

Reconstitution with Bone Marrow Cells is Consistently Defective in the Tibia of Aged Monkeys

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Abstract

Biological aging is associated with a progressive loss of regulation of cellular, tissue and organ interaction, ultimately resulting in senescence. Fat gradually occupies bone cavity with age. Studies of the hematopoietic system using nonhuman primates have provided important information for understanding the mechanism of human hematopoiesis. We previously reported that excellent reconstitution of donor hematopoietic cells in a collagen gel group was observed in the long term in mice. We therefore investigated whether the method would be helpful for reconstituting hematopoiesis in the fatty tibias of monkeys. Nine- to 11-year-old monkeys were used for this study to examine if hematopoiesis could be restored in the fatty marrow of old monkeys. Bone marrow cells were collected from the humerus using the perfusion method, mixed with cultured bone marrow mesenchymal stroma cells in collagen gel, and then injected into the tibia of the same monkey. Clot sections were made from the tibias 1, 2 and 6 weeks after the bone marrow transplantation. However, no bone marrow cells were observed at any of the time points. These results suggested that bone marrow reconstitution is consistently defective in aged monkeys.

Keywords: Cynomolgus monkey; Adipocyte; Bone marrow; Hematopoiesis

Introduction

Bone marrow transplantation (BMT) is one of the most powerful strategies for the treatment of various intractable diseases, including leukemia, aplastic anemia, congenital immunodeficiency, and autoimmune diseases. Studies of the hematopoietic system of nonhuman primates have provided important information for understanding the mechanism of human hematopoiesis [1,2]. Intra bone marrow (IBM)-BMT has been shown to induce rapid recovery of donor hematopoietic cells, to easily induce tolerance in the recipients, reduce the severity of GVHD, and permit a reduction in radiation doses as a pretreatment for BMT [3]. However, our previous studies showed that because of the abundance of the blood vessels in the bone marrow (BM), some of the injected BM cells (BMCs) get into these blood vessels, and thereby into peripheral circulation, even with IBM-BMT. Cellmatrix (Nitta Gelatin, Inc., Yao, Japan) is an acidic soluble type I collagen that is liquid on ice but forms a gel when warm. The BMCs were mixed with the Cellmatrix and then injected by IBM-BMT. This process helped retain more injected donor BMCs in the recipient bone cavity resulting in excellent reconstitution of donor cells [4].

BMCs mainly include hematopoietic stem cells (HSCs) and mesenchymal stroma cells (MSCs). BMSCs are a heterogeneous population of cells that not only support hematogenesis, but also have the capacity for self-renewal, and differentiate into osteocytes [5] and adipocytes [6]. A number of signaling pathways regulate the inverse balance between osteogenesis and adipogenesis [7]. One report

indicated that hMSCs differentiate into adipocytes more often than osteocytes in the aged bone marrow [8]. Marrow cavities in all bones of newborn mammals contain hematopoietic tissue, and the stromal microenvironment supports hematopoiesis. From the early postnatal period onwards, the hematopoietic microenvironment-mainly in tubular bones of the extremities-is progressively replaced by mesenchymal cells that accumulate lipid drops, and the hematopoietic tissue gradually disappears [9,10]. All grafts from patients undergoing foot or ankle fusion were examined and showed that iliac grafts contained active hematopoietic marrow. However, the medullary space of tibia grafts contained fat and little hematopoietic marrow. The hematopoietic microenvironment in tubular bones eventually loses its ability to support hematopoiesis [11]. Our basic hypothesis was that transplanted BMCs should be able to develop functional hematopoiesis in the tibia where adipocytes occupy the bone cavity when IBM-BMT was performed in aged monkeys.

Materials and Methods

Animals

Normal 9-11-year-old male cynomolgus monkeys were obtained from Kearsy (Osaka, Japan). The monkeys were free of intestinal parasites and seronegative for B virus, tuberculosis, herpes B virus, hepatitis A virus, and hepatitis B virus. All surgical procedures and postoperative care of animals were carried out in accordance with the guidelines of the National Institutes of Health for the care and use of primates. The study protocol was approved by the Animal Experimentation, Use and Care Committee, Kansai Medical University. A total of 9 monkeys were used in this study.

BMC harvesting

Monkeys were anesthetized using Ketalar (5 mg; Sankyo Co.Ltd.; Tokyo Japan). The BMCs were harvested from the long bones (humerus) using the perfusion method previously established [12]. In brief, one BM puncture needle was inserted into the proximal side of the long bone and the other was inserted into the distal side. A syringe containing 0.5 ml of heparin was connected to the needle and a syringe containing 30 ml PBS was connected to the other needle. The PBS was gently pushed from the syringe into the BM cavity, and the PBS containing BMCs was collected in the syringe containing heparin.

MSC culture and colony-forming unit-fibroblast (CFU-F) assay

Monkey whole BMCs were obtained from the humerus of monkeys and cultured in α MEM supplemented with 10% FBS. The culture medium containing nonadherent cells was replaced with fresh medium at day 5. The BM adherent cells reached confluence about 2 weeks later. These adherent cells were named MSCs in this experiment. To examine the number of CFU-F, 5x10⁶ BMCs/well (6 well plate, triplicate) were seeded and the colonies counted at day 14.

Flow cytometry

Monkey BMMSCs (1st passage cells) were washed twice with 0.02% ethylene diaminetetraacetic acid (EDTA)-PBS and the MSCs were then detached from the flasks using trypsin-EDTA treatment. The thus-prepared cells were stained with phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) against nonhuman primate CD56 (neural cell adhesion molecules), CD29, CD31, CD11b, CD34 and CD45 (BD Biosciences, San Jose, CA, USA). The stained cells were analyzed using a FACScan (Becton Dickinson, Mountain View, CA, USA).

Methylcellulose assays

The colony-forming ability of the non-adherent cells obtained from the fresh BMCs was assessed using methylcellulose assays. Appropriate numbers (1x10⁴) of these cells were plated in 35 mm dishes in a volume of 1 ml of MethoCult GF H4434 each (Stem Cell Technologies Inc., Vancouver, British Columbia, Canada), consisting of optimal concentrations of human cytokines (SCF, EPO, IL-3, GM-CSF, and G-CSF), 30% FBS, 1% bovine serum albumin, 2 mM L-glutamine, 100 mM 2-mercaptoethanol, and 0.9% methylcellulose (triplicate). The plates were incubated for 14 days and the numbers of colonies then counted.

Induction of monkey BMMSCs to adipocytes and osteoblasts

To induce adipogenic differentiation, the subconfluent BMMSCs were incubated with induced adipogenesis medium using an adipogenesis assay kit (Cayman Chemical Company, MI). The induction medium included isobutylmethylxanthine solution in DMSO (1000x dilution), dexamethasone solution in DMSO (1000x dilution), insulin solution (1000x dilution) and 4.5 mg/ml D-glucose. The medium was changed every 2 days. Adipogenic differentiation was assessed by oil red O staining 4 weeks after the initial adipogenic induction.

Osteogenic differentiation was induced in the subconfluent BMMSCs by incubating them using the STEMPRO osteogenesis differentiation kit (Gibco). The medium was changed every 2 days.

Osteogenic differentiation was assessed by Alizarin red S staining 3 weeks after the initial osteogenic induction.

Clot section and histological examinations

About 2 ml of BM was collected using the aspiration method, and this was then dropped into a dish, fixed with 10% neutral formalin and embedded in paraffin. The sections were prepared and stained with HE. For lipid droplet detection, samples were fixed with 10% neutral formalin for 1 week. Samples were washed and post-fixed with 2% aqueous OsO₄ and 5% potassium dichromate for 16 hours at 4°C. The samples were then dehydrated with serially-increasing concentrations of ethanol (60 to 100%), and then embedded in paraffin.

Preparation of the collagen gel matrix

The collagen gel matrix, Cellmatrix (Nitta Gelatin, Inc., Japan), was prepared following the manufacturer's instructions [4]. That is, solutions A, B, and C were mixed at a ratio of 8:1:1 and were kept on ice to prevent gel formation until use. This is because the mixture is liquid on ice but becomes a gel when it is warmed. Hereafter, this mixture is referred to as collagen gel.

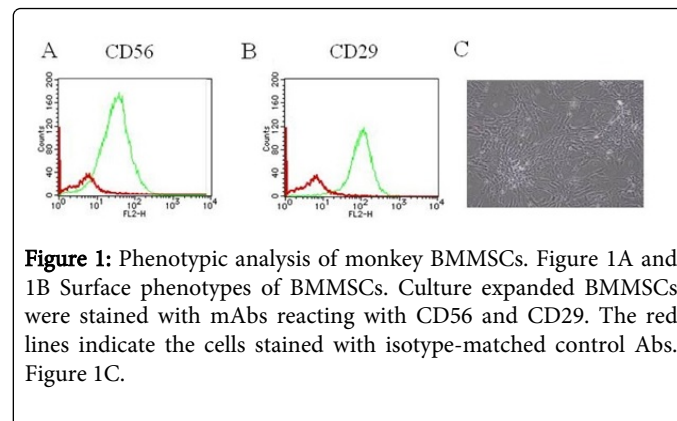
Syngenic transplantation into tibia

The BMCs (1x10⁸) and cultured MSCs (1x10⁷) from the same monkey were suspended in ice-cold collagen gel and then kept at room temperature until injected into the tibia after about 2 ml of BM were taken out from the same tibia.

Results

Immunophenotypic analyses of monkey BMMSCs

Monkey BMCs were collected from cynomolgus monkeys using the perfusion method. After a 2- to 3-week culture, surface markers of the culture-expanded BMMSCs were assessed by flow cytometry. It has been shown that BMMSCs are positive for CD56 [13], so we used it as a marker for BMMSCs. BMMSCs were stained positively with anti-CD56 (Figure 1A) and CD29 (Figure 1B) mAb. CD29 refers to the representative cell adhesion molecules expressed on hemopoietic cells, BM stromal cells, and MSCs. BMMSCs showing uniform fibroblastic morphology were obtained (Figure 1C). These cells were negative for CD31, CD11b, CD34 and CD45 (data not shown).



Morphology of BMMSCs: The spindle-shaped adherent cells were obtained 2 weeks later. Scale bar = 50 μ m. Representative staining patterns of three independent experiments.

Ability of monkey BMMSCs to differentiate into adipocytes and osteoblasts

The BMMSCs were examined to see whether they had any characteristics of MSCs; we investigated their ability to differentiate into adipocytes and osteoblasts. The BMMSCs were cultured in the inductive medium into adipocytes and osteoblasts for 4 and 3 weeks, and then stained with oil red O reagents and Alizarin red S, respectively. As shown in Figure 2A-2D, intracellular lipid droplets were detected by the oil red O staining. The BMMSCs grew quickly in the inductive medium for osteogenesis differentiation (Figure 2E-G). Calcium deposits were detected by Alizarin red S staining (Figure 2H), indicating that the cells had the potential to differentiate into adipocytes and osteoblasts.

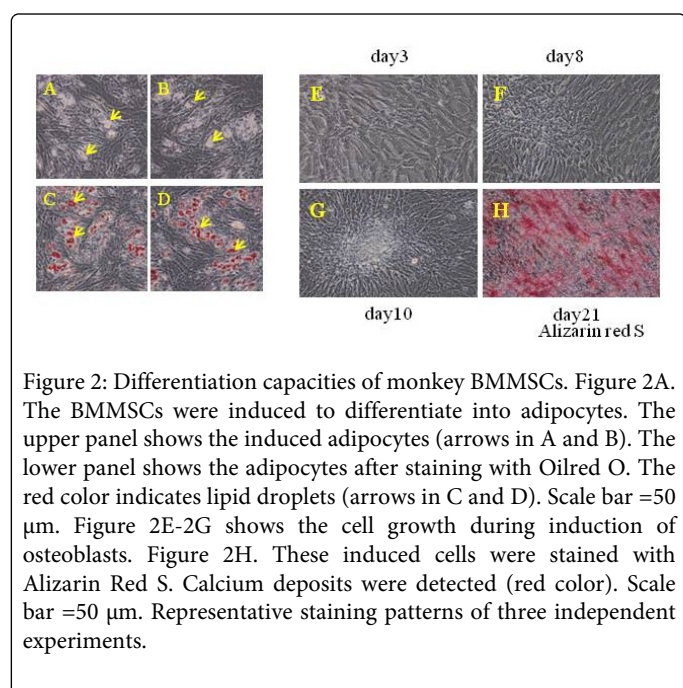


Figure 2: Differentiation capacities of monkey BMMSCs. Figure 2A. The BMMSCs were induced to differentiate into adipocytes. The upper panel shows the induced adipocytes (arrows in A and B). The lower panel shows the adipocytes after staining with Oilred O. The red color indicates lipid droplets (arrows in C and D). Scale bar =50 μ m. Figure 2E-2G shows the cell growth during induction of osteoblasts. Figure 2H. These induced cells were stained with Alizarin Red S. Calcium deposits were detected (red color). Scale bar =50 μ m. Representative staining patterns of three independent experiments.

CFU assay

The CFU-F assay is used as a functional method to quantify MSCs and progenitor cells. BMCs of monkey had the ability to differentiate into erythroid and myeloid lineage cells in the clonal hematopoietic colony assay using MethoCult GF H4434. The numbers of CFU-F and CFU-cultures (CFU-C) are shown in Figure 3A. CFU- erythroid (CFU-E), CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M), and CFU- granulocyte-macrophage (CFU-GM) were detected, and the numbers are shown in Figure 3B.

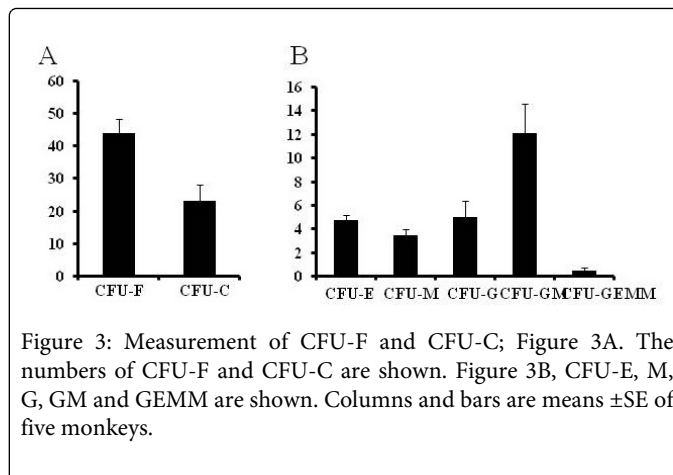


Figure 3: Measurement of CFU-F and CFU-C; Figure 3A. The numbers of CFU-F and CFU-C are shown. Figure 3B, CFU-E, M, G, GM and GEMM are shown. Columns and bars are means \pm SE of five monkeys.

Syngenic transplantation into tibias

Adipocytes mixed with nuclear cells (blue color) were observed in the aged (9-11-year-old) humeri (Figure 4A). However, fewer nuclear cells, which may have been from the peripheral blood and/or lipid droplets (black color), were observed in the tibias (Figure 4B). Although fresh BMCs mixed with cultured BMMSCs (1st passage cells) in collagen gel were syngenicly transplanted into the tibias, almost no mononuclear cells (blue color in Figure 4A) were found in the tibias 1 week (Figure 4D), 2 weeks (Figure 4F) and 6 weeks (Figure 4H) after IBM-BMT, suggesting that transplanted BMCs cannot promote hematopoiesis in the tibia.

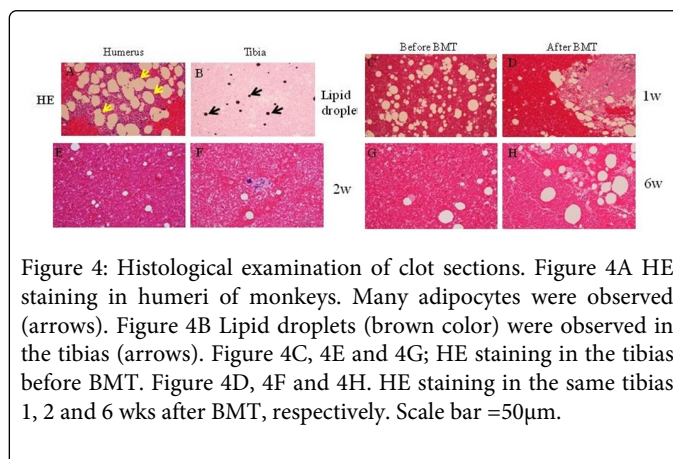


Figure 4: Histological examination of clot sections. Figure 4A HE staining in humeri of monkeys. Many adipocytes were observed (arrows). Figure 4B Lipid droplets (brown color) were observed in the tibias (arrows). Figure 4C, 4E and 4G; HE staining in the tibias before BMT. Figure 4D, 4F and 4H. HE staining in the same tibias 1, 2 and 6 wks after BMT, respectively. Scale bar =50 μ m.

Discussion

Biological aging can negatively influence the regenerative potential of tissue and cellular functions in a variety of organs. Clinical trials as well as animal studies have shown that the generation potential of bone and other tissues declines with age due to a decline in the number or frequency of stem cells present in adult organs [14-17]. In humans, accelerated marrow adipogenesis has been associated with aging and several chronic conditions, including diabetes mellitus and osteoporosis. Several groups have demonstrated that the frequency of CFU is lower in aged donors in a number of species [18]. Aging causes a decrease in the number of bone-forming osteoblasts and an increase in the number of marrow adipocytes. Adipocyte-rich marrow harbors a decreased frequency of progenitors and relatively quiescent stem

cells. Protein malnutrition effect the BMMSCs differentiated into adipocyte, and adipocytes impaired hematopoiesis in the mouse BM [19]. One report has indicated a predominantly suppressive influence of adipocytes on hematopoiesis within the BM microenvironment [20]. We have previously shown that the fatty marrow associated with decreased numbers of BMCs has been found to increase with age in monkeys. The numbers of BMCs varied in the long bones (humerus>femur>tibia) and showed age-dependent decreases when using the perfusion method, whereas they remained similar in the ilium in monkeys aged from 3 to 6 years old. Sufficient numbers of BMCs were still maintained in the humerus and femur in 6-year-old monkeys, while the fatty marrow rapidly replaced red marrow in the tibia [21]. We have previously found that a MHC restriction exists between pluripotent hematopoietic stem cells (HSCs) and stroma cells, and that the recruitment of donor stroma cells facilitates the engraftment of donor BMCs [22,23]. Moreover, we previously reported that IBM-BMT has been proven to be the best method for BMT in the long bones, including humeri and tibiae of 2-4-year-old monkeys [12]. Thus, we chose 9-11-year-old monkeys for this investigation because there is more fat marrow in the tibia of these monkeys.

We previously reported the numbers of CFU-C of young (2-4-year-old) monkeys [12], and that there was no significant difference in the numbers of CFU-C of aged (9-11-year-old) monkeys when compared with young monkeys. The MSCs of these monkeys have the ability to differentiate into adipocytes and osteoblasts, suggesting that they are active even in aged monkeys. No BMCs were observed, but lipid droplets were observed on the clot sections of the tibiae, suggesting that adipocytes filled in the tibiae. Hematopoietic microenvironment cells advance along a three-stage differentiation/maturation pathway. In the first stage, they support hematopoiesis and contain no fat. In the second stage, the cells accumulate fat and no longer support steady state hematopoiesis; however, under conditions of increased hematopoietic requirement, they lose fat and regain their ability to support hematopoiesis. In the last stage, hematopoietic microenvironment cells retain the appearance of yellow BM and do not support hematopoiesis, regardless of the state of hematopoietic requirement [24].

Our previous hypothesis was that BMCs could develop functional hematopoiesis in the fatty microenvironment of tubular bone. We therefore studied the tibiae of aged monkey because the tibiae were filled with adipocytes. First, fresh BMCs were injected into the tibia, but functional hematopoiesis was not observed in the tibia 1wk to 6 wks after injection. Second, since BMMSCs play an important role in the homing, engraftment, self-renewal, and the differentiation of HSCs, and support the growth of HSCs, it has been postulated that stromal damage caused by conditioning regimens may have a profound influence on engraftment kinetics [25]. Cultured BMMSCs mixed with fresh BMCs from the same monkey were injected into the tibia, but again, functional hematopoiesis was also not observed in the tibia 1 wk to 6 wks after injection. Finally, collagen gel containing BMCs proved to be excellent for the reconstitution of donor hematopoietic cells in mice [4]. We mixed cultured BMMSCs and fresh BMCs in collagen gel, and then injected the mix into the tibia. However, no BMCs were observed in the tibiae of the monkeys 1, 2 or even 6 wks after BMT, suggesting that the fatty marrow environment does not support hematopoiesis. Previous reports suggested that BM adipocytes reduced the production of GM-CSF and G-CSF [26]. Adipocytes secrete neuropilin-1 [27], lipocalin 2 [28], adiponectin [29] and TNF- α [30], which impair hematopoietic proliferation.

Future studies will focus on the mechanism of hematopoietic inhibition by BM adipocytes.

We believe that 1) old BMMSCs may not support hematopoiesis *in vivo* in the experiment, even though they do have the ability to differentiate into adipocytes and osteoblasts *in vitro*. 2) Existing marrow adipocytes in the tibia may induce the BMMSCs to differentiate into adipocytes. BM adipocytes prevent hematopoietic progenitor expansion while preserving the stem cell pool [31]. Marrow adipocytes may be self-promotive such that existing marrow adipocytes can induce the differentiation of more MSCs into adipocytes, thereby preventing lineage allocation into other cell lines [32]. 3) Cultured BMMSCs may lose some of their ability to support HSCs. Future studies will confirm whether the old BMMSCs support the growth of HSCs *in vitro*. In conclusion, the present study suggests that BMCs fail to reconstitute in the tibia of aged monkeys.

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