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Non-myeloablative conditioning is sufficient to induce mixed chimerism and subsequent acceptance of donor specific cardiac and skin grafts

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ABSTRACT

Organ transplant recipients have elevated cancer and viral infection risks due to immunosuppression and long-term results of organ transplantation remain unsatisfactory, mainly because of chronic rejection. The purpose of the current study is to establish a nonmyeloablative perioperative regimen, able to induce mixed chimerism and tolerance of allografts. To establish a nonmyeloablative perioperative regimen, we used Busulfan, an important component of many bone marrow transplantation preparative regimens for a variety of non-neoplastic diseases as an alternative to total body irradiation (TBI), and FTY720, a unique immunosuppression agent, inhibition lymphocyte homing. We found that creating a lymphohematopoietic chimera in which donor and recipient hematopoiesis coexist resulted in prolongation of the donor specific heart and skin allografts. Consistent with graft survival, pathological analysis indicated that the allografts from tolerant recipients were free of myocardial injury and had only a few interstitial infiltrates, and obliterative vasculopathy was not observed. Furthermore, we found that Treg cells were increased in the long-term graft acceptance recipients. Our data revealed that the therapeutic potential for using hematopoietic chimerism in non-myeloablated recipients hope the advances in rodent models described above in the development of minimal, nontoxic host conditioning regimens for mixed chimerism induction and subsequent acceptance of donor specific grafts.

1. Introduction

Allograft rejection remains to be one of the major complications after transplantation, in spite of the availability of potent immunosuppressive agents including cyclosporine A, tacrolimus, mycophenolate mofetil and rapamycin [1]. In addition, long-term exposure to pharmacological immunosuppression may cause infections, post-transplant lymphoproliferative disorders and malignancies, which significantly contribute to post-solid organ transplantation morbidity [2]. On the other hand, chronic rejection and other complications from long-term immunosuppressive therapies could be overcome by induction of a state of donor-specific tolerance [3–5]. Therefore, achieving transplantation tolerance, with minimal adverse effects, is the ultimate goal of transplant physicians.

Hematopoietic stem cell transplantation (HSCT) has yet to reach its full potential clinically, because of its side effects associated with the current standard preparative regimens for bone marrow transplantation (BMT) [6]. Myeloablative total body irradiation (TBI) in the setting of autologous transplantation of genetically modified hematopoietic stem cell is associated with several side effects [7]. The mixed chimerism leads to specific tolerance, and permits transplantation of organs matched to the donor bone marrow without immunosuppression [8]. Mixed chimerism has many potential advantages over fully allogeneic chimerism in which it had the ability to achieve mixed chimerism with less toxic preparative regimens and cross MHC barriers, and reduce likelihood of graft verse host disease (GvHD) [6]. A possible solution to these problems is the induction of donor-specific tolerance, which can be reliably achieved through the establishment of mixed chimerism. Recently the successful achievement of long-term acceptance of heart allografts without the maintenance of immunosuppression using non-myeloablative conditioning was reported [9]. The other study showed that the immunosuppressive drugs could last for several weeks before transplantation, once evidence

Abbreviations: BMT, bone marrow transplantation; FCM, flow cytometer; FACS, fluorescence-activated cell sorter; GvHD, graft verse host disease; HSCT, hematopoietic stem cell transplantation; MST, median survival time; Treg, regulatory T; TBI, total body irradiation; PCR, polymerase chain reaction.

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for successful tolerance induction is obtained [10]. Busulfan is an alkylating agent that has been employed as an attentive agent to total body irradiation in HSCT [11]. FTY720 (2-amino-[2-(4-octylphenyl) ethyl]-1,3-propanediol hydrochloride), a synthetic immunosuppressant, showed more potent immunosuppressive activity than cyclosporine A and tacrolimus, which is homing of lymphocyte from blood into secondary lymphocyte tissues beyond high endothelial venules that is highly dependent on the interaction [12–14]. In the present study, we hypothesized that allogeneic mixed chimerism can be achieved without apparent toxicity of the FTY720 and Busulfan conditioned BMT and subsequent prolongation or acceptance of donor specific cardiac and skin grafts.

2. Materials and methods

2.1. Animals

F344 (Fisher) rats were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan), F344/HLA-B27 Tg rats were purchased from Taconic Farms, Inc. (Hudson, NY), and F344/EGFP Tg rats were produced by injecting the purified pCAG-EGFP plasmid DNA into F344 rat fertilized eggs [15]. Double F344/HLA-B27&EGFP Tg rats were the offspring of mating F344/HLA-B27 and F344/EGFP Tg rats, and were identified by PCR. All rats were maintained under standard conditions and fed rodent food and water, in accordance with the guidelines of the Animal Use and Care Committee of the National Research Institute for Child Health and Development (Tokyo, Japan).

2.2. Polymerase chain reaction (PCR)

We extracted total genomic DNA from rat ear skin by using CellEase® Mouse Tail (Kanto Chemical, Tokyo, Japan) according the protocol described. We performed PCR amplification in a 20 μl reaction mixture containing 10 μl AmpliTaq Gold® PCR Master Mix (Life Technologies, NY), 2 μl Betain and 5 pmol of each primer: HLA-B27 (272 bp), forward: gTgAgTgACCCCggCCCg and reverse: gCCCCgCCCCgACCAAC; β-actin (412 bp), forward: CACCACACCTTCTACAATgAgCTg and reverse: TCATCAgg TAgTCAgTgAggTCgC. We used the ASTEC Thermal Cycler PCR system (Fukuoka, Japan) and the "hot start" technique (94 °C for 60 s) to increase specificity. The thermal cycling parameters were denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension for 30 s at 72 °C (35 cycles). PCR products (8 μl) and 6xDNA Loading Dye (2 μl, TaKaRa, Shiga, Japan) were analyzed on 2% TBE agarose gels. We visualized prominent bands of the correct size with ethidium bromide staining.

2.3. Reagents and antibodies

Busulfan was obtained from Otsuka Pharmaceutical Company, Ltd. (Tokyo, Japan) and FTY720 was a gift from the Yoshitomi Pharmaceutical Industries (Osaka, Japan). Monoclonal antibodies specific for CD4 (OX-35), CD45RA (OX-33), and CD45 (OX-1), were purchased from BioLegend (San Diego, CA). CD3 (eBioG4.18) and Foxp3 (FJK-16s) were purchased from eBioscience, Inc. (San Diego, CA).

2.4. BMT, heterotopic cardiac and skin transplantation

F344 recipients received injections with 2×10^8 BMCs re-suspended in 1 ml PBS from tibial and femoral bones of F344/HLA-B27&EGFP Tg donors via tail vein injection.

Three weeks after BMT, heterotopic cardiac transplantation was performed from sex-matched F344/HLA-B27&EGFP Tg donors into F344 recipients by the cuff techniques. The cervical heterotopic rat cardiac transplantation was performed as previously described [16,17]. In brief, after thoracotomy under inhalation anesthesia, the donor heart was harvested. The heart graft was rapidly cooled and flushed with 5 ml physiological saline (4 °C) containing 200 U/ml heparin, which was infused via the aorta and pulmonary artery. The heart graft was preserved in physiological saline at 4 °C.

The cuff was prepared from an intravenous cannula and was then anastomosed in the cervical region of the recipient with arterial and venous cuffs. The total ischemic time for the donor operation and vascular anastomoses did not exceed 30 to 35 min. All procedures were performed under clean but not aseptic conditions. Cardiac graft survival was determined by daily palpation from the skin above the cervical grafted heart. Rejection was considered complete at the time of cessation of a palpable heart beating, and confirmed visually by laparotomy.

Skin transplantation was performed 3 weeks after BMT. Full thickness skin graft from F344/HLA-B27&EGFP Tg (donor) rat was transplanted onto the wild F344 (recipient). The skin grafts were observed daily, and rejection was defined as >50% necrosis of the skin allograft.

2.5. Conditioning regimen and transplantation protocol

Chimeric rat created by HSCT that was used to analyze the duration of irradiation effect on engraftment received a myeloablative conditional regimen consisting of 6 Gy TBI before BMT. Day 1 after irradiation the recipient F344 rats received 2×10^8 F344/HLA-B27&EGFP Tg rat BMCs by lateral tail vein injection. Three weeks after BMT, the rats received skin or heart transplantation. Non-myeloablative conditional regimen consisted of FTY720 and was orally administrated in a dose of 1 mg/kg, and two doses of Busulfan (5 mg/kg or 8 mg/kg) were injected intravenously. Recipient F344 rats were treated with FTY720 combined with Busulfan. FTY720 was administered for four consecutive days (day-3 until day 0), and Busulfan was given at day-3 until day-1, followed by BMT at day 0. Three weeks after BMT, the rats received skin or heart transplantation.

2.6. Flow cytometric analysis

Splenocytes, thymus cells, lymph node cells, bone marrow cells and peripheral blood (1×10^6) were incubated with PE-conjugated mouse anti-rat mAbs to CD25, CD3, CD45RA and CD11b and APC mouse anti-rat CD4 and PECy7 mouse anti-rat mAbs CD45 at 4 °C for 20 min washed once in PBS, and incubated at 4 °C for 20 min in the dark. Cells were washed, fixed and permeabilized in fixation/permeabilization buffer for overnight at 4 °C. After an additional wash, cells were incubated with rat FITC-conjugated anti-mouse/rat Foxp3 for 30 min at 4 °C. Rat FITC-conjugated isotype matched antibodies were used as a control and to set appropriate quadrants. After incubation, cells were analyzed using Attune Acoustic Focusing Cytometer (Applied Biosystems, Santa Clara, CA).

2.7. Blood and bone marrow sampling and analysis of multilineage chimerism

The presence of donor hemopoietic cell lineages in the peripheral blood of BMT recipients was serially and quantitatively evaluated by flow cytometry for GFP (donor cells). Chimerism levels were again assessed every week in all rats after BMT for the first day, followed by every week until 100 days. The percentage of donor cells circulating in host peripheral blood was calculated as described previously [18]. Donor cell lineage analysis was performed at 100 days after BMT by flow cytometry for GFP (donor cells) and CD45, CD3, CD45RA and CD11b markers. For each analysis, approximately 10 μl of peripheral blood was collected in tubes via retro-orbitak vein puncture and diluted to 1 ml with heparinized PBS. The samples were hemolyzed by 10× PBS and pure water. The cells were collected after centrifugation at 1800 rpm for 5 min and subsequently washed in PBS. A minimum of 10,000 events was analyzed for each determination.