-3 activity we measured after cocultures of RPE and activated T cells supports the hypothesis of some T-cell apoptosis. This is in agreement with other work done on hRPE cells and on human fetal RPE cells, in which RPE induction of T-cell apoptosis has also been described.\(^{44,45}\) If the exact molecular mechanisms are still unclear, it is likely that CD40–CD40L interaction could play a role in this process, as it has recently been shown in other cells.\(^{46}\)

**Acknowledgment**

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**References**


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