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Human iris pigment epithelium suppresses activation of bystander T cells via TGFβ–TGFβ receptor interaction

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ABSTRACT

Iris pigment epithelial (IPE) cells from the anterior segment in the eye are able to suppress activation of bystander responder T cells in vitro. The cultured IPE cells fully suppress proliferation and cytokine production by responder T cells via direct cell-to-cell contact. We have now investigated whether primary cultured human iris pigment epithelial (h-IPE) cells that were established from fresh iris tissues can also inhibit the activation of T cells in vitro. We found that cultured h-IPE cells significantly inhibited T cell proliferation and the IFN-γ production by the target T cells from both the allogeneic and autogeneic peripheral blood mononuclear cells (PBMCs). The h-IPE cells also inhibited the activation of $CD4^+$ T cells from patients with active uveitis. The suppression by h-IPE occurred in a completely contact-dependent manner. The h-IPE constitutively expressed transforming growth factor β (TGFβ) and the receptors, and the T cells exposed to h-IPE greatly expressed Smad transcripts. In addition, TGFβ2-siRNA transfected h-IPE failed to inhibit activation of responder T cells. Similarly, h-IPE cells in the presence of anti-TGFβ neutralizing antibodies or recombinant TGFβ receptor blocking proteins failed to inhibit the T-cell activation. In conclusion, cultured human iris pigment epithelium fully inhibits T cell activation in vitro. Our data support the hypothesis that the ocular resident cells play a critical role in immunosuppression in the eye.

1. Introduction

Murine ocular pigment epithelial (PE) cells cultured from different ocular tissues display the capacity to inhibit T cell activation (Streilein, 2003; Sugita and Streilein, 2003; Zamiri et al., 2005; Sugita et al., 2006a). The iris PE (IPE) from the anterior segment also inhibits T cell activation primarily via direct cell-to-cell contact (Streilein, 2003; Sugita and Streilein, 2003). The immunoregulatory cell-surface molecules, which are expressed on murine IPE, play a key role in enabling these ocular cells to inhibit the functional properties of the T cells when they encounter these epithelial cells (Sugita and Streilein, 2003). The IPE cell monolayer covers the posterior surface of the iris and lines the ciliary body that surrounds and extends posteriorly beyond the lens. Various laboratories have demonstrated that the PE of the iris, as well as the ciliary body (CBPE) and retinal PE (RPE) display immunomodulatory features that suggest that this layer of cells contributes to the ocular immune privilege (Jorgensen et al., 1998; Streilein, 2003; Sugita and Streilein, 2003; Sugita et al, 2006a,b; Yoshida et al., 2000; Zamiri et al., 2005). For example, CD86 costimulatory molecules, which are uniquely expressed on murine IPE, bind to the CTLA-4 costimulatory receptors on T cells and inhibit the $CTLA\text{-}4^+$ bystander T cells (Sugita and Streilein, 2003). Freshly explanted RPE, as well as immortalized cell lines from RPE, have been found to secrete immunosuppressive factors (Sugita et al., 2006a; Zamiri et al., 2005), and to express CD95 ligand (CD95L), which triggers apoptosis among $CD95^+$ T cells (Jorgensen et al., 1998). Both the membrane-bound and soluble forms of the transforming growth factor β (TGFβ) are highly expressed on the murine PE cells (Yoshida et al., 2000; Sugita et al., 2006b) and on other ocular tissues (Pasquale et al., 1993; Wilson and Lloyd, 1991), and help to regulate the immune response. If ocular PE cells do contribute to the immune privilege, and if the loss of this immune privilege leads to ocular inflammation, then broad therapeutic implications should result from being able to elucidate the contributions that ocular PE cells make to the immune privilege.

In an effort to understand the molecular bases of the immunosuppressive properties of the ocular PE in immune privileges, we examined whether human IPE (h-IPE) can inhibit the activation of bystander T cells in vitro via a cell contact-dependent mechanism. We succeeded in establishing a primary culture of h-IPE cells from fresh human iris tissue. Our experimental evidence further

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indicates that TGFβ2, a powerful immunoregulatory factor, is constitutively expressed on the h-IPE and, through contact with the TGFβ receptor II (RII)$^+$ T cells, promotes suppression of their activation in vitro similar to that seen for murine IPE cells.

2. Material and methods

2.1. Culture media

Primary cultures of pigmented epithelial cells from human iris (h-IPE) were cultured in RPMI 1640 complete medium composed of RPMI 1640, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20% fetal bovine serum (FBS) (all from BioWhitaker, Walkersville, MD), and 1×10^{-5} M 2-ME (Sigma–Aldrich, St. Louis, MO). Serum-free media were used in the cultures and in the assays involving T cells that were stimulated by anti-CD3 antibodies in order to mimic as closely as possible the intraocular microenvironment inside the blood–ocular barrier. The serum-free media was composed of the RPMI 1640 complete media without the addition of FBS, and supplemented with 0.1% bovine serum albumin (0.1% BSA, Sigma) and 0.2% insulin, transferrin, selenium culture supplement (ITS$^+$) (Collaborative Biochemical Products, Bedford, MA).

2.2. Preparation of cultured human iris pigment epithelial cells

Iris samples were collected after informed consent was obtained from each patient. Iris tissue from a patient with open angle glaucoma was obtained when the patient had trabeculectomy. None of the glaucoma patients had any clinical history of uveitis or systemic diseases. Peripheral iridectomy (about 3×3 mm) was performed in the eye of each patient, with the iris tissue obtained immediately placed in RPMI complete medium. IPE cell cultures were maintained using a modification of a method previously reported for murine IPE (Sugita et al., 2004, 2006a,b). Iris tissue was incubated in PBS containing 1 mg/ml Dispase and 0.05 mg/ml DNaseI (both from Boehringer–Mannheim, Mannheim, Germany) for 2 h. Thereafter, these tissues were placed into 24-well plates and incubated at 37 °C for 4–8 weeks. The medium was changed every five days. All samples were collected after informed consent was obtained from the patients. The research followed the tenets of the Declaration of Helsinki. The Institutional Ethics Committees of Tokyo Medical and Dental University approved this study.

2.3. Flow cytometry with anti-cytokeratin antibodies

Cultured human iris PE cells were stained with anti-pan cytokeratin antibody (Clone PCK-26, Sigma) at the completion of the 28-day primary culture. Before staining, the co-cultured cells were incubated with Fc-receptor block (Miltenyi Biotec, Auburn, CA) for 15 min. The cultured cells were fixed and permeabilized with BD Cytofix/Cytoperm Kits (BD PharMingen, San Diego, CA), incubated with monoclonal anti-human pan cytokeratin antibody (mouse IgG, Sigma) or control mouse IgG isotype at 4 °C for 30 min, and then washed twice. The bound primary antibody was detected with a biotin conjugated anti-mouse IgG (BD PharMingen) antibody and FITC-conjugated streptavidin (BD PharMingen).

2.4. Establishment of target bystander T cells

Target-activated T cells were established from autogeneic or allogeneic T cells from peripheral blood mononuclear cells (PBMCs) of healthy volunteers. T cell growth factors such as X-irradiated (50 Gy) allogeneic PBMCs as the feeder cells, human recombinant IL-2 (rIL-2), and PHA-P (Difco Labs Inc., Detroit, MI) were used. The medium for the culture was RPMI 1640 complete medium. The feeder cells were added to each of the wells along with rIL-2 every 7 days until an outgrowth of cells was observed. The culture period for the T cells was about four weeks. As alternative targets, T cell clones (TCCs) of uveitis patients with Vogt–Koyanagi–Harada disease were established from the ocular fluid obtained via limiting dilution (Sugita et al., 1996, 2006c). All samples were collected after informed consent was obtained from the patients.

For anti-CD3-driven T cell activation, established T cells were added (1.0×10^5 cells/well in a 96-well plate or 5.0×10^5 cells/well in a 24-well plate) to culture wells with and without h-IPE cells. The h-IPE cells were seeded directly into flat-bottomed 96-well plates (2.0×10^4 per well) or 24-well plates (2.0×10^5 per well). T-cell activation was assessed for proliferation by [^{3}H]-thymidine incorporation or IFN-γ production by the target T cells. Anti-human CD3ε antibody (1.0 μg/ml, BD PharMingen) was added to the wells, with the cultures then maintained for 72 h. After 72 h incubation, the cultures were assayed for the uptake of the ^{3}H-thymidine (1 μCi/ml), which was added during the terminal 8 h of the cell culture proliferation period. The T cell supernatants that were exposed to h-IPE were evaluated with IFN-γ ELISA (R&D Systems, Minneapolis, MN). Culture supernatants were collected at 24 h after exposure of the T cells to h-IPE cells, and immediately frozen and stored at −20 °C until use. ELISA was carried out according to the manufacturer's instructions (R&D Systems).

2.5. RNA extraction and PCR analysis

Cellular extracts were prepared from the cultured primary h-IPE or from T cells exposed to h-IPE that were cultured as described above. Enriched T cells obtained from PBMCs of healthy donors were added to cultures of primary h-IPE as described above, but were incubated for 24 h. The cultured h-IPE and T cells were washed twice with PBS, with the total RNA then isolated with Trizol reagent (Invitrogen–Life Technologies, Carlsbad, CA). After cDNA synthesis, PCR was carried out using a standard PCR method. The products were subjected to 35 cycles of PCR amplification. Primers for human TGFβ1 (which resulted in an amplification product of 442 bp), TGFβ2 (which resulted in an amplification product of 279 bp), and TGFβ3 (which resulted in an amplification product of 236 bp) were used as the primer-pair kits in the R&D systems. TGFβ receptor I (TGFβ RI) was 5′-ACGGCGTTACAGTGTTCTG-3′ and 5′-GGTGTGGCAGATATATAGACC-3′, which resulted in an amplification product of 358 bp. TGFβ receptor II (TGFβ RII) was 5′-AGCAACTG-CAGCATCACCTC-3′ and 5′-TGATGTCTGAGAAGATGTCC-3′, which resulted in an amplification product of 688 bp. The forward and reverse primers used for β-actin were the same as has been described previously (Sugita et al., 2006c). The PCR products were electrophoresed in 1% or 1.5% agarose gel and visualized by staining with ethidium bromide.

Cellular extracts were also prepared from T cells exposed to h-IPE, and then cultured to examine the TGFβ signal pathway, Smad2, 3, 4, and 7. The T cells were washed twice with PBS, with the total RNA then isolated with Trizol reagent. After RNA extraction and cDNA synthesis, the products were subjected to 30–35 cycles of PCR amplification. Smad2 was 5′-ATCCTAACAGAACTTCCGCC-3′ and 5′-CTCAGCAAAAACTTCCCCAC-3′, which resulted in an amplification product of 489 bp. Smad3 was 5′-CAGAACGTCAACACCAAGT-3′ and 5′-ATGGAATGGCTGTAGTCGT-3′, which resulted in an amplification product of 308 bp. Smad4 was 5′-GCATCGACAGAGACATACAG-3′ and 5′-CAACAGTAACAATAGGGCAG-3′, which resulted in an amplification product of 484 bp. Smad7 was 5′-GCCCTCTCTGGA-TATCTTC-3′ and 5′-GCTGCATAAACTCGTGGTCA-3′, which resulted in an amplification product of 320 bp. The PCR products were electrophoresed in 1% or 1.5% agarose gel and visualized by staining