Effect of Mycophenolic Acid and Bortezomib on Purified Human B Cells: An In Vitro Study of Long-Term Functionally Stable MICA-Sensitized Renal Recipients

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Abstract

Objectives: We sought to develop a B-cell in vitro culture system and test B cells isolated from sensitized kidney recipients and healthy controls, and assess the effectiveness of proteasome inhibitors and mycophenolic acid on antibody secretion and cell apoptosis.

Materials and Methods: CD19+ B cells and CD19+CD27+ memory B-cell subsets were detected from peripheral blood mononuclear cells obtained from 6 MICA-sensitized kidney recipients and 6 healthy controls. Peripheral blood B cells were isolated and cultured with CpG2006, PMA, MICA antigen, B-cell activating factor, CD40 ligand (CD40L), human recombinant IL-2 (rhuIL-2), rhuIL-10, rhuIL-4, and rhuIL-21. After culturing for 7 days, we tested several variables of B-cell activity including differentiation, apoptosis, and IgM production. We also assessed the effects of 2 immunosuppressive drugs (mycophenolic acid and bortezomib) on antibody secretion and cellular apoptosis.

Results: Kidney recipients had a lower ratio of CD19+ B cells in peripheral blood mononuclear cells than did healthy controls. However, the percentage of CD19+CD27+ B cells was higher in kidney recipients than in healthy controls. In the cell stimulation culture system, the ratio of CD19+ B cells, CD19+CD27+ B cells, and CD19+CD138+ B cells increased after culturing for 7 days compared with unstimulated controls. In addition, the percentage of apoptotic B cells decreased, and antibody production increased in sensitized transplant patients and healthy controls. Treatment with bortezomib or mycophenolic acid induced B-cell apoptosis and inhibited secretion of antibodies.

Conclusions: This study describes establishment of a B-cell in vitro culture system, showing that B cells may be stimulated to secrete antibodies. The study also provides an assay for studying B cells in vitro. This study provides information suggesting that bortezomib and mycophenolic acid can inhibit B-cell antibody secretion.

Key words: B cells, Humoral rejection, Kidney transplant, Immunoglobulin production

Introduction

Antibodies against major histocompatibility complex class I chain-related gene A (MICA) antigen and human leukocyte antigen after transplant remain barriers to long-term survival after kidney transplants.1–3 Multiple protocols have been developed to facilitate successful transplant of sensitized transplant candidates including treatment of antithymocyte globulin, interleukin-2 receptor antagonists, intravenous immunoglobulin, and anti-CD20 antibody injection.4 However, the effectiveness of these approaches to improve long-term survival is unclear owing to the high incidence of acute humoral rejection during the early stages after transplant,5 as well as antibody-mediated injury owing to formation of transplant glomerulopathy.6,7
The most widely used desensitization protocols currently are not sufficient to prevent antibody production by antibody-secreting B cells.\textsuperscript{8,9} Bortezomib is a proteasome inhibitor first approved by the United States Food and Drug Administration in 2003 to treat multiple myeloma. The drug inhibits tumor proliferation by blocking proteasome activity and inducing apoptosis. Previously, it had been evaluated in several trials against various hematologic malignancies, including multiple myeloma. Studies also have demonstrated an inhibitory effect of bortezomib on the formation of antibodies in treating acute graft-versus-host disease, including kidney transplant.\textsuperscript{10-12} Perry and associates found that bortezomib can induce apoptosis of normal human plasma cells, thereby preventing alloantibody production.\textsuperscript{13} Flechner and associates also found that bortezomib exhibits an antihumoral rejection effect in antibody-mediated rejection.\textsuperscript{14} These studies provide information on the reduction of antibody production; however, it is currently unclear whether bortezomib has a direct effect on B cells. Therefore, we evaluated the potential biological effects of bortezomib together with the commonly used antihumoral rejection immunosuppressive drug, mycophenolic acid (MPA), directly on purified B cells.

**Materials and Methods**

**Ethical considerations**
This study was approved by the Research Subjects Review Board at First People’s Hospital. Written, informed consent was obtained from all participants. The study was performed in accordance with the ethical guidelines of the 1975 Helsinki Declaration. Research data were coded so that the subjects could not be identified, directly or through linked identifiers, in compliance with the Department of Health and Human Services Regulations for the Protection of Human Subjects.

**Blood samples**
Major histocompatibility complex class I chain-related gene A is an endothelial cell surface antigen. It has been reported that presensitization of kidney transplant recipients against MICA antigens is associated with an increased frequency of graft loss, and may contribute to allograft loss among recipients who are well-matched for human leukocyte antigen.\textsuperscript{1} Terasaki and associates also provided evidence showing that similar to human leukocyte antigen antibodies, MICA antibodies are associated with graft failures.\textsuperscript{3} However, there has not been an optimal in vitro system for stimulating B cells to secrete antibodies.

In this study, we assessed the serum from 68 long-term survival kidney recipients using Luminex technology (One Lambda, Inc, Canoga Park, CA, USA), and found 11 subjects who were MICA positive. Six MICA-sensitized kidney transplant recipients agreed to join the study, and 6 health volunteer subjects not receiving a transplant were used as controls (anti-MICA antibodies were present in the recipients’ serum, as detected by Luminex single antigen beads based upon the manufacturer’s instructions). The method used is described in our previous study.\textsuperscript{15} All recipients survived for at least 10 years after the transplant and were taking immunosuppressive agents (Table 1) at the time of blood sample collection. Approximately 20 to 50 mL of heparin-treated (50 U/mL) peripheral blood was obtained from each individual.

**Antibodies and cytokines**
Flow cytometry analysis was performed according to standard protocols. Antihuman CD19-APC, antihuman CD27-PE, and antihuman CD138-PerCP-Cy5 antibodies were obtained from BD Biosciences (BD Pharmingen, San Jose, CA, USA). The cytokines used for cell stimulation were human recombinant IL-2 (rhuIL-2; PeproTech, Rocky Hill, NJ, USA), rhuIL-10 (PeproTech), rhuIL-4 (PeproTech), rhuIL-21 (BD Biosciences), CD40L (Alexis Biochemicals, San Diego, CA, USA), BAFF (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel), and MICA-specific antigen (One Lambda, Inc).

**Isolation of human B lymphocytes**
Peripheral blood mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation. B lymphocytes were enriched by negative selection using magnetic beads (B Cell Isolation Kit, Miltenyi Biotec, Cologne, Germany) according to the manufacturer’s instructions. Briefly, cells were incubated with the biotin-labeled antibody mixture for 10 minutes and then mixed with magnetic microbeads coupled with antibodies against biotin for an additional 15 minutes. The labeled cells were removed by passing them through a magnetic cell sorter column type LS (Miltenyi Biotec) that resulted
in an enriched population of B cells. The purity of the isolated B cells was analyzed by flow cytometry using an APC-conjugated monoclonal antibody against CD19 (eBioscience, Inc., San Diego, CA, USA), and the purity was found to be greater than 95%.

B-cell culture and stimulation

Cells were cultured in RPMI 1640 medium (Invitrogen Corporation, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (Sigma-Aldrich Corp. St. Louis, MO, USA) and 100 U/mL penicillin/streptomycin (Invitrogen) at a density of 1 × 10^5 cells/200 μL in round-bottomed 96-well plates (Costar 3799, Corning Costar, Sigma-Aldrich Corp. St. Louis, MO, USA). For B-cell stimulation, we added CpG 2006 (10 ng/mL; synthesized in Sangon Biotech [Shanghai Sangon Biotechnology, Shanghai, China]), MICA antigen (5 μL/well; One Lambda), PMA (10 ng/mL; Sigma-Aldrich), rhuIL-2, rhuIL-10 (50 ng/mL; Sigma-Aldrich), rhuIL-4 (50 ng/mL), rhuIL-21 (10 ng/mL), CD40L (500 ng/mL), and BAFF (75 ng/mL) to the culture system, and the cells were cultured for 7 days. Different dosages of bortezomib (initial concentration: 200 ng/mL; Millennium) and MPA (initial concentration: 50 ng/mL; Sigma-Aldrich) were then added to the cell culture system to determine the direct inhibitory effects on B cells.

Apoptosis assays

After 7 days of culture, the cells were centrifuged at 1500 rpm for 5 minutes at 4°C and then the supernatant was collected. Cell pellets were resuspended in binding buffer for apoptosis assays. Cells were stained according to the instruction of the FITC-labeled Annexin V Apoptosis Detection Kit I (BD Pharmingen). Briefly, 5 μL Annexin V and 5 μL PI in working solution were added in cell suspension and incubated for 15 minutes at room temperature. The cells were then washed and resuspended in binding buffer for flow cytometry analysis. Results were analyzed using FloJo software (Tree Star, Inc. Ashland, OR, USA).

ELISA assay

Antibody concentrations were detected using an ELISA according to the manufacturer’s protocol. Briefly, supernatants recovered from cell stimulation experiments were tested for IgM levels with a standard sandwich ELISA. Plates were coated overnight with a goat anti-IgM (Southern Biotech, Birmingham, AL, USA) diluted in 10 mM Tris pH 9.0 and then blocked with 2% bovine serum albumin (BSA; Sigma-Aldrich) and 0.025% Tween-20 (Sigma-Aldrich) in phosphate-buffered saline (PBS-T). Fifty microliters of supernatants or standard human serum (Southern Biotech) was serially diluted for 60 minutes at 37°C. After washing with PBS-T, biotin-labeled goat anti-IgM (Southern Biotech) diluted in PBS-T was incubated for 60 minutes at 37°C. After extensive washing, streptavidin horseradish peroxidase (Pierce Protein Biology Products [Thermo Fisher Scientific Inc., Rockford, IL, USA]) diluted in PBS-T that contained 1% BSA/PBS-T was incubated for 60 minutes at 37°C. TMB High Sensitivity Substrate solution was added, and the reaction was terminated by the addition of 2N H2SO4 (Sigma-Aldrich) and measured at a wavelength of OD450 nm in an ELISA reader (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). Data were analyzed using Microplate Manager software version 4 (Bio-Rad).

Statistical analyses

Results were analyzed using GraphPad software (La Jolla, CA, USA). A t test and 1-way analysis of variance were used to analyze the data.

**Table 1. Patient Characteristics**

<table>
<thead>
<tr>
<th>Transplant Patient No</th>
<th>Age (y)</th>
<th>Sex</th>
<th>ABO Blood Type (Donor/Recipient)</th>
<th>Months After Transplant</th>
<th>Immunosuppressive Treatment</th>
<th>Allele Specificity of MICA Antibody</th>
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</table>

**Abbreviations:** CsA, cyclosporine; MMF, mycophenolate mofetil; Pred, prednisone.
A *P* value < .05 was considered statistically significant.

**Results**

The constituent ratio of the B-cell subpopulation in peripheral blood in kidney recipients and healthy controls

Peripheral blood mononuclear cells from kidney recipients had a lower ratio of CD19+ B cells compared with those from healthy controls. Additionally, kidney recipients had a higher percentage of CD19+CD27+ B cells compared with healthy controls. The average percentage of CD19+ B cells in PBMC samples was significantly lower in MICA-sensitized transplant patients than it was in healthy controls (3.58 ± 0.80% vs 8.53 ± 1.04%; *P* < .01). In CD19+ B cells, the percentage of memory B cells (CD19+CD27+) in long-term survival sensitized kidney recipients and healthy controls was 49.55% ± 8.42% and 26.33% ± 5.49% (Figure 1; *P* < .01).

The in vitro B-cell culture system: effects of stimuli on purified B-cell differentiation

Peripheral blood B cells from healthy controls were used to establish an in vitro B-cell culture system. Different B-cell stimuli (toll-like receptor-9 ligand ODN-2006 CpG, MICA antigen, PMA, rhuIL-2, rhuIL-10, rhuIL-4, rhuIL-21, CD40L, and BAFF) were add to the culture system to stimulate the B cells. One major limitation for the in vitro differentiation of B cells is the high rate of spontaneous cell death that occurs as the cells acquire a fully differentiated phenotype. In addition, the proliferation of B cells could be associated with cell death, especially in conditions where the stimulation signal is not optimal. Therefore, to further address these issues, we assessed the percentage of different B-cell phenotypes. After 7 days of cell stimulation, the ratio of CD19+ B cells, CD19+CD27+ B cells, and CD19+CD138+ B cells increased compared with controls (Figure 2).

**Effects of stimuli on purified B-cell apoptosis and immunoglobulin production**

The effects of the stimuli on apoptosis and immunoglobulin production of B cells purified from healthy controls and transplant patients were next evaluated by flow cytometry and ELISA. After stimulation the B cells from healthy controls and transplant patients had a low percentage of apoptosis; however, the average percentage of apoptosis of B cells from transplant patients was significantly higher than that of cells from healthy controls (Figure 3; *P* < .01). In addition, IgM is the first antibody produced by B cells after antigen stimulation, and we found that production of IgM was higher in stimulated B cells from transplant patients than from healthy controls (Figure 4).

**Abbreviations:** HC, healthy controls; TP, transplant patients

Kidney recipients have a lower ratio of CD19+ B cells than do healthy controls in the peripheral blood mononuclear cell fraction. The percentage of memory B cells (CD19+CD27+) in the CD19+ B-cell population from transplant recipients was higher than that of healthy controls. *P* < .05.
Bortezomib and mycophenolic acid induce B-cell apoptosis

One possible explanation for the inhibition of antibody production is the induction of apoptosis. To test this hypothesis, B cells from healthy controls were cultured for 7 days with stimulus in the presence or absence of increasing concentrations of MPA and bortezomib (50, 100, and 150 ng/mL; and 200, 500, 1000 ng/mL). B-cell apoptosis was measured by Annexin-V-FITC and PI staining followed by flow cytometry analysis. We found that the percentage of B-cell apoptosis increased in a drug dose-dependent manner (Figure 5). We then fixed the dose of MPA (100 ng/mL) and bortezomib (1000 ng/mL) and treated B cells from both transplant patients and healthy controls. We found that the percentage of viable cells decreased in both groups, and that the percentage of prophase and terminal apoptosis cells in the 2 groups increased compared with untreated controls (Figure 6; P < .01). Therefore, MPA and bortezomib induced a significantly higher percentage of apoptosis in B cells from both patient groups compared with controls.
Effects of immunosuppression on immunoglobulin production

Mycophenolic acid (100 ng/mL) and bortezomib (1000 ng/mL) then were applied to the culture system to determine the effects on antibody secretion. After 7 days’ cell culture, bortezomib and MPA decreased production of IgM in the in vitro stimulation environment (Figure 7).

Discussion

In this study, peripheral blood B cells were purified from 6 MICA-sensitized patients’ and 6 healthy controls’ peripheral blood and were assessed by flow cytometry. We found that the sensitized patients had fewer CD19+ B cells in the total PBMC population than did the healthy controls. Therefore, immunosuppressive agents that are administered to transplant patients seem to alter the constitution of B-cell subpopulations. In our study, all the transplant patients were taking cyclosporine, mycophenolate mofetil, and prednisone, and therefore, B-cell proliferation and cell differentiation might be influenced by the immunosuppressive agents, which may be a benefit for long-term survival. However, more patient data and control groups are needed to confirm this hypothesis. In the total population of B cells, the percentage of CD19+CD27+ B cells was higher in transplant patients than in healthy controls. The CD19+CD27+ B cells are the subset of memory B cells, and therefore, these results suggest that B cells from sensitized patients have the potential ability to secrete antibodies.

In our in vitro B-cell culture system, cell differentiation and function were regulated by multiple pathway stimuli. The stimuli we used in this study have separate and unique functions for B-cell activation. Toll-like receptor-9 ligand ODN-2006 CpG binds with Toll-like receptor-9 to trigger the TLR-9–My88–NF-κB pathway to promote B-cell proliferation and differentiation.17 Interleukin-10 has been shown to increase Toll-like receptor-9 expression18 and stimulate immunoglobulin production19-21 in B cells. Although IL-2 is considered to be a key factor for T-cell proliferation after activation, it also has been shown to sustain B-cell proliferation. Interleukin-21 is mainly produced by follicular helper T cells and is required for plasma cell differentiation and immunoglobulin production in germinal centers.22-24 Therefore, the addition of IL-21 in the culture system most likely promotes the activation of B cells.

CD40 signaling is an important factor for the maturation and proliferation of B cells,25,26 PMA and MICA antigen also can activate the NF-κB pathway for cell proliferation and differentiation. In this study, we stimulated 2 signaling pathways using different combinations of stimuli. CpG2006 combined with IL-2, IL-6, IL-10, and BAFF promote B-cell proliferation,27,28 while CD40L, together with IL-4 and IL-21, induces the differentiation of B cells into antibody secreting cells. Our results show that the levels of IgM in the supernatant of stimulated B cells and the number of viable cells dramatically increased upon stimulation.

In the in vitro B-cell culture system, the average percentage of apoptosis of B cells from healthy controls and transplant patients was 9.73% ± 1.46% and 21.53% ± 3.31%. Because transplant patients are under immunosuppressive regimens, the B-cell population is likely to be compromised, which may alter the apoptosis of B cells under stimulation compared with healthy controls. In the present study, different dosages of MPA and bortezomib were added to the culture system, which resulted in a dose-dependent increase in the average percentage of B cells undergoing apoptosis. At a clinically relevant fixed dose (MPA, 100 ng/mL; and bortezomib, 1000 ng/mL), bortezomib and MPA effectively induced apoptosis of B cells. In addition, both immunosuppressive agents decreased the production of IgM in the stimulation environment, which is consistent with previous studies.29,30 Other studies also have shown that a decrease in donor-specific antibodies strongly correlates with successful treatment of antibody-mediated rejection.8,31,32 Our results suggest that bortezomib and MPA can inhibit B-cell antibody secretion.

Antibodies secreted by antibody-producing B cells in vivo are driven by multiple interactions, including BCR-antigen interactions, costimulatory stimulations, and cytokines. To date, an optimal in vitro system for stimulating B cells to secrete antibodies has not been established. Therefore, optimizing such a system using stimuli to trigger cultured B cells would be a valuable tool in this research setting. In this study, we successfully stimulated B cells with a combination of antigens and cytokine cocktails using purified B cells from MICA-sensitized kidney recipients and healthy controls.
Conclusions

In this study, peripheral blood B cells were isolated from sensitized kidney recipients and found to display a subset bias. In our established B-cell in vitro culture system, stimulation promoted antibodies secretion in vitro. We also found that bortezomib exhibited inhibitory effects on B-cell survival and function, which may provide an experimental basis for future studies.

References

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