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Systemically Transplanted Bone Marrow Stromal Cells Contributing to Bone Tissue Regeneration

S. LI^{1,2}, Q. TU¹, J. ZHANG^{1,2}, G. STEIN³, J. LIAN³, P.S. YANG², and J. CHEN^{1,*}

¹Division of Oral Biology, Department of General Dentistry, Tufts University School of Dental Medicine, Boston, Massachusetts

²School of Stomatology, Shandong University, Jinan, Shandong Province, China

³Department of Cell Biology and Cancer Center, University of Massachusetts, Worcester Campus, North Worcester, Massachusetts

Abstract

Bone marrow stromal cells (BMSCs) are a rich source of osteogenic progenitor cells. A fundamental question is whether systemically transplanted BMSCs participate in bone regeneration. Luciferase and GFP double-labeled BMSCs were transplanted into irradiated mice. Five weeks after transplantation, artificial bone wounds were created in the mandibles and calvaria of the recipients. Animals were sacrificed at weeks 2, 4, and 6 after surgery and the expressions of luciferase and GFP were determined using Xenogen IVIS Imaging System, immunohistochemical staining and RT-PCR. The results demonstrated that transplanted BMSCs can be detected in wound sites as early as 2 weeks and lasted the whole experimental period. Luciferase expression peaked at 2 weeks after surgery and decreased thereafter, exhibiting a similar expression pattern as that of BSP, while GFP expression was relatively stable during the experimental period. In conclusion, BMSCs can migrate to bone wound sites and participate in bone regeneration in orofacial region.

Bone regeneration and tissue engineering are biologically and clinically relevant, particularly in the oral and craniofacial region. The ultimate therapeutic goal for repair of damaged tissues and reconstruction of oral and maxillofacial defects is regeneration of the tissues to a normal or pre-disease state. Such regeneration requires differentiation of reparative cells that produce appropriate amounts of extracellular matrix components, in a precise temporal and spatial manner to form specific types of hard and soft connective tissues (Abukawa et al., 2006; Mao et al., 2006). However, there is still much to be learned of the cellular histogenesis before these approaches for regeneration of the complex tissues that comprise the skeletal structures in orofacial regions become routine clinical procedures.

Within the diverse population of the bone marrow, there exists a subset of stem cells designated mesenchymal stem cells (MSCs) that maintain multipotential differentiative features (Robey, 2000). These cells are capable of self-renewal and can differentiate into several phenotypes including bone, muscle, cartilage and fat tissues (Pereira et al., 1995; Ferrari et al., 1998; Hou et al., 1999; Robey, 2000). When bone marrow is cultured in vitro, adherent cells of nonhematopoietic origin proliferate and exhibit many of the characteristics attributed to bone marrow stroma in vivo (Owen, 1988; Bruder et al., 1997). Under optimal culture conditions, millions of bone marrow stromal cells (BMSCs) can generate from a limited amount of starting

material (Krebsbach et al., 1998; Pittenger, Science). Transplantation of human BMSCs into immunocompromised mice subcutaneously formed lamellar bone containing osteocytes and surface-lining osteoblasts (Gronthos et al., 2000).

Furthermore, cells from the bone marrow stroma compartment have been demonstrated to be able to support hematopoiesis (Yang et al., 2007). The interaction with osteoblastic cells is essential to maintain hematopoietic stem cells (HSCs) and osteoblasts have been demonstrated as a regulatory component of the HSCs niche in adult bone marrow (Calvi et al., 2003; Arai et al., 2004). HSCs have potential to differentiate into all types of blood cells, but the insufficiency of available HSCs limits their application for clinical therapy. For in vitro expansion of HSCs, stromal cells have been considered as a prerequisite (Amsellem et al., 2003) because in vitro expanding HSCs may differentiate rapidly and lose self-renewal capacity without the presence of stromal cells.

Using total body irradiation (TBI) mouse model, it was reported that myeloid and lymphoid progenitors could adhere to plastic within hours and can support survival of lethally irradiated mice (Bearpark and Gordon, 1989). Anklesaria et al. (1987) also reported that hematopoietic recovery from total body irradiation could be achieved after transplantation of a bone marrow stromal cell line. Nilsson et al. (1999) detected fully differentiated, quiescent, donor-derived osteocytes in the femoral cortex of mice receiving marrow grafts. Hou et al. infused marrow stromal cells carrying a reporter gene driven by the osteocalcin promoter into recipient mice and provided evidence for some engraftment. In this study, it was reported that “an adherent and expanded marrow stromal population transplanted systemically can retain competency to (i) engraft, (ii) differentiate into mature osteoblasts and osteocytes after transplantation, and (iii) support reconstitution of hematopoiesis in radiationablated mice” (Hou et al., 1999). These studies demonstrated that bone marrow and BMSCs not only participated in recruitment of hematopoietic stem cells and osteogenic cells but also play a role in metabolism of bone tissues during physiological processes (Bianco et al., 2000). However, to establish clinical relevance, it is necessary to provide evidence that BMSCs possess the potential to recruit and differentiate into osteoblast progenitors in bone defects. In this study, we directly demonstrate that systemically transplanted BMSCs participate in bone regeneration.

Materials and Methods

BMSCs cell culture

The male ACTB-EGFP mice (Jackson Lab) mouse was cross-bred with a homozygote mBSP9.0Luc female mouse (Paz et al., 2005). A Xenogen 200 IVIS Imager was used to confirm the genotypes of the offspring. Mice showing dual reporters were designated as BSP-Luc/ACTB-EGFP. All the animal experimental procedures were approved by IACUC in Tufts-New England Medical Center.

As described previously (Valverde et al., 2005), BMSCs were obtained from 8-week-old BSP-Luc/ACTB-EGFP mice and were cultured under nondifferentiating conditions (DMEM with 20% fetal bovine serum, 100 mg/ml penicillin and 100 mg/ml streptomycin). The effect of osteogenic differentiation on expressions of luciferase and GFP in these cells was determined by culture in nondifferentiating growth medium and osteogenic medium respectively for an additional 7 days. Osteogenic medium consisted of the complete growth medium plus osteogenic growth supplements including 0.5% ascorbic acid, 0.5% dexamethasone, and 1% β -glycerophosphate. The BMSCs were then visualized by the IVIS imaging system to determine the expressions of luciferase and GFP.

BMSCs transplantation

The recipients were 8-week-old CB6F1 male mice (Jackson Laboratory). The radiation and transplantation procedures were based on published protocols (Hou et al., 1999). Briefly, twenty-five CB6F1 male mice were irradiated using a ^{137}Cs Gammacell 1000 Irradiator (Atomic Energy, Canada) with a sublethal dosage of 5.5 Gy twice at an interval of 4 h for a total body irradiation. Each recipient mouse received approximately 2×10^6 cultured BMSCs 4 h after the irradiation. BMSCs were re-suspended in 0.1 ml of DMEM and the entire volume was transplanted by tail vein injection. Five of the irradiated mice without BMSCs systemic transplantation served as a control.

Production of artificial wounds in mandibles and calvaria

Five weeks after irradiation, artificial wounds were created in the recipients. All operations were performed under anesthesia as previously reported (Tu et al., 2005). Ten animals were assigned in each treatment. Briefly, for calvaria, a defect of 4 mm in diameter was created by a dental bur. For mandibles, the right inferior mandibular border up to the mandibular ramus was amply exposed and an osteotomy of 2×2 mm was made. All the generated wounds were subjected to sterile collagen matrix only.

Luciferase and GFP expressions in living animals and exposed bone defects

At weeks 2, 4, and 6 postoperatively, the luciferase expression was measured 10 min after luciferase substrate luciferin injection (1 mg luciferin/5 g body weight) by IVIS Imaging System 200 Series (Xenogen Corporation, Alameda, CA). GFP expression was then determined using the same system under a 488 nm excitation light. After euthanasia the defected mandibles were dissected, scanned by Xenogen and then applied to histological assessment and RT-PCR analysis.

Immunohistochemistry for BSP, GFP, and luciferase

The collected tissues were prepared as described previously (Tu et al., 2006). Tissue sections, 5 μm thick, were mounted on glass slides. Immunohistochemical studies were performed using affinity-purified rabbit polyclonal antibodies against firefly luciferase (Promega, Madison, WI), BSP (a gift from Dr. Larry Fisher, NIH/NIDCR), and GFP (Clontech, Mountain View, CA), with a Histostaining kit (Zymed, South San Francisco, CA). Control sections were incubated with an irrelevant antibody (anti-human CD4 lymphocyte antigen) to estimate background staining.

Morphometric assessment of immunostained sections

Staining data were obtained from light microscope assessments (Diagnostic Instruments, Sterling Heights, MI) of at least nine sections from three different animals. To prevent bias, we used a double-blinded method. BSP was employed to demonstrate the number of the cells which directly participate in new bone formation and GFP was used to quantify the distribution of the implanted cells. Additionally luciferase was used to identify exogenic cells that have undergone osteogenic differentiation, and haematoxylin stain showed the nuclei of all the cells involved in the wound sites. Cell counts were performed with an intraocular grid ($250 \mu\text{m} \times 250 \mu\text{m}$) containing 100 squares of $625 \mu\text{m}$ each (Carl Zeiss). The numbers of BSP, GFP and luciferase positive cells were normalized to the total number of cells in the wound sites, and were used to evaluate the amount of bone formed by transplanted BMSCs.

RT-PCR analysis

Total RNA was extracted and freshly isolated RNA was reverse transcribed with a SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA). The resulting cDNA was then

amplified by PCR with the Platinum PCR supermix (Invitrogen). The sequences of the primers for amplification of BSP and GAPDH were described in our previous publication (Tu et al., 2006); additional primers were: 5'-CCCTGAAGTTCATCTGCACCAC-3' and 5'-ACTCCAGCAGGACCATGTGATC-3' for GFP; and 5'-ATCCAGATCCACAACCTTCG-3' and 5'-AGAACTGCCTGCGTGAGATT-3' for luciferase. Levels of BSP, luciferase, and GFP were normalized with those of the loading control GAPDH in three independent experiments. Images of the amplified products in 1.5% agarose gels were captured with an UVP bioimaging system and were processed by Scion Image Beta 4.02 (Scion Image, Frederick, MD).

Statistical analysis

All results are expressed as means $\pm$ SEM of three or more independent experiments. Chi-squared test and one-way ANOVA were used to test significance using the Statgraphic statistical graphics system software package (STSC, Rockville, MD). Values of P lower than 0.05 were considered statistically significant.

Results

Transgenic mice and characterization of cultured BMSCs

Luciferase imaging demonstrated that all the offspring expressed the luciferase gene. Reporter expression was primarily confined to the regions of limbs, skull and tail where active bone formation takes place in a developing animal skeleton (Fig. 1A). Two of the six pups expressed GFP (Fig. 1B). BMSCs isolated from double-labeled BSP-Luc/ACTB-EGFP animals showed GFP expression in both osteogenic and nonosteogenic cultures (Fig. 1D), but only BMSCs expressed luciferase in osteogenic culture showed luciferase expression (Fig. 1C).

Luciferase and GFP expressions in living animals and exposed bone defects

All the five irradiated mice that did not receive a BMSC transplant died within 7 days, and 16 of the mice that received a BMSC transplant survived. Strong signals from both luciferase and GFP expression were detected in calvarial regions of live animals as early as 2 weeks after surgery and persisted until 6 weeks (Fig. 2A). Distinct signals from both reporter genes were also detected in exposed mandibles. A maximal luciferase signal was detected 2 weeks after surgery, and thereafter it decreased temporally while the GFP signal level was relatively stable throughout the experimental period (Fig. 2B).

Morphometric assessment of immunostained sections

Immunohistochemical analyses showed the existence of BSP, GFP and luciferase positive cells in mandible defects (Fig. 3A). Cell counting demonstrated that at 2-week postoperatively GFP positive cells (transplanted BMSCs) constituted 48% of the cell population. This percentage decreased slightly thereafter but with no statistical significance (Fig. 3B). Among the transplanted BMSCs, 81% (luciferase positive cells/GFP positive cells) were undergoing osteogenic differentiation 2 weeks after surgery. Six weeks postoperatively this ratio decreased dramatically to 26%, demonstrating a similar pattern as the percentage of BSP positive cells (peaked at 82% 2-week postoperatively while decreased to 15% 6-week postoperatively) (Fig. 3C). However, among all the cells undergoing osteogenic differentiation, cells derived from transplanted BMSCs slightly increased from 48% at 2-week postoperatively to 53% at 6-week postoperatively as indicated by the ratio of luciferase positive cells to BSP positive cells (Fig. 3D).

Expression patterns of luciferase, GFP and BSP

Semiquantitative RT-PCR analyses showed that GFP but not luciferase expression could be detected in soft tissues (liver and kidney) isolated from recipient mice (Fig. 4A). The analyses

also demonstrated that in defected mandibles isolated from these mice, luciferase expression, which showed the same pattern as BSP, peaked at 2 weeks after surgery and then decreased (Fig. 4B). However, the expression level of GFP changed minimally during the 6-week period (Fig. 4C).

Discussion

Although it has been demonstrated that local application of BMSCs enhances bone regeneration, it is necessary to establish the extent to which the bone marrow elements engrafting from peripheral circulation contribute to bone regeneration. Furthermore, it is important to determine whether osteoblast progenitors are recruited from bone marrow and participate in bone regeneration. Hou et al. (1999) reported that bone marrow derived stem cells could engraft from peripheral circulation and differentiate into osteogenic cells during normal physiological processes. However, a biologically and clinically relevant question of most of these studies is the competency of blood-borne BMSCs to support bone regeneration.

We, therefore, developed a strategy for transplantation using BMSCs from double-labeled transgenic mice. One genetic marker is a luciferase reporter gene driven by a mouse BSP promoter that regulates the expression of BSP that is specific to mineralized tissues (Oldberg et al., 1988; Fisher et al., 1990; Chen et al., 1996; Paz et al., 2005). Although expression of osteocalcin (OCN) is bone specific, BSP appears to be expressed by osteoblasts earlier than OCN (Ducy et al., 1996; Ganss et al., 1999). Thus, the BSP-Luc chimeric gene can serve as a faithful and sensitive marker for osteogenic differentiation of transplanted BMSCs. Moreover, BSP is expressed in newly differentiated osteoblastic cells that actively synthesize matrix molecules during early bone formation and mineralization (Yao et al., 1994). Thus, detection of luciferase expression reflects the functional stage of the osteogenic cells derived from the infused bone marrow cells. The other genetic marker is GFP driven by a β -actin promoter and a cytomegalovirus enhancer. The integration of both reporters, luciferase and GFP, enabled us to distinguish between cells derived from transplanted BMSCs and from host cells residing in the wound sites. In short, GFP staining was used to track the fate and migration of transplanted BMSCs, whereas luciferase staining served as a marker for osteogenic differentiation of the transplanted BMSCs because the luciferase expression was switched on as these cells differentiated into osteoblasts. Our approach permits the determination of cell origin and how they undergo osteogenic differentiation.

In this study we provided direct evidence that transplanted BMSCs engrafting from peripheral circulation can repopulate the wound sites in bone and participate in the regeneration processes. The systemically delivered BMSCs could undergo osteogenic differentiation and promote wound healing. Hou et al. (1999) reported that transplanted cells were detected in bone and nonosseous tissues of recipient mice as early as 1 month after transplantation and remained engrafted for as long as 12 months. In our study, reporter genes were first detected 2 weeks after bone defects were established (7 weeks after transplantation) and persisted throughout the experimental period (11 weeks after transplantation). Luciferase expression, which marked the transplanted BMSCs undergoing osteogenetic differentiation, showed a similar pattern compared to BSP expression, which peaked 2 weeks following surgery and decreased thereafter. However, GFP expression was relatively stable during the experimental period. These results are consistent with the fact that BSP is an early marker of osteogenic differentiation (Samoto et al., 2003; Paz et al., 2005). Based on the previous studies and our own findings demonstrating engraftment of transplanted cells in unwounded soft tissues, it can be concluded that transplanted BMSCs reside in the whole body after transplantation. Those luciferase positive cells which are exogenous cells undergoing osteogenic differentiation may either come from transplanted cells already residing in the local tissues, or newly engraft from peripheral circulation.

Mesenchymal stem cells without gene therapy have been shown to heal craniofacial and long bone defects (Meinel et al., 2005; Nussenbaum and Krebsbach, 2006). Our results also show that the percentage of osteogenic differentiating cells among transplanted BMSCs was higher than the percentage of BSP positive cells represented in the cell population residing in the bone defect area. These findings indicate that under certain local conditions, systemically recruited BMSCs undergo osteogenic differentiation and may play a major role in bone regeneration than other local cell populations. The time-dependent increase in the ratio of luciferase positive cells to BSP positive cells further indicates that during prolonged regenerative processes, there may be a requirement for recruitment of additional blood-borne BMSCs, perhaps to compensate for a limitation of available local stem cells.

Our study provides evidence that transplanted BMSCs not only take part in but also promote local bone regeneration. Stromal cells can be readily isolated and manipulated and reproduced in vitro. Tissue engineering has emerged as an alternative strategy to regenerate bone. Consequently, in vitro culture of autologous bone marrow to increase the stem cell pool for further use in tissue engineering may provide an important advance in the area of bone regeneration.

To summarize, our results provide novel insight into applications of BMSCs for bone regeneration in the orocraniofacial regions, as well as the manner in which they can be delivered to the regeneration sites. The findings that systemically transplanted BMSCs could engraft into the whole body and participate in bone defects regeneration indicated that systemic BMSC transplantation could be applied to treat multiple bone defects clinically. Future studies in an in vivo setting will enhance our understanding of regulatory mechanisms that are operative as biological controls in BMSC's as well as the potential for therapeutic applications in skeletal rejuvenation.

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Fig. 1.

A,B: Imaging of the offspring of the cross breeding between an ACTB-EGFP transgenic mouse (male, +/-) and an mBSP9.0 Luc transgenic mouse (female, +/+) by Xenogen IVIS imaging system. A: Detection of luciferase activity. All the offspring harbored the luciferase reporter. B: Measurement of GFP fluorescence. Two of the 6 pups were GFP positive (GFP +/-, number 1 and 4, from left to right). The color scale next to the images indicates the signal intensity. C,D: BMSCs from BSP-Luc/ACT-GFP mice with double markers were visualized by an IVIS imaging system. The cells were cultured for 7 days in osteogenic medium (two right plates) or nonosteogenic maintenance medium (two left plates) respectively. C: Detection of luciferase activity; (D) mapping of GFP fluorescence.

Fig. 2.

A: Kinetic expressions of luciferase and GFP in the calvarial defect of an irradiated mouse with BMSCs transplantation which survived until 6 weeks after surgery. B: Expressions of luciferase and GFP in exposed mandibles isolated from wound sides of recipient mice sacrificed at 2, 4, and 6 weeks after surgery.

Fig. 3.

A: Immunohistochemical analyses of BSP, luciferase and GFP in mandibular defects of recipient mice 2, 4, and 6 weeks after surgery (M, mandible; W, wound; NB, new bone; T, tooth; PDL, periodontal ligament. Bar in figures of 2w and 4w: 50 μm. Bar in figures of 6w: 100 μm). B–D: Cell count results of immunohistochemical staining. (B) The percentage of GFP positive cells in total cell population at different time points after surgery. (C) Percentage of BSP positive cells and luciferase positive cells/GFP positive cells ratio at different time points. (D) The ratio of luciferase positive cells to BSP positive cells.

Fig. 4.

A: RT-PCR analysis for luciferase and GFP in soft tissues. GFP but not luciferase expression could be detected in soft tissues (liver and kidney) isolated from recipient mice. B,C: Semiquantitative RT-PCR analysis for BSP, luciferase and GFP in mandibular defect areas. Expression of GAPDH was used as a loading control. (B) Representative RT-PCR experiment. (C) Normalized mRNA expression of BSP, luciferase and GFP.