it is beneficial to culture cells on scaffolds rather than mixing them just prior to the operation.[42] Accordingly, the scaffolds should be highly biocompatible to allow cell attachment, growth, and differentiation of the cells. Since the induction period is two to three weeks, the materials should also be stable for this culture/induction period.

After cell transplantation, the scaffolds are degraded as new bone formation occurs, which continues until the scaffold is completely degraded. This is the phase of actual bone regeneration. When β-TCP granules are used, bone regeneration may continue for up to one year. During this stage, the scaffold seems to be directly degraded by resorption by osteoclastic cells. Compared with the dissolution by interstitial fluid, this process might be favorable since morphology of the tissue is maintained. To accelerate this active resorption process of the scaffold, the scaffold should mimic bone and be presented as a target tissue for osteoclasts. Ceramic-based materials might be advantageous since osteoclastic activities were confirmed from *in vivo* studies.

The third phase starts when the scaffold is completely degraded and the newly formed bone enters the normal remodeling process. For clinical cases, stability of the regenerated bone for a longer period is important. However, factors which affect stability are not well known. It is known that mechanical stimuli such as dental implant placement may help to maintain the volume of regenerated bone. Importantly, the shape of hard tissue should be compatible with the covering soft tissues. Long-term changes of atrophic alveolar bone shape result in a soft-tissue environment that conforms. Accordingly, immediate morphological changes after bone regeneration may lead to increased pressure on the regenerated bone and cause unexpected absorption. To prevent late absorption of the regenerated bone, the resorption of the scaffold should be long enough until the soft tissue surrounding the newly formed bone becomes stabilized. From this point of view, scaffolds with a relatively short degradation time, such as collagen, have disadvantages, and their application is limited to areas without pressure from adjacent soft tissues. Conversely, if scaffold degradation takes too long, the bone regeneration process may be delayed and the mechanical strength of the regenerating tissue may not be enough to support the

dental implant. This is one of the reasons that TCP is preferred for dental implants. One ideal approach for these two differential and contradictory scaffold properties is to implement composite strategies rather than one simple scaffold.

26.3.5 *Prospective Novel Biomaterials for Hard-Tissue Engineering*

Currently used biomaterials for clinical tissue engineering have been used for many years, which is not surprising. Unfortunately, evaluation of newly developed materials takes time and the time lag between material development and clinical application cannot be avoided. However, it is logical to discuss the future of biomaterials as these may replace currently used materials. Since various novel materials are described elsewhere in this book, we have focused on several materials that may potentially overcome the current problems discussed in this chapter.

26.3.5.1 Composite and combined materials

As stated above, successful scaffolds for bone tissue engineering should have two different properties: early resorption to support immediate regeneration of bone and long-term presence to resist pressure from soft tissues and retain their original shape. Accordingly, one ideal approach is to make a composite of two or more different materials, which would play different roles during the regeneration period. A combination of natural polymers with HA and more complex forms of composite such as collagen-PLA-HA have been reported (reviewed by Hutmacher *et al.*[43]).[44–47]

Tissue engineering usually utilizes one type of cell. However, recent studies have shown the benefits of combining two different cell types. Although bone tissue engineering using BMSCs is feasible, the size of the regenerated bone is limited. Grafted cells require oxygen and nutrition to survive, and early neovascularization is considered essential for successful bone tissue engineering.[3] Development of an efficient neovascularization method to sustain engineered transplants is therefore clinically important. For this purpose, endothelial cells (EC) or endothelial progenitor cells (EPC) have been

combined with BMSCs, which enhanced vascularization of the regenerated tissue.[48]

26.3.5.2 Growth factor incorporation into scaffolds

Results from clinical studies have shown that the process of bone regeneration is relatively long and may continue more than one year after cell transplantation. The time for cell transplantation is not a goal but the beginning of more complex phenomena taking place in the body, including cell-to-cell and/or cell-to-biomaterial interactions. Unfortunately, the fates of the transplanted cells and the detailed regenerating process have not been well documented, especially in humans. Transplanted cells play an important role initially, but there are likely many other factors playing roles in later stages. The transplants (transplanted cells and scaffolds) interact with host-derived cells, which affect degradation of the scaffold. The process could also be regulated by local growth factors/cytokines.

An exciting approach for novel biomaterials for bone tissue engineering is the generation of slow-release drug delivery systems for growth factors/cytokines.[49] These are released from the scaffold to establish a favorable environment for bone regeneration, rather than a permissive effect of the location. Growth factors can be directly immersed in ceramic-based scaffolds. When the scaffold is a composite of synthetic polymers, the polymer can function as a slow-release device by controlling the rate of degradation of the polymer. The factors are expected to coordinate with transplanted cells to regenerate bone. Investigations into the mechanism should facilitate this approach in the future.

26.4 Conclusions and Outlook

This article has focused on scaffold design almost exclusively from a clinical point of view, and we selected small-caliber blood vessels and alveolar bone as examples of tissue engineering. It is one of our aims to emphasize the importance of effective discussions between material scientists and clinical teams.

Recent developments in translational and clinical studies should be carefully considered in order to design scaffolds. Materials for small-caliber vascular grafts are still a challenge, and the development of novel materials, with mechanical properties similar to natural blood vessels without loss of their biocompatibility and controllable degradation time, is eagerly anticipated. Scaffold materials for bone tissue engineering have already been applied in clinical studies, although there is a need for more efficient materials. This may depend on investigation of more detailed mechanisms of bone regeneration.

Various scaffolds other than ceramic-based biomaterials are available for bone tissue engineering. Information about novel biomaterials for bone tissue engineering can be found in Parts II, III, VI, and VII and also in recent reviews.[43,50,51]

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Characteristic differences among osteogenic cell populations of rat bone marrow stromal cells isolated from untreated, hemolyzed, or Ficoll-treated marrow

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Running title: Osteogenic cell populations of rat BMSCs

ABSTRACT

Background

Although bone marrow (BM) stromal cells (SC) isolated from adherent cultures of untreated BM are known to contain both committed and uncommitted osteogenic cells, it remains unknown whether BMSCs isolated either by hemolysis or Ficoll centrifugation also contain both of these populations.

Methods

Differences in the osteogenic cell populations of rat BMSCs isolated from untreated, hemolyzed or Ficoll-treated BM were analyzed by *in vivo* transplantation, flow cytometry, alkaline phosphatase (ALP) assay, real-time PCR, and alizarin red staining.

Results

Transplantation of non-cultured samples indicated that the Ficolled BMSCs contained the lowest number of committed osteogenic cells. Flow cytometric analysis of cultured, non-induced samples showed that the percentage of ALP-positive cells was significantly lower in Ficolled BMSCs. Quantitative ALP assays confirmed that the lowest ALP activity was in the Ficolled BMSCs. Hemolyzed BMSCs also contained lower numbers of committed osteogenic cells than did untreated BMSCs, but still greater than Ficolled BMSCs. Interestingly, the Ficolled BMSCs showed greatest levels of osteogenic ability when cultured in osteogenic induction medium.

Discussion

These findings suggest that although Ficolled BMSCs rarely contain committed osteogenic cells, they are able to show comparable or even greater levels of osteogenic ability after induction, possibly because they contain a greater proportion of uncommitted stem cells. In contrast, induction is optional but recommended for both untreated and hemolyzed BMSCs before use, because both these groups contain both committed and uncommitted osteogenic cells. These findings are of significant importance when isolating BMSCs for use in bone tissue engineering.

Keywords: Bone marrow stromal cells, Ficoll, hemolysis, osteogenic cells, stem cells

INTRODUCTION

Bone marrow (BM) stromal cells (SCs) isolated from adherent cultures of untreated whole BM (normal BMSCs) contain an uncommitted stem cell population called mesenchymal stem cells that can differentiate into multiple (osteogenic, chondrogenic, adipogenic, myogenic, and neurogenic) lineages (1, 2). It remains controversial as to whether the multi-lineage differentiation ability of normal BMSCs is solely attributed to uncommitted stem cells, because the BMSC population is composed of a heterogeneous collection of cells that might include committed progenitors of each lineage (3). Nonetheless, at least the osteogenic ability of normal BMSCs depends on both committed and uncommitted cells, as it has been long known that normal BMSCs contain committed osteogenic cells such as pre-osteoblasts (4).

Uncommitted stem cells require induction stimuli to promote osteogenic differentiation (5). Thus, BMSCs are generally cultured in osteogenic induction medium before use in bone tissue engineering. However, the induction process can be omitted or shortened when BMSCs include large numbers of committed osteogenic cells, as committed cells spontaneously differentiate into osteoblasts (4, 5). Thus, the proportion of committed osteogenic cells affects both the protocol design as well as the total cost of bone tissue engineering. In addition, this proportion may significantly affect the

osteogenic ability of BMSCs, since highly osteogenic BMSCs are able to form bone without induction (6). Therefore, it is important to investigate the proportion of committed osteogenic cells when analyzing the osteogenic ability of BMSCs, though few studies have focused on the importance of that population.

In general, BMSCs are obtained from adherent cultures of untreated whole BM because they are able to form adherent colonies unlike non-adherent hematopoietic cells (7, 8). However, this technique may be inefficient for the isolation of BMSCs because untreated BM contains a large proportion of erythrocytes and their presence can interfere with the initial colony-forming ability of BMSCs. Both density gradient centrifugation over Ficoll® (GE Healthcare, UK Ltd., Buckinghamshire, England) and hemolysis treatment with ammonium chloride are the techniques used for the removal of erythrocytes from BM to enhance BMSC adherence, and BMSCs isolated by these techniques have been shown to possess multi-lineage differentiation abilities (9, 10). However, it remains unknown whether these BMSCs have the same differentiative potential as BMSCs isolated from whole BM since the cellular composition of BMSCs may vary with the isolation techniques (11). Differences in cellular composition may greatly influence the osteogenic characteristics of BMSCs, because the osteogenic ability of normal BMSCs depends on at least two different cell populations, *i.e.*, committed

osteogenic cells and uncommitted stem cells (5). Therefore, it is important to investigate the osteogenic BMSC populations following isolation by either Ficoll fractionation or hemolysis before applying these techniques to bone tissue engineering.

In the present study, we isolated BMSCs from untreated, hemolyzed, or Ficoll-treated rat BM and analyzed their differences in osteogenic cell populations, with a special emphasis on committed osteogenic cell populations.

MATERIALS AND METHODS

BM isolation from SD rats

Sprague-Dawley rats (Jcl: SD, male, six weeks old) were purchased from Clea Japan, Inc. (Tokyo, Japan). All experiments were approved by the Animal Ethics Screening Committee of The Institute of Medical Science, The University of Tokyo. After intraperitoneal overdose administration of pentobarbital sodium, the femurs and tibiae were carefully dissected from the sacrificed rats. The tips of the bones were cut with scissors to expose the BM. Thereafter, BM was flushed out from each bone by injecting 7.5 mL of Hanks' Balanced Salt Solutions (HBSS; Wako Pure Chemical Industries, Ltd., Osaka, Japan), with an 18 gauge needle (Terumo, Tokyo, Japan) attached to a 20 mL syringe (Terumo), and collected in a 50 mL centrifuge tube (Becton Dickinson,

Pharmingen, Franklin Lakes, NJ, U.S.A.). Then, tubes were centrifuged at 440 × g for five min at 4°C. After discarding the supernatants, the marrow cells from each rat were resuspended in 10.5 mL of Dulbecco's phosphate buffered saline (DPBS; Nissui Pharmaceutical Co., LTD, Tokyo, Japan), and divided among three different tubes (each tube containing 3.5 mL of marrow cell suspensions).

Isolation of BMSCs from untreated, hemolyzed, or Ficoll-treated BM

To isolate BMSCs, marrow cell suspensions were processed on Ficoll or hemolyzed as described elsewhere (9).

Ficoll separated cells

For Ficoll treatment, 3.5 mL marrow cell suspensions were diluted with 7.5 mL of DPBS and carefully laid over 15 mL of Ficoll-Paque® (GE Healthcare UK Ltd.). After centrifugation (400 × g, 30 min, 20°C), the mononuclear cell fraction was collected and moved to another new tube. Subsequently, cells were washed twice in DPBS. After centrifugation (400 × g, ten min, 4°C), the supernatants were discarded and the obtained cell-pellets were suspended in one mL of physiological saline (Otsuka Pharmaceutical Co., LTD, Tokushima, Japan) for transplantation experiments or suspended in six mL of α-MEM (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10%

fetal bovine serum (JRS, Woodland, CA, U.S.A.) and antibiotics/antimycotics for cell culture analyses. The suspensions were termed, "Ficolled cells".

Hemolyzed cells

For hemolysis treatment, 3.5 mL marrow cell suspensions were mixed with ten mL of lysis buffer [0.15M ammonium chloride (NH_4Cl; Sigma-Aldrich, St. Louis, MO, U.S.A.), ten mM potassium bicarbonate ($KHCO_3$; Sigma-Aldrich), and 0.1 mM ethylenediamine-*N,N,N',N'*-tetraacetic acid, tetrasodium salt, tetrahydrate (EDTA·4Na; Dojindo Laboratories, Kumatomo, Japan)] and incubated for ten min at room temperature on a horizontal shaker. Then, cells were centrifuged (400 × g for ten min at 4°C), and washed twice in DPBS. After centrifugation (400 × g, ten min, 4°C), the supernatants were discarded and the resultant cell pellets were suspended in one mL of physiological saline for the transplantation experiment or suspended in six mL of serum-containing medium for cell culture analyses.

Untreated group

As untreated controls, 3.5 mL marrow cell suspensions were washed twice in DPBS. After centrifugation (400 × g, ten min, 4°C), the supernatants were discarded and the resultant cell pellets were suspended in one mL of physiological saline for

transplantation experiments or suspended in six mL of serum-containing medium for cell culture analyses.

Transplantation of non-cultured cells isolated by different techniques

Committed osteogenic cells spontaneously differentiate into bone-forming osteoblasts (5). Therefore, non-cultured samples of untreated, or hemolyzed, or Ficolled cells were analyzed for their *in vivo* bone forming abilities to investigate differences in committed osteogenic cells. Samples were transplanted to ectopic sites to avoid the effects of recipient nude mouse-derived osteogenic cells. One milliliter of saline containing each sample was mixed with 50 mg of β-tricalcium phosphate (β-TCP) granules (G1 type OSferion®; Olympus Terumo Biomaterials, Tokyo, Japan) in a 14 mL polypropylene tube (Becton Dickinson) and centrifuged (100 × g, five min, 4°C). After careful removal of the supernatants, 100 μL of ten mg/mL fibrinogen solutions (bovine plasma fibrinogen (F8630, Sigma-Aldrich)) and five μL of 100 units/mL thrombin solution (bovine plasma thrombin (T9549, Sigma-Aldrich)) were added to each cell-β-TCP mixture to form a fibrin clot. Thereafter, each clotted cell mixture was transplanted into the subcutaneous space on the backs of six-week-old female BALB/CAJc1-nu/nu mice (Clea Japan, Inc.) under anesthesia with pentobarbital sodium. Transplants were harvested four weeks after the operation.

Histological assessment of the transplants of non-cultured samples

Harvested samples were fixed in 10% buffered formalin, decalcified in Kalkitox™, neutralized in 5% sodium sulfate solution (all purchased from Wako Pure Chemical Industries, Ltd.), and embedded in paraffin. Five μm-thick sections were prepared from the middle of each transplant and stained with hematoxylin and eosin (H&E).

Microscopic examination of H&E stained sections was conducted to determine the number of transplants that contained ectopic bone formation. The percentage of successful transplants (number of transplants containing ectopic bone/ total transplants) was calculated to assess the bone forming potential of untreated, hemolyzed, and Ficolled cells as described elsewhere (12). To investigate the bone forming activity, light microscopic images were captured with a digital camera (Carl Zeiss Japan, Tokyo, Japan) and transferred to a computer. Then, the percentage of the area containing bone (new bone area/ total area) was manually assessed using Image J (Scion Corporation, Frederick, MD, USA), as described elsewhere (13). The extent of bone formation in each transplant was scored on a semi-quantitative scale in a manner similar to that described previously (14). For example, sections prepared from the middle of each transplant were scored on a scale of zero to three: a score of zero corresponded to no bone formation, while a score of three corresponded to abundant bone formation (bone area > 10%) (Table 1). Thereafter,

the average bone score of the transplants (total bone score/ total transplants) was calculated for each group.

***In vitro* expansion of BMSCs isolated from untreated, hemolyzed, or Ficoll-treated marrow cell suspensions**

For further investigations, cells isolated from untreated, hemolyzed, or Ficoll-treated marrow cell suspensions were re-suspended in six mL of serum-containing medium for culture expansion as described above. Cells were plated in three mL/well volumes in six-well plates (Becton Dickinson) and maintained in a 37°C, 5% CO_2 incubator. On the next day, the culture medium was replaced with fresh medium of the same type. Adherent cells were re-fed with fresh medium on the fourth day. After one week of culture, adherent cells were treated with 0.5% trypsin-EDTA (Invitrogen, Carlsbad, CA, U.S.A.), and cells detached within three minutes were passaged and re-plated in 100 mm tissue culture dishes (TPP Techno Plastic Products AG, Trasadingen, Switzerland) at a density of 6.5 x 10^3 cells/cm^2 for flow cytometric analyses or re-plated into multiwell plates (Becton Dickinson) for other analyses.

Flow cytometric analysis

Differences in cell surface marker expression were analyzed with a FACS Aria flow cytometer (Becton Dickinson). Fluorescence isothiocyanate (FITC)-conjugated,

phycoerythrin (PE)-conjugated, or allophycocyanin (APC)-conjugated antibodies targeted against CD45, CD54, CD90 (all from Biolegend, San Diego, CA, U.S.A.), and a biotinylated antibody against ALP (BAM1448, R&D Systems, Inc., Minneapolis, MN, U.S.A.) were used for the analyses. The biotinylated antibody was detected with a streptavidin-FITC conjugate (Biolegend). Propidium iodide (Dojindo, Kumamoto, Japan) was used to detect dead cells.

Cells at passage one were detached with trypsin-EDTA and 1 x 10^6 cells were re-suspended in 50 µL of ice-cold DPBS. Cells were then incubated with individual antibodies for 20 minutes on ice. Thereafter, cells were washed and incubated with a streptavidin conjugate for 20 minutes on ice. Finally, the cells were washed, resuspended in 200 µL of ice cold DPBS, stained with propidium iodide, and analyzed. Data analysis was performed using FlowJo software (TreeStar, Inc, San Carlos, CA, U.S.A.).

Fluorescent immunostaining

Fluorescent immunostaining of CD45 and CD54 was performed to confirm the results of flow cytometric analyses. First passage cells were plated in 24 well plates at a density of 5 × 10^4 cells per well in serum-containing medium, and cultured until 70% confluent. Thereafter, cells were fixed with 4% paraformaldehyde, washed three times with DPBS,