Long-term Existence of Donor Adipose-derived Stem Cells in the Recipient May Modulate the Vascularized Composite Tissue Allotransplantation Survival in a Rodent Model

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Abstract

Our previous studies have demonstrated donor adipose-derived stem cells (ASCs) combined with short-term immunosuppressant therapy could prolong vascularized composite tissue allotransplantation (VCA) survival in a rodent hind-limb model. In this study, we investigated whether the homing and migration of donor ASCs may modulate VCA survival. Orthotopic hind-limb transplants from male Wistar to Lewis rats were performed (day 0). Donor ASCs were propagated from the adipose-tissue and subcutaneous gelatin sponge material harvested from green fluorescent protein (GFP) transgenic Wistar rats. The transplanted rat (GFP-negative Lewis) received the immunosuppressant protocol similar to our previous design including short-term cyclosporine-A (CsA, days 0-+20), anti-lymphocyte serum (ALS; 0.5 ml i.p.; -4, +1), and three rounds of GFP+ASCs (2 × 106 cells/time, i.v. on days +1, +7, and +21). The engraftment assessment of various donor and recipient tissues were performed by using immuno-fluorescent staining. Flow cytometry was performed to quantify the GFP+ASCs. The results revealed allotransplant survival was significantly prolonged (>100 days) exclusively in ASC-ALS-CsA group which was well correlated with long-term existence of donor GFP-positive ASCs in the recipient. Flow cytometric analysis also revealed GFP+ASCs apparent expression in the recipient peripheral blood in 2 weeks post-ASC infusion but subsequent decrease post-transplantation. The immunofluorescent staining performed on tissue biopsy samples revealed the GFP positive cells existed in the alloskin and recipient skin and liver and spleen parenchymal tissues 2 weeks post-ASC injection. However, no apparent expressions of GFP+ASCs were found in recipient tissues at 16 weeks post-transplantation except in recipient spleen parenchyma. These indicated donor ASCs long-term existed in the recipient spleen tissue and circulating blood may lead to prolong allograft survival.

Keywords: Vascularized composite tissue allotransplantation; Adipose-derived stem cells; Engraftment

Introduction

Recent advances in vascularized composite tissue allotransplantation (VCA) represent the essence of reconstructive surgery [1]. Indeed, a successful human hand and partial face allotransplantation have been performed [2,3]. However, VCA transplants are not routinely performed for tissue reconstruction because lifelong administrations of immunosuppressive agents have potentially harmful side effects [4,5]. Furthermore, even if a patient has excellent compliance, conventional immunosuppressive protocols may not be sufficient to prevent chronic rejection. Consequently, researchers have been eagerly seeking alternative methods for establishing lifelong tolerance while minimizing toxicity [6,7].

Mesenchymal stem cells (MSCs) are multipotent and non-hematopoietic progenitor cells in the adult bone marrow and are capable of differentiating into various mesenchymal cell types [8]. Previous studies have demonstrated that MSCs do not express major histocompatibility complex (MHC) class II or co-stimulatory molecules, such as B7-1, B7-2, or CD40. Therefore, they are most likely unable to stimulate allo-specific T cells proliferation [9,10]. Studies revealed that multiple rounds of donor bone marrow-derived MSCs infusion therapies combined with bone marrow transplantation and short-term immunosuppressant could modulate graft-versushost disease and prevent graft rejection in a swine hind-limb VCA model [11]. We demonstrated that administration of donor MSCs without BMT increased allotransplant survival, which may correlate with an increase in regulatory T cell populations [12]. However, the preparation of bone marrow-derived MSCs was time consuming. Adipose-derived mesenchymal stem cells (ASCs) have been found to share characteristics, including multipotency with bone marrow-derived MSCs [13,14]. Additionally, harvesting adipose tissue to obtain ASCs is easier than preparing MSCs from bone marrow and has greater potential for clinical applications [15]. Our previous study showed multiple rounds of donor ASCs infusion therapies combined with short-term anti-lymphocyte serum (ALS, administered 4 days before and 1 day after transplantation) and cyclosporine-A (CsA, day 0-20 post-transplantation) prolonged VCA survival in a rodent (Brown-Norway to Lewis rat) hind-limb VCA model. ASCs alone or short-term of ALS/CsA is also not enough to prevent VCA rejection [16]. Furthermore, the results demonstrated ASCs have immunomodulatory abilities to regulate T cell function, modulate soluble cytokine expression, and prolong allotransplant survival [7,16]. These results...
indicated the protective and promotional effects in combination of immunosuppressant needs for ASCs to migrate and homing into the graft, which may eventually lead to prolong the allograft. Nevertheless, the fate of donor ASCs after composite allografting that survives for long periods is still unclear. Therefore, in this study, we explore the distribution and role of donor ASCs after VCA by using the green fluorescent protein (GFP) transgenic rats.

Materials and Methods

Animals

Twelve male Lewis (LEW, RT1+) rats aged 8-12 weeks and weighing 240-350 g were used as recipients. Orthotopic hind-limb transplants from male Wistar without green fluorescent protein (GFP) expression to Lewis rats were performed (day 0). The Wistar and Lewis rats were purchased from the National Laboratory Animal Breeding and Research Center, Taiwan. ASCs were propagated from the donor adipose-tissue and subculture originated from green fluorescent protein (GFP) transgenic Wistar rats. GFP-transgenic Wistar rats [W-*Tg* (Per1-luc)10a], which sponsored from Osaka university, Japan (Prof. Tsutomu Kurosawa), and are band bred in the Experimental Animal Center of Kaohsiung Chang Gung Memorial Hospital. The animals are maintained under standard conditions with access to rodent chow and water. They were all treated humanely, according to the guidelines provided in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health. The Division of Laboratory Animal Resources at Kaohsiung Chang Gung Memorial Hospital (KCGMH) provided veterinary care to the rodents. The experiments conducted were approved by the Institutional Animal Care and Use Committee (IACUC) at KCGMH.

Culture of GFP+-ASCs

GFP+-ASCs were propagated from the adipose-tissue of GFP-transgenic Wistar and subculture. The isolation of GFP+-ASCs was performed according to published methodologies [16]. Groin adipose tissue was minced, washed, and digested with 0.2% collagenase type I for 30 min at 37°C. The suspension was centrifuged at 500 g for 5 min at room temperature to pellet cells from the stromal vascular fraction. Following RBC lysis with 0.83% NH4Cl, the cells were washed with PBS, resuspended in maintenance medium, and plated in culture dishes for 4 h. The non-adherent cells were removed, and the attached cells were considered to be P0 ASCs. ASCs were cultured in maintenance medium consisting of DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified with 5% CO2 and subcultured prior to confluency. The ASCs were characterized by flow cytometry following positive surface staining for CD44, CD90, MHC-I, CD73, and CD106; but not for CD45, and MHC-II. ASCs were tested as our previously described procedures for their ability to differentiate into various mesenchymal lineages, including adipocytes, osteoblasts, and chondrocytes [16].

Orthotopic hind-limb vascularized composite tissue allotransplantation model

Hind-limb transplantations were performed on the rats according to our previously described procedures [17]. The hind limbs of the donor Wistar rats without GFP expression were amputated at the mid-thigh level and placed in gauze soaked with cold normal saline. The hind limbs of the recipient LEW rats were amputated in a similar manner at the mid-thigh level, following preparation of the recipient vessels. The donor and recipient femurs were then joined by performing osteosynthesis, using an 18-gauge needle as the intramedullary rod. This method was followed by a repair of the ventral and dorsal muscle groups with 4-0 interrupted silk sutures. The sciatic nerve was repaired with 9-0 nylon sutures. The femoral vein and artery were anastomosed with 10-0 nylon sutures under a stereomicroscope (Zeiss®, Germany). The skin was stitched together with 4-0 silk sutures.

Animal experimental design

Twelve orthotopic hind-limb VCA from Wistar to Lewis rats were performed on day 0. Each subgroup included 6 rats. Group 1 (control group) did not receive any treatment. In Group II, transplanted rat was treated with a combination of CsA (days 0-20), ALG (administered 4 days before and 1 day after CTA), and donor ASCs (2 × 106 cells/time on days 1, 7, and 21 after CTA) in combination. The dosage and protocol of ASCs was mimicked from our previous study [16]. Graft rejection was assessed daily.

Flow cytometry assessment

To determine whether donor GFP+-ASC cells migrated into recipient peripheral blood, flow cytometric analysis was performed one week, two weeks, and 16 weeks after ASCs infusion. The cells were incubated with 5 μl of polyclonal antibody against GFP (Abcam, Cambridge, UK) to identify infusion donor ASCs. After incubation for 30 min at room temperature, the cells were centrifuged, washed, and analyzed on a flow cytometer (Becton Dickinson, CA). Data were subsequently analyzed using the BD FACScan TM program.

Immunohistochemical (IHC) staining

Frozen sections were obtained from the allo skin of the grafted limbs, recipient skin, spleen parenchyma, and liver parenchymal tissue on 2 weeks and 16 weeks after the GFP+-ASC infusion. Samples were embedded in Tissue-Tek® OTC (Sakura Fine Technical Co., Ltd.) and stored at -20°C until use. Cryo-sections at 10 µm of thickness were adhered to poly-L-lysine-coated glass cover-slips and fixed with acetone for 10 min. After polyclonal GFP-antibody (Abcam, Cambridge, UK) labeling, these sections were immediately observed for GFP expression by fluorescent microscopy. The sections were also stained with Hematoxylin-Eosin (H&E) according to standard procedures. Photographs of GFP-expressing sections and H&E sections were composed using Photoshop software (Adobe Systems, Japan). The morphologic changes in the GFP-positive tissue component were examined. For semi-quantification of immunofluorescent immunostaining, the tissue sections were analyzed. Four randomly selected areas were then photographed at 400x magnification. All images were captured using a Cool CCD camera. The images were analyzed using the Image-Pro Plus image analysis software (Media Cybernetics, Silverbernetics Spring, MD).

Results

Donor ASCs combined with short-term ALS-CsA prolonged VCA survival

The allotransplanted limbs were rejected between days 8 and 14 post-transplantation in the controls. Recipients treated with multiple dosages of donor ASCs in addition to short-term ALS/ CsA treatment resulted in significant increases in allograft survival (>100 days) of recipients compared to control group (Table 1).

GFP+-ASC existed in recipient circulating blood but skewed decrease

Flow cytometric analysis of recipient peripheral blood revealed
that GFP+-ASC cell populations were significantly increased at one week and two weeks post-transplant in animals treated with GFP+-ASCs and short-term CsA and ALS, compared to the controls. However, the percentage of ASC-GFP+ cell expressions in the recipient peripheral blood skewed decrease post-transplantation. The GFP+-ASC cell expressions in recipient circulating blood still existed at 16 weeks post-transplant in rats treated with GFP+-ASCs, CsA and ALS (Figure 1).

**GFP+-ASC expressed in recipient and donor tissues post-VCA**

The immunofluorescent staining performed on tissue biopsy samples revealed the GFP positive cells existed in the alloskin and recipient skin and liver and spleen parenchymal tissues 2 weeks post-ASC injection (Figure 2). No apparent expressions of ASC-GFP positive cells were found in recipient tissues at 16 weeks post-transplantation except spleen parenchymal tissue still retain GFP positive cells (Figure 3). These results indicated donor ASCs could be long-term existence in the recipient spleen and might play an immunomodulator to prolong allograft survival.

**Discussion**

Our previous study indicated multiple round of donor adipose-derived stem cells (ASC) in combination with transient immunosuppressant (ALS/CsA) significantly prolonged VCA survival and facilitate long-term graft acceptance in a rodent hind-limb VCA model (from Brown-Norway to Lewis inbred strain) [16]. In this study, we mimicked our previous protocol to trace the donor ASCs engraftment. However, no green fluorescent protein (GFP) transgenic BN rats were available. Therefore, in this study, ASCs combined with short term immunosuppressant were applied in different rodent model (Wistar as a donor to Lewis strain). The GFP+-ASCs were propagated from the adipose-tissue of GFP-transgenic Wistar and subculture. The results revealed the multiple rounds of ASCs (days 6, 8, 10 post-transplantation) in addition to short-term ALS/CsA treatment indeed resulted in significant increases in allograft survival as compared to that in controls.

Although the bio-mechanisms as to how ASCs involved modulation of anti-inflammatory cytokine expression and T cell subsets have been proposed [16,18] the fate of donor ASCs after composite allografting that survives for long periods is still unclear. Therefore, the distribution of donor ASCs engraftment after VCA by using the GFP transgenic rats has been investigated.

Studies demonstrated ASC could modulate regulatory T-cells in vitro and in vivo [19]. Our study indicated CD4+/CD25+/Foxp3+...
T cells increased significantly at early time points post-transplant in animals treated with donor ASCs, short term CsA and ALS compared to untreated controls in a rodent hind-limb VCA model [16]. IHC staining revealed significant numbers of Foxp3+ T cells in allo-skin biopsies from animals treated with donor ASC-CsA-ALS compared to the other groups [16]. These results demonstrate that donor ASCs are a promising therapy that could help to lessen rejection of allotransplantation, which is correlated with regulation of T cells.

To investigate the fate of ASC after infusion to recipient, flow cytometry were analyzed the expressions of GFP+-ASCs in the circulating blood. The results revealed GFP+-ASCs apparently transient expression in the recipient peripheral blood in 2 weeks post-ASC infusion but skewing decrease. However, in blood even after 16 weeks post-transplantation, GFP positive graft ASC cells can still be detected. This evidence indicated the donor GFP+-ASCs cell could exist in recipient circulating blood. In contrast, the immunofluorescent staining performed on tissue biopsy samples revealed the GFP positive cells existed in the alloskin and recipient skin and recipient liver and spleen parenchymal tissues 2 weeks post-ASC injection. No apparent expressions of GFP+-ASCs were found in recipient skin and liver tissues at 16 weeks post-transplantation. However, in solid tissues, only recipient spleen parenchyma were existed the GFP positive cells at 16 weeks post-transplantation. This indicated recipient spleen parenchyma retained donor GFP positive cells and limited long-term survival of the graft cells.

Spleen is the most important lymphoid organ to modulate immune cells and function. Our results revealed the donor stem cells could exist in recipient spleen. (B) Semi-quantitative analyses of GFP positive cells in different immunofluorescent staining tissues were counted. The indicated signals were statistically significant (P<0.05). Abbreviations: GFP: Green Fluorescent Protein; HE: Hematoxylin-Eosin; and DAPI: 4',6-diamidino-2-phenylindole.
engraft in recipient spleen parenchyma. This evidence indicated at least in part donor ASCs exist in the recipient tissue and some might transform to mesenchyme tissue in the recipient spleen as an immunomodulator to modulate immune cells regulation after VCA. Therefore, we propose the reason of donor ASCs prolongation allograft survival might through educate the naïve immune cells and regulate their function to decrease the allograft rejection. However, in this study, the long-term results between donor cells and recipient cells redistribution could not be assessed. Although we did not analyze these cells in this study, future studies are needed to address the presence of T cells, B cells and macrophages in the vicinity of the grafted GFP positive cells. Further studies are needed to elucidate the mechanism of mesenchymal stem cells renewal and induction immune modulation.

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