A Clinical Trial Aiming at Tolerance Induction by Adoptive Transfer of Ex Vivo-Induced, Donor-Specific Treg-Like Cells in Clinical Kidney Transplantation

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Abstract

Background: Although the outcome of kidney transplantation has been improved with the recent progress of immunosuppression, various adverse effects of immunosuppressants and chronic rejection remain to be solved. To overcome these problems, the induction of tolerance would be most promising and expected.

Methods: A clinical trial aiming at tolerance induction was conducted by infusing donor-specific regulatory T (Treg)-like cells induced by coculture of the recipient and irradiated donor lymphocytes in the presence of anti-CD80/86 monoclonal antibodies. Cultured cells were infused intravenously to the recipient on 12POD. Mixed lymphocyte reaction (MLR) with donor and 3rd party lymphocytes, the subset of circulating lymphocytes and the graft function were evaluated.

Results: The phenotype of cultured cells was CD4+CD25+CTLA-4+FoxP3+, and cultured cells suppressed MLR with donor lymphocytes in a dose-dependent manner, but not with 3rd party lymphocytes. These ex vivo-induced, donor-specific Treg-like cells suppressed the immunological responsiveness of the recipient to donor antigens so as to reduce immunosuppressants to the almost half of the standard doses, but did not enable the complete withdrawal.

Conclusions: Ex vivo-induced Treg-like cells showed donor-specific hyporesponsiveness in MLR in dose-dependent manner, and induced hyporesponsiveness of the recipient to donor antigens in clinical kidney transplantation, but did not realize immunological tolerance.

Keywords: Tolerance; Regulatory T cell; CD80/86; FoxP3; Kidney transplantation

Introduction

The induction of immunological tolerance is an ultimate goal in organ transplantation. Today, the outcome of organ transplantation has been remarkably improved by the recent progress in many fields, especially in the development of potent and selective immunosuppressants. Many adverse effects of immunosuppressants, however, such as various opportunistic infections which may be fatal sometimes, nephrotoxicity which may lead to renal failure, malignancies and post-transplant lymphoproliferative disorders which may cause the fatal outcome hamper not only graft survivals but also the quality of life. Additionally hypertension, dyslipidemia, impaired glucose tolerance, cataract, glaucoma, osteoporosis and aseptic necrosis influence unfavorably on the quality of life of transplant patients. One of the greatest problems which influence long-term graft survival is a chronic rejection that cannot be successfully treated even with modern potent, selective immunosuppressants.

To overcome aforementioned problems, namely adverse effects of immunosuppressants and chronic rejection, tremendous basic experiments and clinical trials on the induction of immunological tolerance have been reported [1-12]. These trials were divided into two categories, the induction of chimerism by bone marrow transplantation or the infusion of hematopoietic stem cells and the peripheral deletion of donor-responsive clone using alemtuzumab with T cell depleting antibody or immunosuppressants. In case of bone marrow transplantation, capillary leak syndrome (engraftment syndrome) and graft versus host disease (GVHD) remain to be solved [5,6], while in case of hematopoietic stem cells, GVHD has not been observed although chimerism was introduced [8].

To induce the immunological tolerance by the less-invasive method, we tried to induce donor-specific regulatory T (Treg)-like cells by coculture of donor and recipient lymphocytes in the presence of anti-CD80/86 monoclonal antibodies. We reported that the successful induction of the immunological tolerance by infusing ex vivo-induced, donor-specific Treg-like cells in rhesus monkey kidney transplantation [13]. We conducted a clinical trial aiming at tolerance induction in kidney transplantation based upon the new protocol revised for the clinical trial with respect to twice stimulation of donor-specific Treg-like cells by donor lymphocytes (Figure 1).
The purposes of the study are to investigate, (1) whether Treg-like cells can be induced ex vivo also in human by the same methods, (2) whether tolerance can be induced also in clinical kidney transplantation by using ex vivo-induced, donor-specific Treg-like cells?

Materials and Methods

This clinical trial of tolerance induction based upon the protocol shown in figure 1 was approved by the Institutional Review Board (the Ethics Committee of Tokyo Women's Medical University). Nine patients undergoing dialysis therapy were enrolled in this study after the informed consent, who all applied spontaneously to undergo kidney transplantation by this protocol. All kidney donors voluntarily applied to donate the kidney for the clinical trial. Age, gender, original diseases, the duration of dialysis, the relationship to the donor, and the number of mismatched HLA-A, -B and -DR antigens are shown in table 1.

Collection and co-culture of lymphocytes of donors and recipients

Two days before the transplantation, both donor and recipient underwent 3 hours continuous density gradient lymphocytapheresis using Haemonetics CCS (Haemonetics Japan, Tokyo, Japan) and COBE Spectra (Terumo BCT Inc., Tokyo, Japan), and 2 × 10^6 peripheral blood mononuclear cells (PBMCs) per body and 1 – 2 × 10^8 PBMCs per kilogram of body collected from donor and recipient, respectively. Recipient lymphocytes(0.5 ~ 0.8 × 10^10) were co-cultured with 0.2 × 10^10 30 Gy-irradiated donor lymphocytes in the presence of 12 mg of anti-human CD80 (2D10; eBioscience Inc., San Diego, CA) and 12 mg of anti-human CD86 monoclonal antibodies (IT2.2; eBioscience Inc.) in 87-301A-100N culture bag (Nipro Inc, Osaka, Japan) containing 1,000 ml of ALyS-505N medium (Cell Science & Technology Institute Inc., Sendai, Japan) supplemented with 14 ml of recipient heat-inactivated plasma at 37°C in a humidified 5% CO2 atmosphere. After a 7-day co-culture (5 POD), viable cells were collected, counted and co-cultured again with 0.2 × 10^10 30 Gy-irradiated donor lymphocytes obtained by the second lymphocytapheresis in the presence of anti-CD80/86 monoclonal antibodies (8 mg each) under the same condition for further 7 days at 37°C in a humidified 5% CO2 atmosphere.

After total 14 day co-culture, viable cells were collected, washed and centrifuged several times, tested for bacterial contamination and endotoxin (Endotoxin Single test Wako, Wako Pure Chemical Industries Ltd., Osaka, Japan), and suspended in 100 ml of physiological saline for intravenous administration to the recipients. All these procedures were done in the Cell Processing Center of Tokyo Women’s Medical University. A sampling of cultured cells was used to study for lymphocytes subsets by Flow cytometry and mixed lymphocyte reaction (MLR) with lymphocytes from donor and 3rd party.

Kidney transplantation and inoculation of cultured cells

Laparoscopic donor nephrectomy and kidney transplantation in the iliac fossa along with laparoscopic splenectomy of the recipient were done. Immunosuppressants regimen consists of 8 mg/kg/day of cyclosporine (CsA), 2,000 mg/body/day of mycophenolate mofetil (MMF) and 5 ~ 10

Table 1: Demography of patients enrolled in the clinical trial.

<table>
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<th>gender</th>
<th>y/m/d</th>
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<th>HD duration</th>
<th>donor</th>
<th>blood type</th>
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Figure 1: Protocol of clinical trial of tolerance induction in clinical kidney transplantation. CPA: cyclophosphamide, Lymph: lymphocytes, CsA: cyclosporine, MMF: mycophenolate mofetil, MP: methylprednisolone.
Immunohistochemical staining of the biopsied specimen of the kidney grafts

The biopsied specimen was fixed in 10% buffered formalin and embedded in paraffin. H&E and PAS stains were performed for histological examination. The immunohistochemical staining assay using anti-CD4, -CD8, -CD20, -CD25, -FoxP3 and -granzyme B antibody was performed with iView DAB Detection Kit (Roche Tissue Diagnostics Japan Ventana, Tokyo, Japan) on a VENTANA BenchMark GX automated staining system (Roche Diagnostics Japan Ventana, Tokyo, Japan) to investigate the characteristics of infiltrated cells into kidney grafts.

Briefly, the tissue sections were deparaffinized with EZ Prep (Roche Tissue Diagnostics Japan Ventana) at 75°C, heat pretreated in Cell Conditioning 1 (CC1; Roche Tissue Diagnostics Japan Ventana) using “standard cell conditioning” for antigen retrieval at 100°C, and then incubated with anti-CD4 and -CD8 rabbit monoclonal antibody (Roche Tissue Diagnostics Japan Ventana), anti-CD20 primary antibody (Roche Tissue Diagnostics Japan Ventana), anti-CD25 mouse monoclonal antibody (NICHIREI Biotechnology Inc., Tokyo, Japan), anti-human Foxp3 antibody (BioLegend Japan, Tokyo, Japan) and anti-granzyme B polyclonal antibody (Roche Tissue Diagnostics Japan Ventana) as a primary antibody, respectively, for 32 min at 37°C after inactivation of the endogenous peroxidase with hydrogen peroxide for 4 min. The slides were then blocked using Endogenous Biotin Blocking Kit (Roche Tissue Diagnostics Japan Ventana), incubated with a biotinylated Ig secondary antibody for 8 min, and incubated with a streptavidin-HRP conjugate for 8 min at 37°C. The immunolocalized CD4, CD8, CD20, CD25, FoxP3 and granzyme B were visualized using a copper-enhanced DAB reaction. The slides were counterstained with Hematoxylin II (Roche Tissue Diagnostics Japan Ventana) for 4 min and Bluing Reagent (Roche Tissue Diagnostics Japan Ventana) for 4 min and coverslips were applied by an automated coverslipper (Tissue-Tek Film Automated Coverslipper; Sakura Finetek Japan, Tokyo, Japan).

Results

The number of viable cells obtained by co-culture of the recipient and irradiated donor lymphocytes in the presence of anti-CD80/86 monoclonal antibodies was 0.8 – 1.5 x 10^6 in case that the culture period was within 14 days, while the number of viable cells was decreased as the period of culture was extended to more than 14 days (Table 2). Cultured cells expressed CD4, CD25, FoxP3, and granzyme B were visualized using a copper-enhanced DAB reaction. The slides were counterstained with Hematoxylin II (Roche Tissue Diagnostics Japan Ventana) for 4 min and Bluing Reagent (Roche Tissue Diagnostics Japan Ventana) for 4 min and coverslips were applied by an automated coverslipper.

Table 2: Cyclophosphamide dosage and the number of infused cells (TReg-like cells).

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Peripheral blood samplings of recipients were treated with the same procedures and assayed for flow cytometry.
Figure 2: Phenotype of cultured viable cells, Treg-like cells expressing CD4, CD25, CTLA-4 and Foxp3.

Figure 3: Mixed lymphocytes reaction (MLR) with donor and third party lymphocytes in the presence of cultured cells, which depressed MLR with donor lymphocytes in dose-dependent manner, while did not with 3rd party lymphocytes.

20 ~ 30 mg/kg of CPA. In three patients, cultured cells were given on 19, 33 and 21 POD (0.8 × 10⁹, 0.075 × 10⁹, 0.3 × 10⁹), respectively, because of the development of postoperative complications, as cites related to preoperative CAPD in patient No.4, delayed urination due to postoperative ATN in patients No.7, and pancreatic fistula after splenectomy in patient No.9 (Table 2).

All patients well tolerated the administration of CPA, while leukocytopenia and the loss of hair were observed after the administration of CPA. Any adverse effects attributable to the intravenous infusion of cultured cells, such as fever, hypotension, and rash have not been observed when cultured cells were infused intravenously in all patients.

The phenotype of circulating lymphocytes of the recipients after infusing Treg-like cells is shown in figure 4. The incidence of CD4+CD25+CTLA-4+Foxp3+ cells in circulating lymphocytes was increased with time and reached the peak about 4 weeks after infusing cultured cells.

The ratio of MLR with the donor to 3rd party lymphocytes was depressed with time after the intravenous infusion of cultured cells to 0.006 ~ 0.22 in 8 patients, whereas not depressed to 1.0 or less in remaining one patient (patient No.9, table 3) from 1 month to 12 months after the transplantation. Thereafter in three patients (patient No.1, 4 and 7), the ratio of MLR with the donor to 3rd party lymphocytes was increased to 0.49-0.720 (Table 3).
Table 3 shows the number of mismatched antigens, the dose of CsA, MMF and MP, the Banff grade, the type and the time of rejection episode and its treatment in addition to the ratio of MLR with the donor to 3rd party lymphocytes. Six out of 9 patients developed rejection episode between 220 and 378 POD, cellular rejection (IA) in 4 patients (patient No. 3, 4, 6 and 8), and mixed type (IIB) in two patients (patient No.1 and 5).

Figure 5 shows the postoperative course of patient No.2, in which a dose of CsA and MMF was reduced to 25 mg/day and 250 mg/day following discontinuing steroid, respectively. In case of patient No.3, after reducing CsA dose to 25 mg/day following discontinuing steroid and MMF, a cellular rejection occurred in 330 POD. After the recovery by the treatment, we started again low-dose CsA and MMF (Figure 6). Figure 7 shows the postoperative course of patient No.8. After reducing CsA dose to 10 mg/day following discontinuing steroid and MMF, a cellular rejection occurred in 307 POD. After it was relieved by the treatment, we started again low-dose triple regimen. Other two patients (patient No.1 and 5) developed rejection episode on 302 POD and 378POD, respectively, during reducing the doses of CsA and MMF following the discontinuation of steroid. All rejection episodes were relieved by steroid pulse with or without muromonab CD3 and low-dose triple regimen was started.

The average daily doses of CsA and MMF in the study group during the postoperative time course are compared to those in the control group (Figure 8). The control group consists of living-related ABO-compatible kidney transplantation performed during the same period in which basiliximab was given at the transplantation. The average daily dose of CsA and MMF in the study group was 40.3% and 45.6% of that in the study group at 12 months, and 51.2% and 51.0% of that in the study group at 18 months after the transplantation, respectively.

Figure 9 shows the immuno-histochemical findings of a biopsy specimen of kidney graft (case 2, 100 POD), showing CD4+, CD25+ and FoxP3+ cell infiltration. Despite the infiltration of CD8+ cells, the granzyme+ cell was not observed.

Table 3: The number of HLA mismatch antigens, the ratio of MLR with donor to 3rd party lymphocytes, anti-HLA antibody, immunosuppressants, Banff grade, the type and the time of the rejection, and its treatment.

*: donor-specific antibody

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Figure 4: Phenotype of circulating lymphocytes of the recipients after infusing Treg-like cells.

**Figure 5:** Clinical course of patient No.2 (53 y.o., male, 1 mismatched against donor HLA-antigens). The minimum ratio of MLR with the donor to the 3rd party was 0.07. Immunosuppressants were reduced and maintained with 25 mg/day of CsA and 250 mg/day of MMF without any rejection episode.

**Figure 6:** Clinical course of patient No.3 (41 y.o., female, 2 mismatched against donor HLA-antigens). The minimum ratio of MLR with the donor to the 3rd party was 0.02. When reducing CsA to 25 mg/day following the discontinuation of MP and MMF, cellular rejection (IA) occurred on 330 POD, which was resolved by steroid pulse therapy and maintained with a reduced dose of CsA and MMF.

**Figure 7:** Clinical course of patient No.8 (34 y.o., male, 2 mismatched against donor HLA-antigens). The minimum ratio of MLR with the donor to the 3rd party was 0.06. When reducing CsA to 10 mg/day following the discontinuation of MP and MMF, cellular rejection (IA) occurred on 307 POD, which was resolved by steroid pulse therapy and maintained with a small dose of triple regimen.

**Figure 8:** Average daily dose of CsA (A) and MMF (B) at given time (postoperative months) in study group as compared to control group (living-related ABO-compatible kidney transplantation performed during the same period in which basiliximab was given at the induction).

**Figure 9:** Immunohistochemical findings of patient No.2 (100 POD), showing CD4+, CD25+ and FoxP3+ cell infiltration. Despite the infiltration of CD8+ cells, granzyme+ cell was not observed.

**Discussion**

Antigen recognition without co-stimulatory signals is known to lead to T cell anergy. Anti-CD80/86 antibodies inhibit CD28 signaling and promote CTLA-4 ligation of CD80/86, leading to express Treg-specific transcription factor, forkhead box P3 (FoxP3), which suppresses IL-2 transcription via interacting with AML-1/Runx1 (leucine zipper domain), histone de-acetylases (aminoterminal repressor domain), and NF-AT (forkhead domain) [14,15].

We reported that anergic T cells induced by the co-culture of recipient lymphocytes with irradiated donor lymphocytes in the presence of anti-CD80/86 antibodies, expressed CD4, CD25, CTLA-4 and FoxP3 and demonstrated donor-specific hypo-responsiveness in MLR in a dose-dependent manner, named ex vivo-induced, donor-specific Treg-like cells, and that tolerance induction was successfully achieved in rhesus monkey kidney transplantation by infusing ex vivo-induced Treg-like cells intravenously to the recipient [13].
We conducted the clinical trial of tolerance induction in the kidney transplantation to investigate, (1) whether Treg-like cells can be produced also in human by the same methods, (2) whether tolerance can be induced also in clinical kidney transplantation by using ex vivo-induced, donor-specific Treg-like cells?

Viable cultured cells obtained by the co-culture of recipient lymphocytes with irradiated donor lymphocytes in the presence of anti-human CD80/86 antibodies expressed CD4, CD25, CTLA-4 and FoxP3 and depressed MLR with donor lymphocytes in a dose-dependent manner, while did not depressed MLR with 3rd party lymphocytes, suggesting that donor-specific Treg-like cells can be introduced also in human subjects (Figure 2.3).

The ratio of MLR of the recipient with donor lymphocytes to that with 3rd party lymphocytes is considered to indicate the specificity of the suppressive effect of Treg-like cells. On conducting the clinical trial, we considered the decrease in the ratio of MLR with donor lymphocytes to 3rd party lymphocytes would be a key indicator of the immunological responsiveness of the recipient to donor antigens, and therefore a possible indicator of reducing immunosuppressants.

In the process of reducing immunosuppressants referring to the ratio of MLR with the donor to 3rd party lymphocytes, a cellular rejection (IA) occurred on 330 POD after reducing CsA dose to 25 mg/day following discontinuing steroid and MMF in case 3 (Figure 6), although the ratio of MLR with the donor to the 3rd party was suppressed to 0.02. Also in case of patient No.8, after reducing CsA dose to 10 mg/day following discontinuing steroid and MMF, a cellular rejection (IA) occurred on 307 POD although the ratio of MLR with the donor to the 3rd party was suppressed to 0.06 (Figure 6). Additionally, in case of patient No.5, a mixed type rejection occurred on 378 POD after reducing CsA and MMF to 25 mg/day and 250 mg/day, respectively following discontinuing steroid, even though the ratio of MLR with the donor to the 3rd party was depressed to 0.006.

Finally in 6 out of 9 patients, the rejection occurred on 220 – 378 POD in the process of reducing CsA and/or MMF following reducing and/or discontinuing steroid and MMF, even though the ratio of MLR with the donor to 3rd party lymphocytes were depressed to 0.006 – 0.22. While in case 4, although triple regimen had been maintained (CsA: 125 mg/day, MMF: 500 mg/day, MPS: 2 mg), a cellular rejection (IA) occurred on 220 POD (Table 3).

From above-mentioned facts several problems to be solved arise. Can the ratio of MLR with the donor to 3rd party lymphocytes be a key indicator of immunological responsiveness of the recipient to donor antigens? And can it be an indicator of reducing immunosuppressants? Can infect donor-specific Treg-like cells suppress in vivo the immunological responsiveness of the recipient to donor antigens enough to reduce and/or withdraw immunosuppressants in human subjects, same as rhesus monkey? [13] Can ex vivo-induced, donor-specific Treg-like cells continue to function in vivo and expand in the recipient for long period?

Regarding the point at issue, “To what extent the dose of immunosuppressants can be reduced?” would depend upon “To what extent MLR with donor can be depressed compared to that with 3rd party?”, it could not be settled in this study. The intravenous infusion of ex vivo-induced, donor-specific Treg-like cells suppressed the immunological responsiveness of the recipient to donor antigens enough to reduce immunosuppressants to some extent, but not enough to withdraw them completely in the clinical kidney transplantation.

The suppressive activity of CD4+CD25+Treg cells was also reported to lead to the development of an additional CD4+T suppressor cell population (CD4+Treg cells), which emerge from the CD4+CD25-T cell population and suppress the proliferation of freshly isolated conventional CD4+T cells [17].

Finally, the occurrence of the rejection might be related to memory cells probably generated by encountering donor antigens before the infusion of Treg-like cells. It is likely that initial immunosuppression, the triple regimen consisting of CsA, MMF, and MP, would not be enough to inhibit producing memory T and/or B cells against donor antigens. We are reconsidering a new protocol including the administration of a small dose of thymoglobulin and rituximab on POD to inhibit the production of T and B memory cells, based upon outcomes of the pilot study adding thymoglobulin to the previous protocol, and of the in vitro culture of Treg-like cells with thymoglobulin and human plasma containing the complement.

Conclusion

Ex vivo-induced Treg-like cells by co-culture of donor and recipient lymphocytes in the presence of anti-CD80/86 monoclonal antibody showed donor-specific hyporesponsiveness in MLR in a dose-dependent manner. The infusion of Treg-like cells to recipients induced hyporesponsiveness of the recipient so as to reduce immunosuppressants approximately by 50% in clinical kidney transplantation, but did not realize immunological tolerance. To induce immunological tolerance, further study was required.

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