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Research paper

Immunoregulatory effects of sirolimus vs. tacrolimus treatment in kidney allograft recipients

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ABSTRACT

The difference in immunoregulatory effects between sirolimus and tacrolimus on kidney transplantation remains unclear. In this study, a total of 18 living-donor-related kidney transplant recipients received sirolimus ($n = 8$) or tacrolimus ($n = 10$) treatment. Kidney function, acute rejection, peripheral blood $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs), $CD19^+CD5^+CD1d^+$ regulatory B cells (Bregs), and panel reactivity antibody were analyzed after one and three years. Th1/2 cell polarization was also determined at one year. The proportion of Tregs in the recipients receiving tacrolimus significantly decreased to 3.69% and 2.49% at one and three years, respectively, compared to 6.59% in controls, whereas the proportion in the recipients receiving sirolimus remained at 6.67% and 5.66%, respectively. However, no differences in kidney function, acute rejection, proportion of Bregs, panel reactivity antibody, or the frequencies of Th1/2 cells were identified. In conclusion, unlike tacrolimus, sirolimus maintains the proportion of Tregs in kidney transplant recipients.

1. Introduction

Current immunosuppressive strategies following kidney transplantation often consist of a triple drug regimen composed of calcineurin inhibitors (CNIs), mycophenolate mofetil (MMF), and hormone-based treatment. CNIs, including cyclosporin A and tacrolimus, greatly reduce the occurrence of acute organ rejection and improve the short-term survival rate of transplant recipients. However, CNIs can cause chronic renal allograft dysfunction due to nephrotoxicity, which may diminish their overall benefits for long-term graft survival [1,2]. CNIs also lead to cardiovascular diseases and an increased incidence of malignant tumors [3]. The serious side effects often associated with CNIs have led to a decline in their use. Therefore, many trials are currently looking at minimizing or avoiding the use of CNIs.

Sirolimus, a drug that inhibits mammalian target of rapamycin, is an effective immunosuppressive agent with reportedly low nephrotoxicity [4]. Sirolimus and tacrolimus share a similar molecular structure, but have different mechanisms of action [5,6]. Unlike tacrolimus, which inhibits the release of calcium by T cells though calcineurin, sirolimus inhibits the activation of T cells. In addition, it is also reported that sirolimus displays a strong antiproliferative effect on cancer cell growth [7]. Importantly, sirolimus has the ability to induce and maintain peripheral tolerance in transplantation models [8–10].

Previous studies have compared the immunoregulatory effects of sirolimus and CNIs, but the research has focused mainly on dendritic cells and regulatory T cells (Tregs). It has been reported that CNIs may hamper the induction of tolerance by interfering with the induction of Tregs in allograft recipients [11,12], whereas sirolimus can potently suppress proliferation of T effector cells while sparing Tregs [13–15]. In addition, sirolimus inhibits the expression of costimulatory molecules, such as CD80 and CD86, on dendritic cells, which impairs the maturation of dendritic cells and reduces their ability to present antigens [16]. A number of studies have demonstrated that sirolimus can suppress the functional activation of dendritic cells both *in vitro* and *in vivo*, and significantly prolong alloantigen-specific graft survival [16–18]. Thus, the difference in immunoregulatory effects between sirolimus and CNIs remains to be clarified, especially in the context of human organ transplantation.

Regulatory B cells (Bregs) are a newly described subset of B cells, first introduced by Mizoguchi et al. [19] who identified Bregs as an interleukin (IL)-10-producing B cell subset. Studies

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have shown that Bregs are involved in induction and maintenance of tolerance during organ transplantation [20]. The mechanisms by which B cells induce tolerance are not well established, but it is thought that Bregs function via IL-10 secretion and activation of the CD40 pathway [20]. However, few studies have been performed to explore the immunoregulatory effect of long-term use of CNIs and sirolimus on Bregs in allograft recipients.

In this study, the proportion of peripheral blood Tregs and Bregs were analyzed in living-donor-related kidney transplant recipients, and antibody production and T-helper cell polarization upon activation by donor-specific antigen were investigated. The objective of this study was to assess the impact of sirolimus and tacrolimus on the immunoregulatory cells of living-donor-related kidney transplant recipients.

2. Methods and materials

2.1. Subjects and grouping

A total of 18 consecutive kidney transplant recipients treated by our group from the period of January 2008 to December 2011 were enrolled in this study. All the transplanted kidneys came from living-related donors. The study protocols were approved by the Ethics Committee of the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, China. Patients with a concomitant infectious disease or malignancy were excluded from the study.

All enrolled patients were primary transplant recipients, and none of these patients received induction therapy. All of the recipients were given a standard triple regimen (tacrolimus, MMF, and prednisolone) following the transplantation procedure. Tacrolimus (Astellas Pharma, Inc., Tokyo, Japan) was administered at a starting oral dose of 0.1 mg/kg/d and adjusted based on trough level. MMF (Cellcept; Roche Diagnostics, Basel, Switzerland) was given at a median dose of 1 g/d. Oral prednisone was started at 1 mg/kg/d, and the maintenance dosing was 10 mg/d in both groups.

All recipients presented stable renal function, without any signs of acute rejection throughout the follow-up. Among the 18 recipients, 10 remained on the tacrolimus treatment protocol at a maintaining trough level of 6–8 ng/mL, defined as the tacrolimus group; 8 were converted from tacrolimus to sirolimus (Wyeth of Pfizer, Inc., New York, NY, USA) at 3 mo post-transplantation with a starting dose of 2 mg/d and a maintaining trough level of 7–10 ng/mL, defined as the sirolimus group. The individuals who donated their kidneys to the recipients were included in the study as controls. The recipients were followed-up at the timepoints of one year and three years after receiving tacrolimus or sirolimus for evaluation of kidney function and immune status.

2.2. Clinical monitoring

After transplantation, serum creatinine (Cr) was evaluated for kidney function in recipients. Acute rejection was clinically defined as an increase in Cr of 25% within 24 h with oliguria, artery resistance index > 0.75 by ultrasound Doppler, fever, and kidney distension pain. Meanwhile, blood was routinely collected for white blood cells (WBC) counting as a parameter of immune status.

2.3. Isolation of peripheral blood mononuclear cells (PBMCs)

After collecting 10 mL of heparinized whole blood from the recipients and donors, PBMCs were isolated from PBS-diluted whole blood (1:1) by using Ficoll density gradient (Dakewe Biotech Co., Ltd., Shenzhen, China). PBS-diluted whole blood and Ficoll were centrifuged together at 1800 rpm/min for 30 min, and the lymphocyte interface was collected as described by the manufacturer's protocol.

2.4. Absolute lymphocyte count

The absolute lymphocyte count was determined by flow cytometry analysis and calculated based on the percentage of lymphocytes in automated complete blood counts from peripheral blood: absolute lymphocyte count $\times$ percentage of $CD4^+CD25^+FOXP3^+$ Tregs from the total Tregs.

2.5. Flow cytometric analysis

The concentration of PBMCs was adjusted to 1×10^6/mL before analysis. Three-color flow cytometric analysis was performed on 10^4 cells using a FACScalibur machine (BD Biosciences, Franklin Lakes, NJ, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-human CD4, allophycocyanin-conjugated anti-human CD25, and phycoerythrin-conjugated anti-human FOXP3 antibodies were used for the detection of Tregs. For intracellular FOXP3 staining, a cell membrane permeabilization step was performed prior to fluorescent staining following the manufacturer's instructions. Allophycocyanin-conjugated anti-human CD19, FITC-conjugated anti-human CD5, and phycoerythrin-conjugated anti-human CD1d antibodies were used for detection of Bregs. All antibodies were obtained from eBioscience, Inc. (San Diego, CA, USA). Isotype-matched antibodies were used as negative controls.

2.6. Enzyme-linked immunospot (ELISPOT) assay

The ELISPOT assay was performed for the detection of human IL-10 or interferon (IFN)-γ (eBioscience, Inc.) according to the manufacturer's instructions. Briefly, lymphocytes were collected from donor and recipient blood. Lymphocytes isolated from donors were used as stimulator cells containing donor antigen, and were inactivated by incubation with 25 μg/mL mitomycin C for 30 min at 37 °C. Next, 5×10^5/100 μL lymphocytes from recipients (responder cells) were mixed with stimulator cells at a 1:1 ratio, and incubated in the coated ELISPOT plate. The PBMCs in medium alone or stimulated with 5 μg/mL phytohemagglutinin (Sigma–Aldrich Corp., St. Louis, MO, USA) were used as negative and positive controls, respectively. ELISPOT assays were set up using duplicate wells. The number of spots per well was counted on an automated ELISPOT reader (Cellular Technology Ltd., Shaker Heights, OH, USA), and the frequency of T cells producing IFN-γ or IL-10 was calculated by subtracting the negative control values.

2.7. Panel reactive antibody (PRA) detection

PRA detection was performed by incubating 20 μL test serum with FlowPRA® Class I and/or Class II beads (One Lambda of Thermo Fisher Scientific, Waltham, MA, USA) in a 1.5 mL microcentrifuge tube for 30 min in the dark at room temperature with gentle shaking. Next, 1 mL of wash buffer was added to each tube prior to centrifuging at 9000*g* for 2 min, and the supernatant was aspirated and discarded; 100 μL of 1 $\times$ FITC-conjugated goat anti-human IgG (diluted in wash buffer) was added to the beads. After vortexing, the beads were incubated for 30 min in the dark at room temperature with gentle shaking. Finally, 0.5 mL of 1$\times$ fixing solution was added to the tube for flow analysis.

2.8. Statistical analysis

All statistical analyses were performed using Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Quantitative data are

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