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ORIGINAL ARTICLE

ALLOGENEIC CHIMERISM INDUCED BY LOW DOSE OF PERIPHERAL BLOOD STEM CELLS COMBINED WITH SPLENOCYTES IN SUBLETHALLY IRRADIATED MICE

Naoki Hashimoto, Shunji Narumi, Yukihiro Itabashi, Kenichi Hakamada, and Mutsuo Sasaki

Abstract Background: We previously demonstrated that a high level of chimerism was induced by low dose of bone marrow cells (BMCs) combined with splenocytes (SPLCs), while the same dose of BMCs alone was insufficient for tolerance induction. In the present study, we investigated whether stable mixed chimerism and donor specific tolerance could be established in sublethally irradiated mice following intravenous injection of various dosages of fully allogeneic peripheral blood stem cells (PBSCs). In addition, validity of combination of donor splenocytes in the peripheral blood stem cell transplantation (PBSCT) as well as in bone marrow transplantation for tolerance induction were examined. **Methods:** Sublethally irradiated C57BL/6 recipient mice were injected $10x10^6$ PBSCs mixed with various numbers of granulocyte-colony-stimulating factor (G-CSF)-mobilized SPLCs harvested from BALB/c donor mice. Three months later, the degree of chimerism in peripheral blood lymphocytes and in SPLCs was examined by FACS analysis. Mixed lymphocyte responses (MLR) and skin grafting were performed to confirm donor-specific tolerance. **Results:** Recipients receiving $10x10^6$ PBSCs mixed with $30x10^6$ G-CSF-mobilized SPLCs showed a higher level of chimerism. High percentage chimeric mice all showed donor specific tolerance *in vitro*, MLR and *in vivo*, and skin grafting. **Conclusions:** PBSCT mixed with SPLCs was proven to be useful for induction of donor-specific tolerance. Admixture of SPLCs reduces the amount of PBSCs needed for induction of tolerance.

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Key words: peripheral blood stem cells; chimerism; mice; splenocytes; tolerance.

原 著

低用量末梢血幹細胞と脾細胞混合移入による非致死的照射マウスへのキメリズムの導入

橋本直樹　鳴海俊治　板橋幸弘　袴田健一　佐々木睦男

抄録　背景：以前我々は，単独では免疫寛容を誘導できない少量の骨髄細胞でも，脾細胞(SPLCs)と混合移入することでキメリズムを誘導できることを明らかにしてきた．今回，非致死的照射レシピエントマウスにドナー末梢血幹細胞(PBSCs)を静注し，キメリズム及びドナー特異的免疫寛容が得られるか検討した．さらにPBSCsにSPLCsを混合して移入し，脾細胞混合の有効性を検討した．**方法**：ドナーBALB/cマウス 10×10^6 PBSCsを，様々な用量のドナーG-CSF-mobilized SPLCsと混合し，非致死的照射レシピエントC57BL/6マウスに静注した．3ヶ月後，末梢血及び脾細胞でのキメリズムをFACSで検討した．また混合リンパ球試験及び皮膚移植を施行し，ドナー特異的免疫寛容であるか確認した．**結果**：10×10^6 PBSCsを30×10^6 G-CSF-mobilized SPLCsと混合して移入すると，高いレベルのキメリズムが得られ，それらはドナー特異的であった．**結論**：脾細胞混合末梢血幹細胞移植はドナー特異的免疫寛容誘導に有効である．脾細胞混合により，免疫寛容誘導に必要なPBSCsを減らすことができる．

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Second Department of Surgery, Hirosaki University School of Medicine
Correspondence: N. Hashimoto
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Introduction

Induction of donor-specific tolerance to foreign antigens on the transplanted organ would eliminate the need for non-specific immunosuppression. Recipients of bone marrow transplants (BMT) achieve this state of donor-specific tolerance, and do not require life-long immunosuppression[1]. We previously demonstrated that a high level of chimerism was induced by low dose of bone marrow cells (BMCs) combined with splenocytes (SPLCs), while the same dose of pure BMCs was insufficient for tolerance induction[2]. Combination with SPLCs might bring hematopoietic cell transplantation for tolerance induction closer to clinical use. However, BMT itself is hampered clinically because of the difficulty of stem cell collection particularly from living related donor and the risk of graft-versus-host disease (GVHD)[3]. Recently, peripheral blood stem cell transplantation (PBSCT) instead of BMT is being increasingly used for the treatment of hematologic malignancies. Advantages of PBSCT over BMT are rapid recovery of hematopoiesis after transplantation, and decrease in hospital stay[4, 5]. In addition, collection of peripheral blood stem cells (PBSCs) averts an invasive procedure of the bone marrow.

In the present study, we investigated whether stable mixed chimerism and donor-specific tolerance can be established in sublethally irradiated mice following intravenous injection of various doses of fully allogeneic PBSC. In addition, validity of combination of donor splenocytes in PBSCT for induction of donor-specific tolerance was examined.

Materials and Methods

Mice

BALB/c donor mice (H-2^d), C57BL/6 recipient mice (H-2^b), and C3H/He third-party mice (H-2^k) (Charles River Laboratories Japan, Ibaraki, Japan), all 8~12-week-old females, were housed in filtered tops cages with controlled light and dark cycle and given free access to drinking water and to a high protein, high fat "breeder diet" (Oriental Yeast Co., LTD., Tokyo, Japan). This study was carried out in accordance with the Guidelines for Animal Experimentation, Hirosaki University.

Preparation of PBSCs and granulocyte-colony-stimulating factor (G-CSF)-mobilized SPLCs

According to a modified method of Neben et al.[6], donor mice were given cyclophosphamide in a dose of 200 mg/kg by intraperitoneal injection on day 0, G-CSF 250 μg/kg twice a day by subcutaneous injection on day 2, 3, 4, 5 and 6. Whole blood was harvested by cardiac puncture on day 7, and diluted into an equal volume of phosphate-buffered saline (PBS) and centrifuged through a Ficoll-Hypaque gradient (Pharmacia Biotech AB, Uppsala, Sweden) to remove erythrocytes. All nucleated cells between the lower erythrocyte fraction and upper serum fraction were collected and washed three times with PBS and RPMI 1640 medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL). On day 7, spleens were also harvested. G-CSF-mobilized SPLCs, obtained by crushing the spleens between glass slides, were resuspended in 4℃ RPMI 1640 medium supplemented with 10% FBS, and filtered through the 70 μm nylon mesh (Becton Dickinson, Franklin Lakes, NJ). PBSCs and G-CSF mobilized SPLCs

were stained with fluorescein isothiocyanate (FITC)-labeled rat anti-mouse Ly-6A/E (Sca-1) monoclonal antibodies (mAbs) (Pharmingen, SanDiego, CA) and measured the number of hematopoietic stem cells by FACS analysis.

PBSCs and SPLCs transplantation

C57BL/6 recipient mice were irradiated at 7.5 Gy (1.11 Gy/min) and on the same day received the donor cells in 1.0 ml of RPMI 1640 medium, which was administered through the tail vein of C57BL/6 recipient mice using a 27 gauge needle. Experimental groups were divided into two groups, PBSC groups, and SPp groups. PBSC groups, which were injected varying numbers of PBSCs included PBSC-I, $15x10^6$ PBSCs; PBSC-II, $10x10^6$ PBSCs; PBSC-III, $3x10^6$ PBSCs. SPp groups, which were injected varying numbers of G-CSF-mobilized SPLCs with a fixed number of $10x10^6$ PBSCs, included SPp-I composed of $30x10^6$ G-CSF-mobilized SPLCs with $10x10^6$ PBSCs, and SPp-II composed of $15x10^6$ G-CSF-mobilized SPLCs with $10x10^6$ PBSCs.

Chimerism analysis of peripheral blood cells and splenocytes

Recipients were anesthetized by diethyl-ether, and peripheral blood was collected from the tail artery and partial splenectomy was performed 3 months after PBSCs injection. Cells were stained with FITC-labeled mAbs to recipient H-$2D^b$ antigens (Pharmingen, SanDiego, CA) and phycoerythrin (PE)-labeled mAbs to donor H-$2D^d$ antigens (Pharmingen). Suspensions were incubated for 30 min at 4℃ in FACS analysis tubes and then removed red blood cells (RBCs) by 0.125% Tris-NH_4Cl buffer for 5 min and washed twice with PBS supplemented with 2% FBS, and resuspended in 400 μl PBS with 1% paraformaldehyde. Ten thousand cells were analyzed using FACScan (Becton Dickinson).

Mixed lymphocyte reaction (MLR)

Responder cells (1x105/100 μl/well) obtained from recipient mice were cultured in 96-well flat-bottomed plates in triplicates, with equal number of irradiated (30 Gy) stimulator cells obtained from donor and third party mice. Plates were incubated at 37℃ in 5% CO_2 for 72, 96 and 120 hours. 5-bromo-2'-deoxyuridine (BrdU, Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England) was added for the last 24 hours before harvesting. The peroxidase-labeled anti-BrdU was added to the wells and these were incubated for 90 minutes. The immune complexes were detected by the subsequent substrate reaction, and the resultant color was read at 450 nm in a microtitre plate spectrophotometer. Stimulation index (SI) was calculated by comparing anti-donor and anti-third-party responses with anti-host responses, which were similar to background counts.

Measurement of cytokine production in the MLR

Production of cytokines was measured in supernatants of MLRs at 72, 96 and 120 hours. All supernatants were stored at −70℃ before assay. Concentration of cytokines was determined using ELISA kits with specific antibodies for interleukin-2 (IL-2), IL-4, and interferon-γ (IFN-γ) (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England). Fifty-microliter samples were added to 96-well flat-bottomed plate coated by an antibody specific for each cytokine in duplicate. Incubating at 37℃ in 5% CO_2 for 2 hours and washing away any unbound sample proteins, an enzyme-linked antibody specific for each cytokine was added to wells. Following another incubation at 37℃ in 5% CO_2 for 1 hour or more and a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to

wells. After incubation for 30 minutes at room temperature, absorbance at 450 nm was measured and the concentration of each cytokine was determined by a standard curve using control cytokines.

Skin transplantation

A full thickness donor skin graft (5x5mm) was harvested from the tail of donor and the third party mice and grafted onto the dorsal thorax of recipient mice. Grafts were covered by plastic bandage to prevent shearing. The bandage was removed on the 7th postoperative day, and daily inspection defined rejection as loss of over 80% of the grafted tissue.

Statistics

Statistical analysis was performed using a computer program Statview 4.5 (Abacus Concepts Inc., Berkeley, CA). All data were expressed as means ± standard error of the mean (SEM). Skin graft survival in different groups was compared using the log-rank test. Other statistical differences were determined according to analysis of variance (ANOVA). *P* values less than 0.05 were considered significant.

Results

Degree of chimerism in PBSC group

PBSCs included around 10% anti-Sca 1 Ab positive cells, while peripheral blood monocytes harvested from naïve mice included 0.15% anti-Sca 1 Ab positive cells by FACS analysis. Recipients receiving less than $15x10^6$ PBSCs showed failed chimerism both in the PBLs and in the SPLCs, which was just similar to background observed in normal control mice (Table 1).

Degree of chimerism in SPp group

G-CSF mobilized SPLCs included around 3% anti-Sca 1 Ab positive cells, while SPLCc harvested from naïve mice included 0.15%

Table 1 The chimerism of peripheral blood lymphocytes and splenocytes in PBSC Group (FACS)[a]

experimental groups	dose	peripheral blood lymphocytes (%)	splenocytes (%)
PBSC-Ⅰ	15×10^6 PBSC	0.9±0.4 (n=4)	0.9±0.7 (n=4)
PBSC-Ⅱ	10×10^6 PBSC	0.6±0.1 (n=4)	0.6±0.2 (n=3)
PBSC-Ⅲ	3×10^6 PBSC	0.9±0.4 (n=3)	0.8±0.2 (n=3)

[a] Mean±SEM.

Table 2 The chimerism of peripheral blood lymphocytes and splenocytes in SPp Group (FACS)[a]

experimental groups	dose	peripheral blood lymphocytes (%)	splenocytes (%)
SPp-Ⅰ	10×10^6 PBSC+30×10^6 G-CSF-mobilized SPLCs	99.4±0.1 (n=6)[b]	98.1±1.0 (n=6)[b]
SPp-Ⅱ	10×10^6 PBSC+15×10^6 G-CSF-mobilized SPLCs	1.2±0.6 (n=4)	0.5±0.2 (n=3)

[a] Mean±SEM.
[b] $P<0.0001$, compared with SPp-Ⅱ group.

anti-Sca 1 Ab positive cells by FACS analysis. Recipients receiving $30x10^6$ G-CSF-mobilized SPLCs failed chimerism both in the PBLs and in the SPLCs, however over $45x10^6$ G-CSF-mobilized SPLCs induced a high level of chimerism (deta not shown). As shown in Table 2, recipients receiving $10x10^6$ PBSCs mixed with $30x10^6$ G-CSF-mobilized SPLCs (SPp-I group) showed a higher level of chimerism than those receiving $10x10^6$ PBSCs mixed with $15x10^6$ G-CSF-mobilized SPLCs (SPp-II group) in the PBLs (99.4 ± 0.06%; $P<0.0001$) and in the SPLCs (98.1 ± 1.0%; $P<0.0001$). This chimeric state persisted for more than 90 days.

MLR

Lymphocyte proliferation in SPp-I group lymphocytes was significantly depressed against donor BALB/c alloantigen compared to control group ($P<0.05$), whereas reaction against the third party C3H/He alloantigen was similar to control group at 72, 96, and 120 hours (Table 3, 4). To the contrary failed chimeric mice did not show donor-specific unresponsiveness.

Cytokine productions in cellular responses against donor alloantigens were measured to determine which type of T-helper cells (Th)

Table 3 Mixed lymphocyte reaction in PBSC Group (Stimulation Index)[a]

	72 hours		96 hours		120 hours	
	vs. BALB/c	vs. C3H	vs. BALB/c	vs. C3H	vs. BALB/c	vs. C3H
control	1.17±0.08	1.39±0.17	1.71±0.25	1.46±0.12	1.23±0.06	1.49±0.31
PBSC-Ⅰ	1.12±0.02	1.15±0.18	1.83±0.80	1.29±0.52	1.29±0.46	1.10±0.29
PBSC-Ⅱ	1.16±0.05	0.90±0.01	1.24±0.18	1.03±0.16	1.48±0.10	1.08±0.09
PBSC-Ⅲ	1.13±0.16	1.29±0.41	1.17±0.19	1.38±0.16	1.07±0.19	1.47±0.43

[a] Mean±SEM, not significant compared with control.

Table 4 Mixed lymphocyte reaction in SPp Group (Stimulation Index)[a]

	72 hours		96 hours		120 hours	
	vs. BALB/c	vs. C3H	vs. BALB/c	vs. C3H	vs. BALB/c	vs. C3H
control	1.17±0.08	1.39±0.17	1.71±0.25	1.46±0.12	1.23±0.06	1.49±0.31
SPp-Ⅰ	0.93±0.05[b]	0.96±0.05	0.90±0.06[b]	1.01±0.05	0.86±0.04[b]	0.99±0.03
SPp-Ⅱ	1.85±0.35	1.81±0.54	1.57±0.09	1.85±0.32	1.78±0.07	2.34±0.49

[a] Mean±SEM.
[b] $P<0.05$, compared with control.

were dominant in SPp-I group. As shown in Fig. 1, production of IL-2 in supernatent of MLR was significantly lower in SPp-I group than in control mice at 72 and 96 hours ($P<0.05$). By contrast, IL-4 production was significantly higher in SPp-I group than in control at 72 and 96 hours ($P<0.05$). There was no significant difference of IFN-γ production between SPp-I group and control.

Survival of donor skin grafts and third party skin grafts

As shown in Table 5, striking prolongation

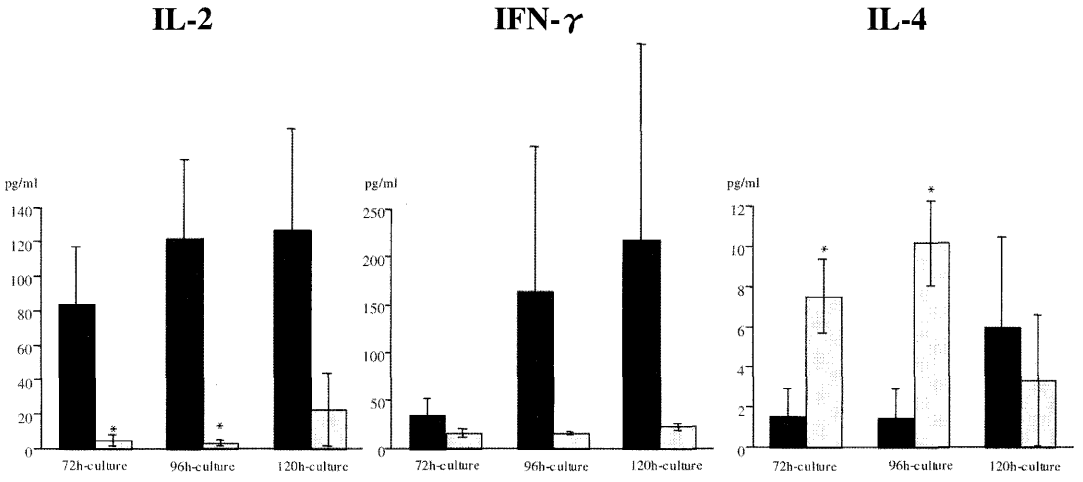

Figure 1 Cytokine production in MLR of splenocytes from SPp-I group and control mice in response to the donor splenocytes. Closed bars represent the production of the indicated cytokine by the splenocytes from control mice. Dotted bars represent production of the indicated cytokine by the splenocytes from SPp-I group. * $P<0.05$ compared with control.

Table 5 Skin graft survival (days)

experimental groups	n	vs. BALB/c skin graft	vs. C3H skin graft
control	3	8.3±0.3	9.7±0.3
PBSC-Ⅰ	4	8.8±1.1	8.3±0.9
PBSC-Ⅱ	3	8.7±0.5	8.0±1.1
PBSC-Ⅲ	3	9.7±1.1	7.8±0.7
SPp-Ⅰ	4	>90×4[a]	7.5±0.5
SPp-Ⅱ	3	9.5±1.5	10.5±0.5

[a] $P<0.05$, compared with control.

of graft survival was observed in chimeric mice ($P<0.001$). Graft prolongation was specific for BALB/c, as C3H/He (third-party) grafts were rejected in normal fashion. No prolongation in donor skin graft survival was observed in failed chimeric mice. GVHD was observed in a few mice in SPp-I group, in which weight loss and skin rash appeared 2 months after transplantation.

Discussion

Bone marrow transplantation has been reported as a useful modality of induction of donor-specific tolerance[1]. However, BMCs harvesting procedure is very invasive especially for living-donor, leading to increasing risk of complications[3]. In place of BMCs, intravenous injections of G-CSF-mobilized peripheral blood mononuclear cells are being used increasingly for hematopoietic reconstitution because of the advantages of faster engraftment and the ease of stem cell collection[5, 6]. Therefore, there is great possibility that PBSCs will become an alternative to BMCs for tolerance induction.

Intravenous injection of G-CSF-mobilized peripheral blood mononuclear cells (PBMC) markedly prolonged heart allograft survival in rat hosts given posttransplant total lymphoid irradiation and anti-thymocyte globulin, but those showed non-chimeric tolerance and developed chronic rejection[7]. Supplemented with megadose of G-CSF-mobilized PBSCs mice could lead to allogeneic chimerism by injection of T cell-depleted BMCs without development of GVHD[8, 9]. Thus, a megadose of donor PBSCs was necessary to establish stable mixed chimerism and allograft tolerance using non-myelosuppressive regimens[10]. In a large animal model, it was also intended to develop a cytokine mobilization and automated leukapheresis technique to enable acquisition of larger numbers of PBSCs[11]. However, this megadose technique is costly and may increase the risk of complications in donors. It is, therefore, necessary to identify an optimal strategy for reduction in number of PBSCs required for stable tolerance induction. This approach is clinically leading to a reduction of cell load given to a patient and prevention of GVHD without changing the property of tolerance induction.

Mice receiving a single injection of $15x10^6$ PBSCs with sublethal irradiation failed to establish chimerism in our experiment. Dose of PBSCs was hardly increased, since the amount of PBSCs obtained from one mouse was very small. Our previous observations demonstrated that effective chimerism was established by a low dose of bone marrow cells (BMCs) if combined with splenocytes (SPLCs)[2]. In the present study, in addition to PBSCs, G-CSF-mobilized SPLCs were utilized, which has sometimes been used for an alternative source of PBSCs[12-14]. Mice infused with $10x10^6$ PBSCs mixed with $30x10^6$ G-CSF-mobilized SPLCs established a high level of chimerism, while a single injection of $10x10^6$ PBSCs was rapidly rejected. This result indicated that a mixture of SPLCs enabled induction of donor specific tolerance with a small amount of PBSCs as well as BMCs.

Donor splenic T cells reportedly facilitate allogeneic engraftment in a fully major MHC-mismatched mice skin transplantation model[15] and rat heart transplantation model[16]. Our previous studies demonstrated active effects of splenic T cells were more important to induce donor specific tolerance than those of splenic B cells[17]. PBSCs have been reported to contain over 10 times larger number of mature T cells, B cells, monocytes and NK cells as BMCs[18, 19]. In allo-PBSCT, 10- to 20-fold more T cells are required compared to

allo-BMT[18]. T cells from G-CSF-treated donors maintained their ability to eliminate residual host hematopoietic cells and to sustain complete long-term donor engraftment in a murine model of allogeneic transplantation across MHC barriers[12]. In the present model, therefore, it was possible that not only splenic T cells but also peripheral T cells might play an important role to establish chimerism and donor specific tolerance. One mechanism that must be considered as the explanation is graft-versus-host reactivity (GVHR) that suppress alloresponsive host cells either by directly attacking the host hematopoietic cells or by indirect effects (e.g., via cytokines)[16, 20].

G-CSF mobilization polarizes PBSC donors and transplant recipients towards Th2 cytokines which accounts for the absence of an increased incidence of acute GVHD[21, 22]. These observations were consistent with our results in which low secretion of IL-2 and high secretion of IL-4 in the supernatant of MLR were demonstrated. This suppression of the function of donor-reactive Th1 cells via a dominant influence of their Th2 counterpart also likely maintained donor-specific tolerance and enhanced allograft survival in portal venous immunization[15, 23]. This phenomenon was also observed in intravenous immunization by blocking co-stimulatory signals for T cell activation using monoclonal antibodies to ICAM-1 and LFA-1[24].

Our present study demonstrated that combination with SPLCs makes PBSCT less invasive particularly for living-donor by reduction in number of PBSCs required for tolerance induction. In addition, PBSCT is proven to be useful for tolerance induction as well as BMT. Usage of mesenteric lymph node instead of the spleen could be investigated as an alternative for potential clinical use to achieve induction of tolerance. Still, further studies in large animals are necessary to optimize the most suitable conditioning protocol of recipients and donors, as well as an appropriate dosage of PBSCs and G-CSF-mobilized SPLCs for induction of donor-specific tolerance.

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