Bone Marrow-Engrafted Cells after Mice Umbilical Cord Blood Transplantation Differentiate into Osteoblastic Cells in Response to Fracture and Placement of Titanium Screws

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Abstract: As the in vivo function of bone marrow-engrafted umbilical cord blood (UCB)-derived mesenchymal cells (UCBCs) after UCB transplantation is unknown, we examined in vivo osteoblastic differentiation using mouse UCB transplantation and fracture models. UCBCs obtained from GFP transgenic mice were intravenously injected into irradiated C57BL/6 mice. After three months, the in vivo osteoblastic differentiation potential of bone marrow-engrafted UCBCs was examined histologically using a mouse fracture model. GFP-positive UCBCs were detected in the bone marrow of recipient mice. On day 7, UCBCs were observed in the fracture gap and surrounding the titanium screws of the fixation device. The UCBCs were also positive for alkaline phosphatase and von Kossa staining. By day 14, UCBCs were observed in the fracture gap and surrounding the titanium screws of the fixation device. The UCBCs were also positive for alkaline phosphatase and von Kossa staining. Our findings suggest that UCBCs contribute to the fracture healing process after bone marrow engraftment and that UCBC transplantation can fully reconstruct not only hematopoietic cells but also mesenchymal cell lineages.

Key words: in vivo, mesenchymal cells, osteoblastic differentiation potential, transplantation, umbilical cord blood

Introduction

Umbilical cord blood (UCB) is an attractive source of hematopoietic stem cells (HSCs) and has many advantages over bone marrow transplantation, including a larger available donor pool size, enriched population of hematopoietic progenitor cells [27], low content of mature T cells capable of mediating graft-versus-host reactions [2, 8, 16], and the absence of potential cytomegalovirus infection [10]. UCB transplantation fully reconstitutes hematopoietic lineage cells [18], and T and B lymphocytes derived from UCB-HSCs are fully immunologically competent [19]. UCB also contains mesenchymal stem cells (MSCs) that have the potential to differentiate into multilineage cells, including mesenchymal lineage cells, but can also form neurons, skeletal muscle, and myocardium [1, 4, 5, 9, 20]. Human UCB-derived MSCs promote skin, bone, and blood vessel
regeneration [3, 11, 14, 15] and are therefore considered potentially useful for regeneration therapy. A recent study reported that the cotransplantation of UCB-derived MSCs and CD34 (+) cells promotes HSC transplantation and hematopoietic recovery in vivo [7]. Moreover, we previously demonstrated that mouse UCB-derived mesenchymal cells (UCBCs) engraft in bone marrow after mouse UCB transplantation [23]. However, it remains unclear whether engrafted mesenchymal cells are functional in vivo.

Endogenous bone marrow mesenchymal cells participate in several healing processes, including fracture and vascular healing, and epithelial regeneration [6, 11, 22]. Recently, we also reported that bone marrow cells differentiate into osteoblastic cells in response to fractures that are rigidly fixed using a mouse plate fixation device [24]. Therefore, the reconstitution of mesenchymal cells may be important for not only the acceleration of HSC engraftment, but also several healing processes after UCB transplantation. However, the in vivo function of bone marrow-engrafted UCBCs after UCB transplantation is largely unknown.

Here, to clarify the in vivo function of bone marrow-engrafted UCBCs, we investigated whether these cells differentiate into osteoblastic lineages during fracture healing using a mouse UCB transplantation model, which facilitates studies on the basic biology, engraftment, and therapeutic potential of UCBCs [18, 19, 23], and mouse fracture models, which are useful for evaluating fracture healing under standardized mechanical and biological conditions [24, 25].

Materials and Methods

Animals

All animal experiments were performed in accordance with the “Institutional Guidelines for Animal Care” of Kitasato University. Female C57BL/6 (B6: H2b) mice were purchased from Charles River Laboratories Japan, Inc. (Tokyo, Japan). C57BL/6 TgN (act-EGFP) OsbY01 mice (H2b) (referred B6-GFP in this paper) were bred in our animal facility. F1 fetuses (B6-GFP × B6) were used as the source of UCBCs because transgene homozygous cells emit excessively strong fluorescence during flow cytometry analysis such that negative and positive cells cannot be observed in the identical plotted field [18]. Mice were housed in a semi-barrier system with a controlled environment (temperature of 23 ± 2°C; humidity of 55 ± 10%; 12-h light/dark cycle) throughout the study. All mice were fed a diet of standard rodent chow (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan).

Mouse umbilical cord blood cells

To obtain UCB cells, female B6 mice were first mated with male B6-GFP mice overnight. The following morning, the female mice were separated from males and examined for vaginal plugs as evidence of mating. Eighteen days later, uteri were removed from pregnant females under anesthesia. The cleaning of fetuses and collection of blood were performed as previously described [18].

Mouse umbilical cord blood cell transplantation

Six eight-week-old B6 mice were irradiated using an MBR-1505R X-ray irradiator (Hitachi Medical Corporation, Tokyo, Japan) equipped with a filter (0.5 mm Cu, 2 mm Al) for monitoring the cumulative radiation dose. After the recipient mice received 10.5 Gy irradiation, UCB-derived mononuclear cells that had not been subjected to in vitro culture were intravenously injected into the irradiated mice [18, 19, 23].

Flow cytometric analysis of peripheral blood after transplantation

Single-cell suspensions were obtained from the peripheral blood of irradiated B6 mice three months after the intravenous injection of UCB-derived mononuclear cells. Blood cells (1 × 10^5 cells) were analyzed by flow cytometric analysis on a FACSCalibur instrument (Becton Dickinson and Co., Mountain View, CA, USA).

Creation of a mouse fracture model

We used a rigid plate of the MouseFix™ system (AO Development Institute, Davos, Switzerland), which is a locking plate system with good angular stability, as an internal fixator of fractured mouse femurs [25]. Prior to the plate implantation and fracture of femurs, a mixture of 1 part midazolam (Sandoz, Tokyo, Japan), 3 parts Domitor™ (Nippon Zenyaku Kogyo Co., Ltd., Fuku-shima, Japan), and 1 part Vetorphale™ (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) was intramuscularly injected into the upper limbs of mice at an optimal dose of 0.05 ml/100 g to induce anesthesia. Plates were implanted on the left femur. After the medical grade titanium plate and screws were implanted, the bone was cut gently using a wire saw to create a gap [17]. The success
of the surgical procedure was confirmed radiographically. After the surgery, the mice were allowed to use their fractured legs without restriction.

**Preparation of undecalcified fresh-frozen sections of femurs**

Mice were sacrificed by the administration of an excess of CO$_2$ gas on days 7 and 14 after fracture induction. After blood was removed by cardiopuncture to avoid contamination of the fracture site, the left femurs were excised with the surrounding muscle. The implanted plate and screws were carefully removed, and the collected femurs were then immersed in a carboxymethyl cellulose (CMC) gel, transferred to hexane, and completely frozen using solid CO$_2$. The femur samples were then cut into 6-µm thick sections using a CM3050S IV cryomicrotome (Leica Microsystems GmbH, Wetzlar, Germany) [23]. After the sections were dried, the distribution of UCBCs at the fracture site was examined by fluorescence microscopy. Serial sections were also stained with hematoxylin and eosin (HE), alkaline phosphatase (ALP) for detection of the differentiation of immature osteoblastic cells into UCBCs, and von Kossa stain for the detection of mineralized bone (Kureha Special Laboratory Co., Ltd., Tokyo, Japan) using standard procedures. Staining for type 1 collagen was also performed using rabbit anti-mouse type 1 collagen polyclonal antibody (AbD Serotec, Raleigh, NC, USA) and the streptavidin-biotin-peroxidase system with 3,3-diaminobenzidine (DAB) for the detection of osteoblastic cells.

**Results**

To identify UCBCs, peripheral cells collected from GFP-chimeric C57BL/6 mice were analyzed using a FACSCompur one month after UCBC transplantation. In the 6 transplanted mice, chimerism was approximately 93% (Fig. 1). As determined by fluorescence microscopy, GFP-positive cells were confirmed in the bone marrow, whereas the cells of bone tissue were GFP negative (Fig. 2). The cortical surfaces and cortical bone in the femoral diaphysis of C57Bl/6J and non-fractured UCBC-transplanted mice were positive for ALP- and von Kossa staining, respectively (Fig. 2). In contrast, almost no cells in the bone marrow were stained by ALP or von Kossa staining (Fig. 2).

On day 7 after the fracture of femurs and implantation of a rigid plate, abundant fibrous tissue was observed in the fracture gap (Fig. 3A and 3B). The cells in the fibrous tissue were GFP positive (Fig. 3C and 3D), and a portion of the cells was stained by ALP (Fig. 3E and 3F) and type I collagen (Fig. 4A). In the bone marrow, the accumulation of GFP-positive cells was also observed around the titanium screws (Fig. 5B). These cells were also positive for von Kossa (Fig. 5D) and type I collagen staining (Fig. 4B).

On day 14, the HE-stained sections revealed remodeling of woven bone in the fracture gap and the formation of a small intramedullary callus and a new bone layer (Fig. 6). In the fluorescence images, GFP-expressing cells were observed around the formed callus (Fig. 6B). A small number of GFP-expressing cells were observed under the periosteum, in concurrence with the ALP and type I collagen staining. The newly formed bone consisted of ALP- and von Kossa-positive cells (Fig. 6B and 6C). These observations were seen in all six examined animals.
Discussion

In the present study, fracture healing in UCB-transplanted mice was examined by monitoring the fate of UCBCs obtained from GFP transgenic mice. We demonstrated that woven bone formed within the fracture gap and around the titanium screws of the fixation plate within 14 days of fracture. Notably, we detected that the cells within the fracture gap were ALP, type I collagen, and GFP positive, whereas only GFP- and von Kossa-positive cells were detected around screws. These results suggest that bone marrow-engrafted UCBCs differentiate into osteoblastic cells in response to environmental-specific cues in vivo.

Endogenous bone marrow cells participate in several healing processes [6, 21, 22]. For example, Taguchi et al. [21] reported that endogenous bone marrow cells contribute to fracture healing and differentiate into osteoblasts, while our previous studies showed that under rigid fixation, transplanted bone marrow-derived cells mainly contribute to callus formation [24]. Here, GFP-positive cells were clearly observed in the fracture gap.
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and callus, and were positive for ALP and type I collagen staining on day 7. By day 14, GFP-positive cells were observed around the callus and under the periosteum, in concurrence with the ALP and type I collagen staining. Moreover, von Kossa-positive cells were observed on day 14. In our previous studies, similar cellular events were observed within the same time period in wild-type C57BL/6J mice and a GFP bone marrow chimeric mouse [24, 25]. Taken together, our results suggest that bone marrow-engrafted UCBCs contribute to fracture healing in a manner similar to endogenous bone marrow-derived cells.

On day 7 after fracture, GFP- and von Kossa-positive cells were observed around the titanium screws of the fixation plate. Several studies have reported that titanium stimulates osteoblastic differentiation in mesenchymal and osteoblastic cells [10, 12, 13, 26]. For example, Wall et al. [26] reported that titanium surfaces accelerate osteogenic differentiation of mesenchymal stromal cells in vitro. Moreover, in an in vivo study examining the

Fig. 4. Immunostaining of type I collagen in the GFP-positive area in the region of the fracture site and around the titanium screw. (A) Fracture gap on day 7, (B) in the vicinity of the titanium screw on day 7, and (C) under the periosteum on day 14. Arrows indicate type I collagen-positive areas. The scale bar indicates 100 µm.

Fig. 5. Localization of GFP-positive cells around the titanium screw of the fixation plate on day 7. (A) Hematoxylin and eosin-stained section, (B) fluorescence micrograph, (C) alkaline phosphatase-stained section, and (D) von Kossa-stained section. Arrows indicate Von Kossa-positive areas that overlap with GFP-positive areas. The scale bar indicates 200 µm.

Fig. 6. Localization of GFP-positive cells in the fracture site on day 14. (A) Hematoxylin and eosin-stained section, (B) fluorescence micrograph (arrows indicate GFP-positive cells located under the periosteum, while arrowheads indicate GFP-positive cells located around the formed callus), (C) alkaline phosphatase-stained section (arrows indicate ALP-positive cells located in the same location as GFP-positive cells), and (D) von Kossa-stained section. The scale bar indicates 200 µm.
placement of titanium implants in mouse femurs, bone formation was observed juxtaposed and parallel to the titanium implant surface [12]. Our present results suggest that bone marrow-engrafted UCBCs also differentiate into osteoblastic cells in response to the placement of titanium screws. Thus, we propose that bone marrow-engrafted UCBCs may respond to several cues, similar to those of bone marrow mesenchymal cells.

A limitation of this study warrants mention. Total GFP-positive UCBCs were transplanted into C57BL/6J mice. As UCBC contains several cell types, including mesenchymal and hematopoietic stem cells and hematopoietic lineage cells, it was not possible to deduce the cell type(s) that contributed to callus formation in this investigation.

In conclusion, we found that bone marrow-engrafted UCBCs differentiate into osteoblastic lineages in response to fracture and titanium. Thus, UCBC transplantation has the potential to fully reconstruct not only hematopoietic cells, but also mesenchymal cell lineages.

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