·Basic Research·

Preliminary findings of immunological characteristics in primary human corneal endothelial cells

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Abstract

• AIM: To evaluate the function of primary human corneal endothelial cells (HCEC) serving as immunological cells.

• METHODS: Expression of HLA-DP, -DQ, -DR, CD40, CD80, and CD86 was determined by immunohistochemical methods. Meanwhile, purified peripheral blood mononuclear cells (PBMC) were cocultured with primary HCEC which were pre-treated with and without γ-IFN respectively. The activation of lymphocytes was determined by FACS analysis.

• RESULTS: In coculture system, T lymphocyte was activated by primary HCEC, HLA-DP, -DQ, -DR and CD40 expression were increased by γ-IFN induction. Costimulatory molecular CD80 was shown on the endothelial cells.

• CONCLUSION: Primary HCEC are assumed to be involved in the corneal transplantation rejection process as potential antigen presenting cells (APC).

• KEYWORDS: antigen presenting cells; primary human corneal endothelial cells; peripheral blood mononuclear cells; costimulatory pathway; FACS analysis

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INTRODUCTION

Prevention and therapy of allograft rejection is still one of the most challenging fields of present keratoplasty. It has been concluded that corneal graft rejection, a T cell mediated immune process, is mainly dependent on activation of alloreactive T lymphocytes [1,2]. The human leukocyte alloantigens (HLA) are known to be the main targets for the first signal in immune reaction [3]. Besides T cell activation, the development of an effective immune response also requires a costimulatory signal. This costimulatory signal is mediated by B7-1 (CD80) or B7-2 (CD86) interaction with their receptors CD28 and CTLA4 on the surface of T cells. Other T cell surface molecules, such as CD154 (receptor of CD40), CD2, LFA1 and ICAM1, contribute to the second signal. It is known that corneal endothelial rejection occurs more frequently than epithelial rejection, leading to the hypothesis that the interaction between the corneal endothelial cells and infiltrating lymphocytes is involved in the mechanism of corneal graft rejection.

MATERIALS AND METHODS

Coculture of HCEC and PBMC The cultures of primary human corneal endothelial cells (HCEC) were established in the laboratory as described elsewhere [4]. Approximately 15mL blood was collected from healthy human volunteer donors with informed consent according to the ethical standards of the Institutional Review Board on human experimentation. 10mL Histopaque-1077 were overlaid with 10mL fresh blood, centrifuged at 400g for 30 minutes. The opaque interface was centrifuged at 250g for 10 minutes. The cell pellet was resuspended in RPMI 1640 medium (Gibco Invitrogen) supplemented with 2mmol/L L-glutamine, 1mmol/L Hepes solution (Gibco), 100mL/L fetal calf serum (Gibco) and penicillin streptomycin (50IU/mL). After each preparation, cell integrity and cell number were evaluated by Trypan Blue dye exclusion test. Fresh peripheral blood mononuclear cells (PBMC) preparations were used for each repetitive experiment.

Immunohistochemical Staining 2×10^4 primary HCEC were seeded into chamber slides (Nunc LabTek). Parts of the cells were supplied with 1 000U/mL γ-IFN. After three days, freshly purified PBMC (1×10^6) and anti-CD28 monoclonal antibody (1mg/L, Pharmingen International) were applied to the cell cultures. PBMC cultured with

immobilized anti-CD3 antibody (4mg/L, Pharmingen international) and anti-CD28 antibody served as positive control, PBMC cultured without any supplement served as negative control. All cocultures were maintained in RPMI 1640 medium applied with 100mL/L fetal calf serum, and incubated for further 2 days. Then the cocultured PBMC cell suspension was harvested for FACS analysis, while the primary HCEC attaching on the chamber slides and residual PBMC adherent were fixed with 700mL/L ethanol in 50mmoL glycin buffer or 40g/L formaldehyde. The cells were incubated with monoclonal antibodies against CD80 (Ancell cooperation, U.S.A), CD86 (DAKO, Denmark) molecular respectively. Each antibody was diluted as 1:100 and cells were incubated at room temperature for 30 minutes. The cells which did not show first antibody were served as negative control. Combined antibody was detected using LSAB-2 kit (Dako Cytomation) according to the manufacturer's instructions.

Primary HCEC without any pre-treatment were fixed likewise and also analyzed immunohistochemically for expression of HLA-DP, -DQ, -DR (Dako, Denmark), CD40 (DPC, Biermann) as described above. Staining was rated as: - (negative), + (weak, <25%), ++ (intermediate, 25%-50%), +++ (strong, 50%-75%), ++++ (very strong, 75%-100%).

FACS Analysis Two-color analysis was performed by means of FACS analyzer (Becton-Dickinson FACS-Calibur). Cocultivated cell suspension were analyzed for expression of CD3 (T lymphocytes) and CD69 (activated T cells) using anti-CD3FITC and anti-CD69PE monoclonal antibodies (Pharmingen International) to quantify the percentage of activated T lymphocytes (control isotypes from Becton-Dickinson). Cells were incubated with saturating amounts (1μg/106 cells) of anti-CD3FITC and anti-CD69PE antibodies or isotype control antibody for 30 minutes at 4℃ in the dark. CellQuest software (Becton Dickinson) was applied for calculation.

RESULTS

HLA and CD40 Expression on HCECHL A class-II antigens play a major regulatory role in interaction between immunocytes and represent the first signal pathway in immunological recognition and rejection processes. Without inducement of γ-IFN, there was no staining observable on corneal endothelial cells. After stimulation with γ-IFN, upregulation of HLA-DP, -DQ, -DR antigen expression was shown, more than 95% of the primary HCEC presented positive staining. In most of these samples, positive staining could be observed either on the cell surface or in the cytoplasm. Likewise, γ-IFN treatment further enhanced CD40 expression as well as HLA-DP, -DQ, -DR.

FACS Analysis Scatter regions for gating of lymphocytes and monocytes based on their morphologic features (forward scatter for size) and staining by propidium iodide were initially optimized and thereafter used for all FACS analyses. Expression of CD69 as a marker for activated T lymphocytes was measured after cocultivation of PBMC with primary HCEC; 10% activated lymphocytes were harvested in cocultured primary HCEC and PBMC system. Similarly, 11% CD69 positive lymphocytes were detected in PBMC cocultured with γ-IFN pre-stimulated primary HCEC.

CD80, CD86 Expression in Coculture System CD80/CD86, CD40 and its receptor CD154 are the ligands of the second or costimulatory signal additional to HLA signalling, which is required for T cell activation, proliferation and cytokine secretion. Almost 50% CD80 positive staining was observed on corneal endothelial cells with and without γ-IFN inducement, no CD86 positive endothelial cells were found.

DISCUSSION

In the clinical field, it has been shown that HLA class-II antigen expression appears to be limited to Langerhans cells and endothelial cells lining limbal blood vessels. However, HLA antigens are also expressed on other corneal cells after allograft rejection[5]. The CD154:CD40 pathway is important in the generation of cell-mediated immunity and has been recognized as a key pathway for T cell activation as well. CD154 binds to CD40, induces a signaling pathway that leads to increased expression of CD80 and CD86 [6-8]. Accordingly, blocking the CD40:CD154 pathway was demonstrated to be an effective prolongation in graft survival in several animal transplantation models [9-12]. The evidence highlight the role of the B7:CD28 pathway of T cell costimulation in transplant rejection: drugs that block the interaction between B7 and CD28 molecules can induce long-lasting non-responsiveness of T cells to alloantigens in vitro. Also, combined administration of anti-CD80 and anti-CD86 monoclonal antibodies after orthotopic corneal allograft transplantation was effective in prolonging corneal allograft survival[13,14].

In present study, the contact between PBMC and primary

HCEC could boost costimulatory pathway. FACS analysis further demonstrated that primary HCEC stimulated human T lymphocytes to express CD69 at a higher level. It could be concluded that T cell receptor recognize the peptide/HLA complex on the corneal endothelial cells to get signal 1, the CD28 antibody was provided in the system bind to CD80 molecules on corneal endothelial cells. Both signal 1 and signal 2 lead to a positive signal to stimulate T cell activation and proliferation. The same mechanism is as well as CD40-CD154 second pathway. Our results indicated primary HCEC functioned as potential APC, the induced HLA class-II alloantigen expression on the corneal endothelium may be a contributing factor in the rejection of corneal allografts, the alloimmunological reaction could be triggered by HCEC.

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