THE HEMATOPOIETIC STEM CELL THERAPY FOR EXPLORATION OF SPACE

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ABSTRACT

Astronauts experience severe/invasive disorders caused by space environments. These include hematological/cardiac abnormalities, bone and muscle losses, immunodeficiency, neurological disorders and cancer. While the cause of these symptoms are not yet fully delineated, one possible explanation could be the inhibition of hematopoietic stem cell (HSC) growth and hematopoiesis in space. HSCs differentiate into all types of blood cells, and growing evidence indicates that the HSCs also have the ability to transdifferentiate to various tissues, including muscle, skin, liver, neuronal cells and possibly bone. Therefore, a hypothesis was advanced in this laboratory that the hematopoietic stem cell-based therapy, herein called the hematopoietic stem cell therapy (HSCT), could mitigate some of the disorders described above. Due to the magnitude of this project our laboratory has subdivided it into 3 sections: a) HSCT for space anemia; b) HSCT for muscle and bone losses; and c) HSCT for immunodeficiency. Toward developing the HSCT protocol for space anemia, the HSC transplantation procedure was established using a mouse model of β-thalassemia. In addition, the NASA Rotating Wall Vessel (RWV) culture system was used to grow HSCs in space condition. To investigate the HSCT for muscle loss and bone loss, donor HSCs were genetically marked either by transfecting the β-galactosidase-containing plasmid, pCMV.SPORT-β-gal or by preparing from β-galactosidase transgenic mice. The transdifferentiation of HSCs to muscle is traced by the reporter gene expression in the hindlimb suspended mice with some positive outcome, as studied by the X-gal staining procedure. The possible structural contribution of HSCs against muscle loss is being investigated histochemically. Since there are reports that hindlimb suspended mice show decreased immunity, an ability to eliminate bacterial infection by the host immune system may be compromised in these mice. To prove this, we have transformed *Escherichia coli* with the plasmid, pCMV.SPORT-β-gal, which were then used as the gene-marked bacteria to infect control and the hindlimb suspended mice. Preliminary results by the X-gal wholmount staining procedure indicate that the hindlimb suspension unloading indeed cause the immunodeficiency and the HSCT could help eliminate the reporter gene-marked *E. coli*.

DESIGN PROBLEM

To maintain astronauts’ homeostasis in space using hematopoietic stem cell therapy, so as to enable them to "go anywhere at any time".

INTRODUCTION

Several reports indicate that astronauts develop hematological abnormalities, including space flight anemia, abnormal red cell morphology and structure, thrombocytopenia, 5-20% reduction in red blood cell mass, decreased hemoglobin concentration and hematocrit, and lowered serum erythropoietin levels (1-5). It is likely that stem cell self-renewal is also inhibited in space, reducing the total number of totipotent stem cells in bone marrow. These abnormal hematopoiesis in 0/µG could adversely affect the astronauts' homeostasis. One avenue to overcome the abnormal hematopoiesis might be to supply normal hematopoietic stem cells (HSCs) periodically, e.g. once in two weeks, to astronauts. As the abnormal blood cells will be destroyed in time, the newly supplied HSCs could differentiate to normal blood cells, thus, maintaining the healthy hematological status of the astronaut for some duration. This process could be repeated and made as routine throughout the long-duration space mission so as to maintain this equilibrium state. This is the essence of our proposed hematopoietic stem cell therapy (HSCT) in space; namely, the objective is to
prevent disorders to take place, rather than to repair already damaged tissues: the Preventive Medicine over the Reparative Medicine. And thus, the HSCT could be applied to various disorders in space, including space anemia, immunodeficiency and muscle and bone losses, as discussed below. It must be emphasized that the HSCs should be prepared on the ground from individual astronaut's blood preflight and kept frozen. The HSCs should be designated to each astronaut and should not be intermixed. During the flight the HSCs would be thawed and grown and expanded. Following the culture, a half of the HSCs would be used for transplantation and the other half frozen for the next culture. In this way, the individual HSCs would be maintained as a normal stock during the long-term space flight. A robotic culture system and stem cell injection machine would ease the astronaut's operation, which we have recently initiated. The transplanted HSCs should be astronaut's own, i.e. autologous transplantation, avoiding the danger of graft-versus-host disease (GVHD). Hence, no myeloablation by toxic chemicals, such as cyclophosphamide and busulfan, would be necessary.

Growth of Hematopoietic Stem Cells in Space

The HSCT in space will necessitate establishing an optimal condition for growing normal HSCs in 0/μ gravity from the frozen state. The growth and expansion of HSCs in space could be achieved by the use of NASA Rotating Wall Vessel (RWV) system. Thus, in this paper we describe a part of our efforts to culture HSCs in the RWV system with some positive outcome. The RWV, aside from the low gravity simulating effect, seems to have a beneficial effect on the growth of HSCs, since according to our experience these cells require constant mixing in a low shear force environment and spatial colocation of participating cell populations or growth factors. We believe that such a condition may reflect in vivo status of HSCs, since the body is a constantly moving environment to them. In consideration of these facts, we have been collaborating with the NASA-NIH Center for Three-Dimensional Tissue Culture (J. Zimmer-berg, Director), conducting several experiments to establish the HSC culture in RWV system (6,7). While the initial study indicated severe inhibition of the HSC growth in the RWV system, by changing the conditions we recently succeeded to grow and expand the HSCs (see Fig. 4, below). Other laboratories have also reported the use of RWV culture system for mouse HSCs (8) and erythroleukemia cell line (5), with apparent inhibitory effect of the RWV on the cell growth and differentiation. As the methods of isolation and culture of HSCs are different in each laboratory, it is necessary to delineate the optimal condition of growth for the individual preparation. In addition, since multiple steps are involved in HSCT, and gene therapy in future, it is necessary to develop conditions, which suit individual needs, so that one controlled flow system is available in the laboratory.

HSCT for Space Anemia

Space anemia may manifest in two forms. One is the anemia experienced by astronauts when they return to earth from μ gravity conditions (4). The other occurs in long duration flight in μ gravity, since studies on hematopoiesis in space indicated that both proliferation and differentiation of blood cells were severely inhibited. Erythropoiesis was more affected than myelopoiesis (2). In our lab, studies on HSCT for space anemia are coupled with studies on the transplantability of HSCs, since the ability of the recipient to express donor hemoglobin is indicative of successful transplantation and differentiation of HSCs. In addition, successful expression of donor hemoglobin indicates that HSCT is successful in mitigating anemic conditions. Transplantability of cultured HSCs can be analyzed by using β-thalassemic mice (9). The β-thalassemic mice, which colony we have established in this institution, have been quite useful for us to establish the transplantation procedure and also to evaluate the quality of HSCs for transplantation. Since the hemoglobin molecule of the animal clearly differs from those of wild type mouse and heterozygotes, as analyzed by the cellulose-acetate electrophoresis, the transplantation can be assessed by characterizing hemoglobin species in the transplanted recipients. Thus, transplantability of HSCs grown in RWV system can be analyzed by the β-thalassemic mouse transplantation system. Regarding the β-thalassemic mouse, it is noteworthy that despite the difference in basic mechanisms between the β-thalassemia and spaceflight, there are uncanny similarities in their phenotypes. Namely, the β-thalassemic mouse shows abnormal red cell morphology, reduced hemoglobin concentration and hematocrit, decreased body weight and size, and brittle bones. It may be that the common underlining cause is a hypoxic condition in the body due to reduced hemoglobin concentration, which both disorders display. Thus,
HSCT for Muscle and Bone Loss

Emerging reports indicate an extraordinary plasticity of HSCs; namely the HSCs, the so-called adult stem cells, can differentiate not only to all types of blood cells but also to muscle, skin, liver, neuronal cells, and possibly bone (10-18). According to Blau et al., as much as 15% of muscle cells could be derived from the transplanted HSCs in normal mouse (17). With regard to bone repair, Cobb's group showed that not only a fractured bone but also completely disconnected bone gap was repaired by bone marrow derived mesenchimal stem cells (12,18). If this holds true in space, the HSCs could be useful to countermeasure various space-caused symptoms, especially muscle and bone losses (19). Since one of the aims of our HSCT is to maintain the homeostasis of muscles and bones, as in hematopoiesis above, during long-duration space missions such as Mars exploration, our working hypothesis is that combined with exercise, periodic autologous HSC transplantation might prevent muscle and bone losses of the astronauts during the long-term exposure to 0/μ G, the differentiating HSCs contributing to the repair of these atrophying tissues. We are investigating these possibilities, using a mouse hindlimb suspension unloading model (20). Since this model is frequently used to simulate astronauts’ bone and muscle losses in space, as well as bed-rest patients on earth, information obtained from this investigation may shed light for the countermeasures. Our experimental design involves the use of transgenic (tg) mice which harbor ubiquitously expressing β-galactosidase (LacZ) gene (21) or green fluorescent protein (GFP) gene (22). The HSCs are prepared from these mice and being transplanted to isologous wild type mice that are hindlimb suspended. If the LacZ-HSCs differentiated to muscles and bones, then examination of these tissues for β-galactosidase expression, which can be detected by X-gal (23), blue-color staining, would signify the possibility. Similarly, GFP-HSC can be monitored by the fluorescence emission. While these are the initial studies, more refined anatomical/histological examinations would be necessary to ensure the actual integration of grafted cells to existing tissues. In space situation, our hope is that the earth-programmed HSCs would either form new muscle cells of ground type or fuse to the existing cells to make ground type fibers. As to the frequency of HSC transplantation, the interval could be determined by the rate of muscle fiber transition from slow to fast type (19, 24, 25). The incoming HSCs should prevent this remodeling and thus, this myosin heavy chain (MHC) isoform change can be the determining factor for frequency.

While it is premature to speculate the contribution of HSCs for repair of bone loss and muscle loss in space, the participation of HSCs for needed repair is apparent from the above reports. In addition, the localization of HSCs to bones and muscles might in the future make it possible to perform HSC-mediated ex vivo gene therapy in space (6,7,27), using insulin-like growth factor 1 (IGF-1) gene which would promote growth of bones and muscles (26) in an autocrine/paracrine fashion. A few words need to be added on the muscle derived stem cells (MDSC), which subject is currently actively pursued by several investigators (28-30). Although we are also working on the MDSC in a mouse system, reproducing pre-plating methods of Huard's laboratory (29), there may be a potential difficulty of this approach to space program because of the invasive operations needed to prepare MDSC: namely, muscle specimens from the astronauts have to be obtained before the flight. Unrelated individual's MDSC would result in graft-vs-host-disease. Thus, the MDSC approach may not be applicable to the space-based stem cell therapy, at least at the current level of technology. Compared with this situation, hematopoietic stem cells can be prepared from the astronauts' blood samples, as is commonly done.

HSCT for Immunodeficiency

Studies on hematopoiesis in space using human HSCs (CD 34+ cells) indicated a decrease in both erythropoiesis and myelopoiesis (2). This decrease in myelopoiesis can then lead to decrease in immunity at μ gravity conditions. Other studies have indicated alterations of several immunological parameters, including leukocyte blastogenesis, cytokine production and leukocyte subset distribution (32-33). Since HSCs have the potential to differentiate into all types of blood cells, including leukocytes, HSCT should be able to mitigate these abnormalities. Since one of the aims of our HSCT is to maintain the homeostasis of immunity during long-duration space missions such as Mars exploration, our working hypothesis is that periodic autologous HSC transplantation might prevent immunodeficiency of
the astronauts during the long-term exposure to \(0/\mu\) gravity, the differentiating HSCs contributing to the maintenance of the immune parameters. We are investigating these possibilities, using a mouse hindlimb suspension unloading model (20). Since this model is frequently used to simulate effects of spaceflight on physiological changes of the body, information obtained from this investigation may shed light for the countermeasures. Our experimental design involves the use of wild type (C57BL) mice which are then intraperitoneally infected with *E. coli* harboring the plasmid, pCMV.SPORT-β-gal. Examination of the tissues for β-galactosidase expression, which can be detected by X-gal (23), blue-color staining, would signify the level of immunity of host system: with blue-color staining being inversely proportional to the ability of mice to eliminate the bacteria: the more intense blue-color staining indicative of a decrease in immunity. The second stage would then be to mitigate this immunodeficiency via HSCT. The HSCs are prepared and transplanted to isologous hindlimb suspended mice. If the HSCs mitigated the immunodeficiency, then examination of these tissues for β-galactosidase staining, would signify the possibility of HSCT.

**MATERIALS AND APPROACH**

**Experimental animals:**
β-thalassemic mouse, C57BL/6Hbb\(^{th}/Hbb^{th} \); the breeding pairs were purchased from the Jackson Laboratory, ME and thereafter bred in this institution to establish a colony. Breeder pairs for LacZ-mouse, B6;129S-Gtrosa26 and GFP-mouse, C57BL/6-TgN (ACTbEGFP)1Obs were also purchased from the Jackson Laboratory and bred in this institution. The latter two mice express the respective reporter genes ubiquitously, except for erythrocytes and hair in the GFP-mouse. The animals were handled and experimented according to the protocols of the Howard University IACUC and IBC.

**Purification of mouse HSCs:**
The HSCs were prepared as described previously (31). Briefly, the mice were sacrificed and the tibiae and femora harvested; no treatment with 5-fluorouracil (FU) was done, unless otherwise noted. The marrow cells were obtained by flushing the bones with Hank's Balanced Salt Solution (HBSS) with 2% FBS. The bone marrow (BM) HSCs were enriched/purified by Histopaque 1077 (Sigma Chemical Co.; \(\rho = 1.077 \text{ g/mL}\) density gradient centrifugation, followed by negative and positive immunomagnetic purging. Primary antibodies used for this purpose were rat anti-mouse L3T4 and anti-mouse Lyt2 antibodies (Becton Dickinson) for negative selection, and rat anti-mouse Thy 1.2 antibody (ibid.) for positive selection. Magnetic goat anti-rat IgG Ab (BioMag; Advanced Magnetics, Inc.) was used as the secondary antibody in both cases, and the immuno-positive cells were selected by a magnet (BioMag Separator). In our hand, the purified Thy-1.2° Lin° stem/progenitor cells account for 0.1 to 0.4% of initial bone marrow cells. In some instances, the Sca-1 selection was also applied to further purify the stem cell population, yielding Thy-1°Lin°Sca-1° (34).

**Long-term liquid suspension culture of mHSCs in static culture:**
The static culture serves as the control to the RWV culture on the ground-based experiments. The condition of static culture is as follows: purified HSCs was seeded in a membrane-vented culture flask in IMDM + 20% FBS, supplemented with 10 ng/ml of recombinant stem cell factor (SCF), Epo (2 U/ml), IL-3 (50 U/ml), IL-6 (2 U/ml), GM-CSF (2 U/ml), Penicillin, Streptomycin, and 0.1% starch. The cells were incubated at 37°C with 7% CO\(_2\) in a 100% humidified chamber. Cell growth kinetics was monitored by counting Vital Red-stained cells every three days for more than one month. Medium is replenished once a week. The HSCs sustained vigorous growth in this culture system for more than 12 months.

**Culture of mHSCs in the Rotating Wall Vessel:**
The RWV culture is conducted in collaboration with L. B. Margolis and W. Fitzgerald in the NASA-NIH Center for Three-Dimensional Tissue Culture, who operates the NASA RWV culture system. The RWV culture was conducted with 12 rpm and the medium described as for the static culture. Expansion of HSC was determined by cell counting as well as by measuring the Thy-1°Kit° population by flow cytometer (with FITC- anti-Thy-1.2 antibody (Ab) and Rhodamin-anti-cKit Ab).

**Engraftment assay for the cultured HSCs using β-thalassemic mice:**
To eradicate recipient's marrow cells in preparation for HSCTP, Cyclophosphamide (CP) and Busulfan (BS) were used, rather than γ-irradiation. The drug-treatment is chosen over the whole body irradiation,
because the latter method is not commonly used for bone marrow transplantation (BMT) in humans with hemoglobinopathies. CP (200mg/kg, i.p.) and BS (80 mg/kg, p.o.) in Hank’s Balanced Salt Solution (HBSS) were administered to mice on two consecutive days. Twenty-four hours later, 10 day-precultured HSCs (apx. 1 x 10^6 cells) in HBSS were injected into ocular veins. Contrary to our concern, the β-thalassemic mice (C57BL/6-Hbbth/Hbbth) could tolerate the drug regimen quite well, even better than the wild type. Both static cultured and the RWV-cultured HSCs, usually after 7-10 days of the culture, were harvested, resuspended in HBSS, and injected into eye vein of the marrow ablated β-thalassemic mice. As the nil control, another group of β-thal mice were injected with HBSS alone. The three groups of mice were maintained in isolation cages for an extended period for testing blood samples.

HSC transplantation (HSCTP):
The transplantation was carried out according to the procedure described (7,35). For LacZ-mouse, strain 129S was used as the recipient to prevent GVHD. While our routine method of HSC injection is through ocular vein, intramuscular injection to thigh is also being tried, aiming to deliver HSCs directly to leg muscles and bones.

Hemoglobin typing by cystamine-cellulose acetate electrophoresis:
Approximately 0.1 mL of peripheral blood was collected from the retroorbital sinus of mice using a 2 mg/mL Na-heparin as an anticoagulant. Blood cells were lysed with 2 vol. of 'Hb Elution Solution' (ISOLAB; 0.05% KCN, 1% Triton X-100, Na-azide) and the sample reacted with an equal volume of cystamine solution [66.7 mM cystamine, 1.33 mM dithioerythritol, 0.1 M (NH4)OH]. The samples were run on a cellulose acetate electrophoresis (7,36).

LacZ staining Procedure:
The HSCs in culture were harvested by centrifugation, fixed with formalin/glutaraldehyde and stained with X-gal-Fe cyanide solution (23). The differentiation of HSCs to muscles was assessed by the LacZ marker. Harvested tissues were stained according to the procedure of Schmidt et al. (21).

RESULTS

Purification and the liquid suspension culture of mouse hematopoietic stem cells (mHSCs)
Characteristics of mouse hematopoietic stem cells. A: The purified mouse hematopoietic stem cells. Mouse HSCs were purified/enriched by immunomagnetic selection and suspended in the MEM/20% fetal bovine serum. The picture was taken under the Leitz inverted phase microscope. Magnification: 250x. B: Liquid suspension culture of hematopoietic stem cells. The purified HSCs were incubated in a liquid suspension culture medium in a vented 25 cm² flask for 10 days at 37°C, 7% CO₂ in a humidified chamber and observed under the microscope as in A.

Differentiation of mHSCs to various blood cell lineages
Basic characteristics of hematopoietic stem cells is an ability to differentiate to various blood cells. In the methylcellulose clonogenic assay system in the presence of erythropoietin (Epo) and IL-3, our HSCs did differentiate to Burst forming unit-erythroid (BFU-E), Colony forming unit-granulocyte, erythroid, megakaryocyte (CFU-GEM) and Colony forming unit-monocytes (CFU-M) (Fig. 2), thus satisfying pluripotential nature of stem cells. BFU-E colony was reddish colored due to hemoglobin production in the cells; CFU-GEM also contained some hemoglobinized cells.

Kinetics of HSC growth in liquid suspension culture
1) Static culture: When cultured in our liquid suspension culture system, which does not contain stromal cells, the HSC numbers in the static culture oscillated, having growth phase and apoptic phase (Fig. 3). Arrows in the figure indicate medium changes during the culture; however, this oscillating pattern appears to be independent of the medium change. Thus, the self-regulating growth pattern is quite unique to the stem cell culture. Flow cytometric analysis for Thy-1.2 + cells increased
from 4.5% to 70% and c-Kit+ cells increased from 25% to 90%, indicating the expansion of stem cells. During the growth phase, it is possible to subculture and expand the stem cells. In one attempt, we have kept the liquid culture for 18 months.

Fig. 3. Growth kinetics of hematopoietic stem cells in liquid suspension culture. The purified mHSCs were grown in the liquid suspension culture as in Fig. 1 B and the cell numbers were counted as indicated in the graph. The cell numbers were determined by using hemocytometer after staining cells with Vital Red. Medium change (MC) is indicated by arrows. Duplicate cultures are shown.

2) RWV culture: Fig. 4 shows the growth pattern of mHSCs in the RWV culture. As reported by others, the HSC growth was severely inhibited in the Biorector system (Fig. 4A), compared with the static culture. However, the inhibition could be overcome by two independent mechanisms: the use of High aspect ratio vessel (HARV) system, as well as by the “1 G exposure” regimen (Fig. 4B). The latter operation is to periodically stop the rotation of RWV culture. Flow cytometric analysis during the culture indicated 98% Kit+ cells and about 4% Thy 1.2+ cells. The detailed study will be published elsewhere.

Fig. 4. Growth kinetics of mouse hematopoietic stem cells in the NASA RWV culture. A: The purified mHSCs were grown either in a static flask culture or the RWV system. Note the inhibitory effect of the RWV system on the HSC growth. However, modification of the culture condition has led to the growth of HSCs. B.

HSC transplantation to β-thalassemic mice

1) β-thal hemoglobin (Hb) types: When hemoglobin is treated with cystamine, the chemical undergoes disulfide interchange reaction with hemoglobin, adding extra positive charges (as cysteamine) to Hbs (36). Since the mouse βββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββbeta
genetically marked, using reporter genes. We have attempted two methods: one is to transfect HSCs with a plasmid harboring β-galactosidase (β-gal) reporter gene, and the other is to isolate HSCs from β-gal transgenic mice. Fig. 6A shows the β-gal plasmid-transfected HSCs, which were subsequently stained by X-gal staining procedure, while Fig. 6B shows the HSCs isolated from the transgenic mice, similarly stained with X-gal. The transfection resulted in more than 50% of HSCs to be marked with β-gal, while 100% of HSCs from the transgenic mice were positive in X-gal staining.

Transplantation of the marked HSCs for HSCT for muscle loss

To initiate the HSC therapy for bone and muscle losses, we set up the mouse hindlimb suspension unloading system in this laboratory. Currently, β-galactosidase-marked HSCs are being transplanted to the isologous hind limb suspended mouse and differentiation of the HSCs to muscles are investigated by X-gal staining procedure. GFP-marked HSCs will be also used in the future. Effect of exercise on the HSC engraftment and differentiation is being investigated. If the engraftment/differentiation were proven, further studies, such as integration/participation of HSCs to existing muscle structure, will be conducted. Effect of HSCT and exercise on the prevention of slow- to fast-type muscle fiber is under investigation utilizing myosin heavy chain (MHC) isoform analysis (24,25,37).

Transplantation of HSCs for HSCT for immunodeficiency

To initiate the HSC therapy for immunodeficiency, we set up the mouse hindlimb suspension unloading system. Examination of hindlimb suspended mouse, following E. coli /β-gal injection indicated an increase in the intensity of blue-color compared to control mice, when the tissues are stained with X-gal. This indicates a decrease in the ability of hindlimb suspended mice to eliminate E. coli and therefore a decrease in immunity. Observation of hindlimb suspended mice injected with isologous HSCs indicated an increase in agility and alertness of mouse. When these mice were injected with E. coli/β-gal and the tissues subsequently stained with X-gal, less stain was detected compared to hindlimb suspended mice that are not treated with HSCs, indicating regaining of immunity by HSCT. This

2) Correction of β-thalassemia by HSC transplantation: The successful bone marrow transplantation (BMT) of β-thalassemic mice with HSCs from C57BL/6J will result in conversion of hemoglobin type in the recipient blood from Hβ^− to Hbβ. Since this combination often killed the β-thal recipients, probably due to the GVHD, we opted to transplant HSCs from heterozygote to β-thal mice (Fig. 5B). The figure shows the chimerism of hemoglobin species in the recipients. The longer period of post-transplantation results stronger donor hemoglobin bands (compare 3 weeks vs. 5 weeks).

Genetic marking of HSCs

In order to trace the transplanted HSCs in the hindlimb suspended mice, HSCs were designed to be genetically marked, using reporter genes. We have attempted two methods: one is to transfect HSCs with a plasmid harboring β-galactosidase (β-gal) reporter gene, and the other is to isolate HSCs from β-gal transgenic mice. Fig. 6A shows the β-gal plasmid-transfected HSCs, which were subsequently stained by X-gal staining procedure, while Fig. 6B shows the HSCs isolated from the transgenic mice, similarly stained with X-gal. The transfection resulted in more than 50% of HSCs to be marked with β-gal, while 100% of HSCs from the transgenic mice were positive in X-gal staining.

Transplantation of the marked HSCs for HSCT for muscle loss

To initiate the HSC therapy for bone and muscle losses, we set up the mouse hindlimb suspension unloading system in this laboratory. Currently, β-galactosidase-marked HSCs are being transplanted to the isologous hind limb suspended mouse and differentiation of the HSCs to muscles are investigated by X-gal staining procedure. GFP-marked HSCs will be also used in the future. Effect of exercise on the HSC engraftment and differentiation is being investigated. If the engraftment/differentiation were proven, further studies, such as integration/participation of HSCs to existing muscle structure, will be conducted. Effect of HSCT and exercise on the prevention of slow- to fast-type muscle fiber is under investigation utilizing myosin heavy chain (MHC) isoform analysis (24,25,37).

Transplantation of HSCs for HSCT for immunodeficiency

To initiate the HSC therapy for immunodeficiency, we set up the mouse hindlimb suspension unloading system. Examination of hindlimb suspended mouse, following E. coli /β-gal injection indicated an increase in the intensity of blue-color compared to control mice, when the tissues are stained with X-gal. This indicates a decrease in the ability of hindlimb suspended mice to eliminate E. coli and therefore a decrease in immunity. Observation of hindlimb suspended mice injected with isologous HSCs indicated an increase in agility and alertness of mouse. When these mice were injected with E. coli/β-gal and the tissues subsequently stained with X-gal, less stain was detected compared to hindlimb suspended mice that are not treated with HSCs, indicating regaining of immunity by HSCT. This
area of research is currently subject to further investigation and more detailed testing.

**Fig. 6.** Lac Z marking of HSCs. HSCs were purified either from A: wild type C57 BL/6J or B: β-galactosidase-transgenic mouse and cultured in a liquid suspension culture system. The wild type HSCs were then transfected with pCMV.SPORT.β-gal (LifeTechnology, Inc.) by Lipofectamine method and stained by X-gal. With regard to the HSCs from the β-gal transgenic mice, the culture was expanded for 3 weeks and an aliquot of the liquid suspension culture was stained with X-gal. The pictures were taken under the Leitz inverted phase microscope. Magnification: x 250.

**Fig. 7.** Transplantation of β-gal-HSCs to a hindlimb suspended mouse. HSCs from a β-gal transgenic mouse were isolated and expanded, and thereafter about 2x 10^5 cells in 0.25 ml of HBSS was injected to thigh and gastrocnemius regions of the right leg of a hindlimb suspended mouse. Two days later, the mouse was sacrificed and various tissues harvested and stained by X-gal stain. Note that segments of the large and small intestine (a) were strongly stained, while the portions of left leg (b) and right leg (c) were positive by the stain. Pictures were taken by Nikon Coolpix 5000.

**DISCUSSION**

We have hypothesized that the hematopoietic stem cell-based countermeasures, hematopoietic stem cell therapy (HSCT), might be effective in maintaining health condition of astronauts during long-duration space missions, such as Mars exploration. While there are several known symptoms which astronauts experience in 0/μ G, we are focusing three areas at this time, namely hematological disorders, immunodeficiency and muscle loss. To formulate the relevant techniques and protocols, two animal models are being used in the ground-based experiments: β-thalassemic mouse and mouse hindlimb suspension system. The goal is to countermeasure/cure hematological abnormalities of β-thalassemic mouse as well as muscle loss and immunodeficiency of the unloaded mouse. In this paper, some of the success of correcting β-thalassemic mouse as studied by the hemoglobin change is presented (Fig. 5B). Successful cure of β-thalassemia by HSCT may normalize various hematological parameters. These would include white blood cell counts, lymphocyte counts, hematocrit, reticulocytes counts, RBC volume (MCV), and hemoglobin content (MCHC) (38). Therefore, these parameters are being measured in the marrow transplanted β-thalassemic mice in comparison to mock treated mice. This transplantation system has been quite useful in this laboratory to establish transplantation procedures as well as to evaluate transplantability of HSCs. Therefore, it is in our plan to test the quality of HSCs grown in the NASA Rotating Wall Vessel (RWW) culture by the β-thal transplantation system. Thus, the HSCT protocols derived from this animal model would be highly relevant to the HSCT for space-caused hematological abnormalities.

With regard to the study on efficacy of HSCT for muscle loss, the current goal is to delineate participation of HSCs in repair/prevention of muscle and bone losses in the hindlimb suspended mouse. The reporter gene marked HSCs are useful to trace transdifferentiation in the transplant recipient. We have successfully marked the HSCs with β-galactosidase expression by two methods, transfection and transgenic mouse, which are presented in Fig. 6A,B. The transfection method will be useful for marking human HSCs in the future clinical trials. Further physicochemical analyses for the actual countermeasure will be performed. These will include: 1) Measurement of muscle weight; 2) Study of prevention of MHC transition by HSCT; 3) Measurement of muscle strength; 4) Other biochemical marker studies (19). Insulin-like growth factor (IGF)-1 treatment of HSCs is being considered to stimulate the muscle growth (19,26). In addition,
using the hindlimb suspension model, effect of exercise on the HSCT for muscle loss can be investigated. Combined with proper exercise regimen, periodic HSC transplantation might prevent bone and muscle losses. These studies are under way in this laboratory.

Thus far, our preliminary results have successfully indicated that not only do hindlimb suspended mice suffer immunodeficiency, but also that the HSCT has the capability of mitigating these symptoms. Our current goals are now to quantify the degree of immunodeficiency exhibited in the hindlimb suspended mouse and then to possibly establish the process of HSCT for immunodeficiency. Since astronauts suffer a decreased immunity similar to that of hindlimb suspended mice, the HSCT protocols derived from this animal model would be highly relevant to the HSCT for immunodeficiency in astronauts.

**FUTURE PLANS**

One major spin-off of the HSCT could be an opportunity to develop stem cell-mediated *ex vivo* gene therapy in the future (6,7). As our expertise lies on adeno-associated virus (AAV)-mediated gene therapy, the vector containing IGF-1 could be constructed and used for muscle gene therapy in space. The adeno-associated virus, serotype 2 (AAV-2), is a human parvovirus, which contains a linear single-stranded DNA of 4,675 nucleotides (nt), having broad host-range and tissue-specificity (39,40). Because the virus is a non-pathogen, the virus promises to be a safer vector for gene therapy than other pathogenic viruses, such as, adenovirus, retrovirus and herpes virus. This non-pathogenicity of AAV, compared with other vectors, is particularly important for the space program, because of the enclosed environment in a spaceship. Exploiting these advantages of AAV-2, starting in 1985, we have constructed recombinant AAVs (rAAVs) that harbor human globin genes as well as an anti-HIV-1 *gag* ribozyme (anti-*gag* Rz) for eventual use in gene therapies of the hemoglobin disorders, the β-thalassemia and sickle cell disease, and AIDS, respectively (31,35,41,42). Putting all the techniques together, we are currently trying to cure mouse model of human β-thalassemia (35). Once these mice are cured by gene therapy, we will then move to possible clinical trials for human patients. Many laboratories worldwide are now using the rAAV system for various gene therapy projects with promising results (43-45). With regard to the space program, this system promises to be a good model system for developing space-based gene therapy, and our long-term plan is to conduct the model experiment on International Space Station.

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**REFERENCES**


