Allogenic Peripheral Blood Derived Mesenchymal Stem Cells (MSCs) Enhance Bone Regeneration in Rabbit Ulna Critical-Sized Bone Defect Model

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ABSTRACT: Mesenchymal stem cells (MSCs) were demonstrated to exist within peripheral blood (PB) of several mammalian species including human, guinea pig, mice, rat, and rabbit. Whether or not the PB derived MSCs (PBMSCs) could enhance the regeneration of large bone defects have not been reported. In this study, rabbit MSCs were obtained from mononuclear cells (MNCs) cultures of both the PB and bone marrow (BM) origin. The number of PBMSCs was relatively lower, with the colony forming efficiency (CFE) ranging from 1.2 to 13 per million MNCs. Under specific inductive conditions, PBMSCs differentiated into osteoblasts, chondrocytes, and adipocytes, showing multi-differentiation ability similar to BMMSCs. Bilateral 20 mm critical-sized bone defects were created in the ulnae of 12 6-month-old New Zealand white rabbits. The defects were treated with allogenic PBMSCs/Skelite (porous calcium phosphate resorbable substitute), BMMSCs/Skelite, PBMNCs/Skelite, Skelite alone, and left empty for 12 weeks. Bone regeneration was evaluated by serial radiography, peripheral quantitative computed tomography (pQCT), and histological examinations. The X-ray scores and the pQCT total bone mineral density in the PBMSCs/Skelite and BMMSCs/Skelite treated groups were significantly greater than those of the PBMNCs/Skelite and Skelite alone groups ($p < 0.05$), respectively. Histologically, newly formed bone was evident in the PBMSCs/Skelite and BMMSCs/Skelite treated groups. The findings demonstrated that the rabbit PBMSCs possessed multidifferentiation potential comparable with BMMSCs, allogenic PBMSCs seeded onto porous calcium phosphate resorbable substitutes enhanced bone regeneration in the rabbit ulna critical-sized bone defect model, suggesting allogenic PBMSCs may be a new source of circulating osteogenic stem cells for bone regeneration and tissue engineering. © 2006 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 24:610–618, 2006

Keywords: mesenchymal stem cells (MSCs); peripheral blood; critical-sized bone defect; bone regeneration; cell therapy

INTRODUCTION

Bone marrow derived mesenchymal stem cells (BMMSCs) are multipotent cells capable of differentiating into osteoblasts, chondrocytes, adipocytes, fibroblasts, tenocytes, and myoblasts,[1–5] which are considered as a cell source for various tissue repair and regeneration. It is supposed that transit of these cells through circulation might be one of the mechanisms for reparative cells homing and recruitment. Although evidence has been documented for the presence of PBMSCs in normal human adults, results were conflicted and not always be reproducible because of variations in the methods of cell isolation, purification, and culture conditions.[6]

Early work by Ojeda-Uribe and colleagues failed to identify mobilization of MSCs into the peripheral blood following chemotherapy and/or growth factors treatment, which would be routinely utilized to recruit hematopoietic stem cells (HSCs) in humans.[7] Wexler and coworkers were unable to demonstrate PBMSCs in normal peripheral blood stem cell collections.[8] Lazarus and colleagues also failed to recover MSCs in peripheral blood from cancer patients undergoing chemotherapy, by either in vitro and in vivo assays.[9] However, some attempts to isolate PBMSCs have been successful. Luria and colleagues demonstrated that fibroblast precursors circulated among the nucleated cells of peripheral blood of guinea pigs.[10] Piersma and coworkers found that phenylhydrazine-induced haemolytic anaemia in mice resulted in a threefold increase in the blood CFU-f numbers accompanied by a rise in the hematopoietic cells, with colony forming efficiency 5.3 ± 0.8 per million nucleated

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cells.[11] Huss and coworkers isolated CD34 negative adherent fibroblastlike cells from peripheral blood of dogs, and found that following injection these cells homed to the bone marrow as bone lining cells.[12] Using an aortic pouch allograft as a trapping device, Wu and colleagues isolated rat MSCs from circulation, which exhibited multipotential for differentiating into various lineages. In vivo, these cells vigorously migrated into the site of allograft rejection in a rat cardiac allograft model.[13] Zvaifler and colleagues estimated the number of PBMSCs in the adult human to be as low as 0.5 to 5 per million mononucleated cells.[14] Kuwana and coworkers reported that the induction of CD14+ monocyte derived mesenchymal progenitors to differentiate along multiple mesodermal lineages resulted in specific phenotype expression for osteoblasts, chondrocytes, adipocytes, and skeletal myoblasts, suggesting as a source of progenitors for mesenchymal tissues.[15] Kuznetsov and coworkers confirmed the physiological existence of circulating skeletal stem cells by the isolation of adherent, clonogenic, fibroblastlike cells with osteogenic and adipogenic potential, from four mammalian species including guinea pig, mouse, rabbit, as well as human, which indicated that the PBMSCs may serve as a new cell source for bone regeneration or remodeling.[16]

In orthopedic clinic, large bone defects are usually treated by autografts or allografts, but their clinical applications are limited by insufficient amount of bone that can be harvested. BMMSCs have been considered as an alternative because they could regenerate a critical-sized bone defect in several animal models including mouse, rat, rabbit, and dog.[17–20] However, the requirements of aspiration of bone marrow from the patient will cause pain and morbidity of the donor sites. It will be very convenient if PBMSCs could be harvested and expanded to enough numbers, with their osteogenic capacity maintained in a clinical permitted period. This study aimed to investigate whether or not the use of allogenic PBMSCs could enhance bone regeneration in a rabbit ulna critical-sized bone defect model.

MATERIALS AND METHODS

Peripheral Blood and Bone Marrow Cell Culture

Mononuclear cells from peripheral blood (PB) and bone marrow (BM) were isolated from New Zealand white rabbit by density gradient centrifugation method, plated at a density of $1 \times 10^5/cm^2$ in T-75 flasks with basal culture media containing α-MEM (alpha modified Eagle medium), 15% fetal bovine serum (FBS), 2 m*M* L-glutamine, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2.5 μg/mL fungizone (Gibco, UK), and cultured in a humidified atmosphere at 37° with 5% CO_2. When MSCs colonies formed and became confluent in the primary culture, both the single colonies and confluent cultures were passaged respectively for further expansion. The single colonies derived MSCs were cultured under specific inductive conditions. The osteogenic inductive conditions (OIC) contained basal culture media with additional supplements including 50 μg/mL L-ascorbic acid, 10 m*M* β-glycerophosphate, 10^{-8} *M* dexamethasone (Sigma Diagnostics Inc, UK). The adipogenic inductive conditions (AIC) were composed of basal culture media with 0.5 m*M* 3-isobutyl-1-methylxanthine (IBMX), 1 μ*M* dexamethasone, and 50 μ*M* indomethacin (Sigma Diagnostics Inc, UK). The chondrogenic inductive conditions (CIC) used serum free medium supplying with 10 ng/mL TGF-β1 (PrepTech, UK), Premix ITS+ (BD Biosciences, Discovery Labware), and 1 m*M* pyruvate (Sigma). The culture media were replenished every three days for total 21 days.

Colony Forming Efficiency (CFE) Assay

The CFE was originally defined as number of MSC colonies per 1×10^5 marrow nucleated cells in the original marrow cell suspension.[21] Here, the CFE was employed to quantity the number of MSCs in per million primary PB mononuclear cells (PBMNCs). Colonies formed in all flasks were counted at 14 days from the day when the original flasks were set up.

Cytochemistry Staining

Alkaline Phosphatase (ALP) Staining

At day 21, OIC cultured cells were stained with a Sigma alkaline phosphatase kit (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's instructions.

Mineralized Nodules and Alizarin Red S (ARS) Staining

At day 35 to 42, mineralized nodules in the OIC treated cultures were fixed with 95% ethanol, then treated for 30 min with 0.1% ARS dissolved in Tris-HCl buffer at pH 8.3 to stain the calcium salts, and observed under light microscope.

Alcian Blue Staining

At day 21, nodulelike structure appeared in the CIC treated cultures were fixed with 95% ethanol and stained with 1% alcian blue in 3% glacial acetic acid solution.

Oil-Red-O Staining

Following 14 to 21 days in the AIC cultures, cells were washed with PBS, fixed with 10% neutral buffered

formalin for 30 min, stained with 0.5% Oil-red-O in isopropanol:distilled water (60:40) for 30 min, then counterstained with hematoxyline, rinsed in tap water, air-dried, and mounted on a cover slip with aqueous mounting gel for light microscopic observation.

Preparation of Cell Seeded Implants

Sterilized Skelite (porous calcium phosphate resorbable substitute, EBI) were coated with fibronectin (100 μg/mL) prior to cell seeding. The third passage allogenic rabbit PBMSCs or BMMSCs (all treated with basal culture media without osteogenic or chondrogenic conditions) were trypsinized with 0.25M EDTA and 0.05% trypsin (Sigma) on the day of implantation. The rabbit PBMNCs were isolated by LymphPrep density gradient centrifugation from freshly harvested peripheral blood by cardiac puncture under anesthesia. After a final wash, the above three type of cells were resuspended in α-MEM at 7.5×10^6 cells/mL. Five milliliters of cell suspension was added to a 50 mL polyethylene tube containing the Skelite implants. All the cell seeded implants were incubated at 37°C with 5% CO_2 for 3 h to allow cell attachment, and then delivered to the operating room. This procedure was similar to that had been used previously.[19]

Rabbit Bilateral Critical-Sized Ulna Bone Defect Model

All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act (1986) at the Queen's University Belfast Biomedical Research facility. Ulna critical-sized bone defects, 20 mm in length, were created in the mid-portion of bilateral ulnar diaphysis of 12 6-month-old New Zealand white rabbits. The bone defects were treated with PBMSCs/Skelite (porous calcium phosphate resorbable substitute, EBI) ($n = 5$), BMMSCs/ Skelite ($n = 5$), PBMNCs/Skelite ($n = 5$), Skelite ($n = 5$), and empty control ($n = 4$). The muscle and skin were then reapposed and sutured. The animals were fully able to walk and active 24 hours postoperation.

Radiographic Examination

Serial radiographs were taken at the day of operation, and 4, 8, and 12 weeks postoperation, using a high-resolution digital radiography system (Faxitron MX-20 with DC-2 option, Faxitron X-ray Corporation). The exposure condition was 32 kV, 10 ms at 1× magnification. The percentage areas of the ulnae segmental defect occupied by newly formed bone was scored by two independent and blinded observers as previously reported.[22] The percentage of bone regeneration area filled the spaces of segmental defect and porous materials was graded from 1 to 4 on the radiographs at 12 weeks postoperation. The grades were defined as: (1) <25% space of segmental defect and/or porous materials filled with new bone; (2) 25% to 50% space of segmental defect and/or porous materials filled with new bone; (3) 50% to 75% space of segmental defect and/or porous materials filled with new bone; (4) >75% space of segmental defect and/or porous materials filled with new bone. The average score from the two observers was taken for each set of radiographs. After decoding the animal groups, the means of the scores of each group were calculated and compared.

Peripheral Quantitative Computed Tomography (pQCT)

To assess the volumetric density of the regenerated segmental defect, the excised bone specimens were scanned with a Stratec XCT 960M (Norland Medical System, Fort Atkinson, WI) with the software version 5.10 (Norland Stratec Medizintechinik GmbH, Birkenfeld, Germany). Briefly, prior to scanning, calibration of the pQCT was routinely performed with a set of hydroxyapatite standards. The specimens were then placed in the holder and the centers of the regenerated segmental defect were identified with the scout view window. Three slices were scanned, including the central slice, and one slice each 5 mm proximal and distal from the central one. All slices were analyzed for total volumetric bone mineral density (BMD) using the manufacturer-supplied software program XMICE v1.3. A threshold of 1.300 attenuation units was selected, based on sampling of all scans, to include mineralized tissue and exclude soft tissue. A density threshold of 275 mg/cm^2 was used to differentiate bone from soft tissues. The mean volumetric BMD of the regeneration area from the three slices per sample were calculated and compared.

Histological Observation

All rabbits were killed at 12 weeks postsurgery. A 4 cm segment of bone including the defect site were removed from both ulnae. Specimens were then fixed with 10% neutral buffered formalin for 48 h, decalcified in neutral buffered 20% formic acid (pH 6.0) for 3 weeks, and then embedded in paraffin. Five micrometer sections were cut and processed for hematoxylin and eosin (H&E) staining.

Immunohistochemistry Staining

PBMSCs cultured on chamber slides under specific inductive conditions were rinsed in PBS, fixed with 95% ethanol for 20 min, then treated with 3% H_2O_2 for 5 min, and blocked in 10% normal swan serum, 0.1% bovine serum albumin (BSA) in PBS for 20 min. The cells were incubated sequentially for 1 h with or without the primary antibody diluted 1:100, including collagen type I, osteocalcin, and collagen type II, then the biotinylated secondary antibody diluted 1:100, and a Vectastain Elite ABC-HRP reagent. The bound ABC-HRP complex was detected using a Vector-AEC substrate kit for peroxidase, and counterstained with hematoxylene.

Statistical Analysis

The high-resolution digital radiography scores, and the pQCT total volumetric bone mineral density were expressed as the mean ± standard deviation, the tests

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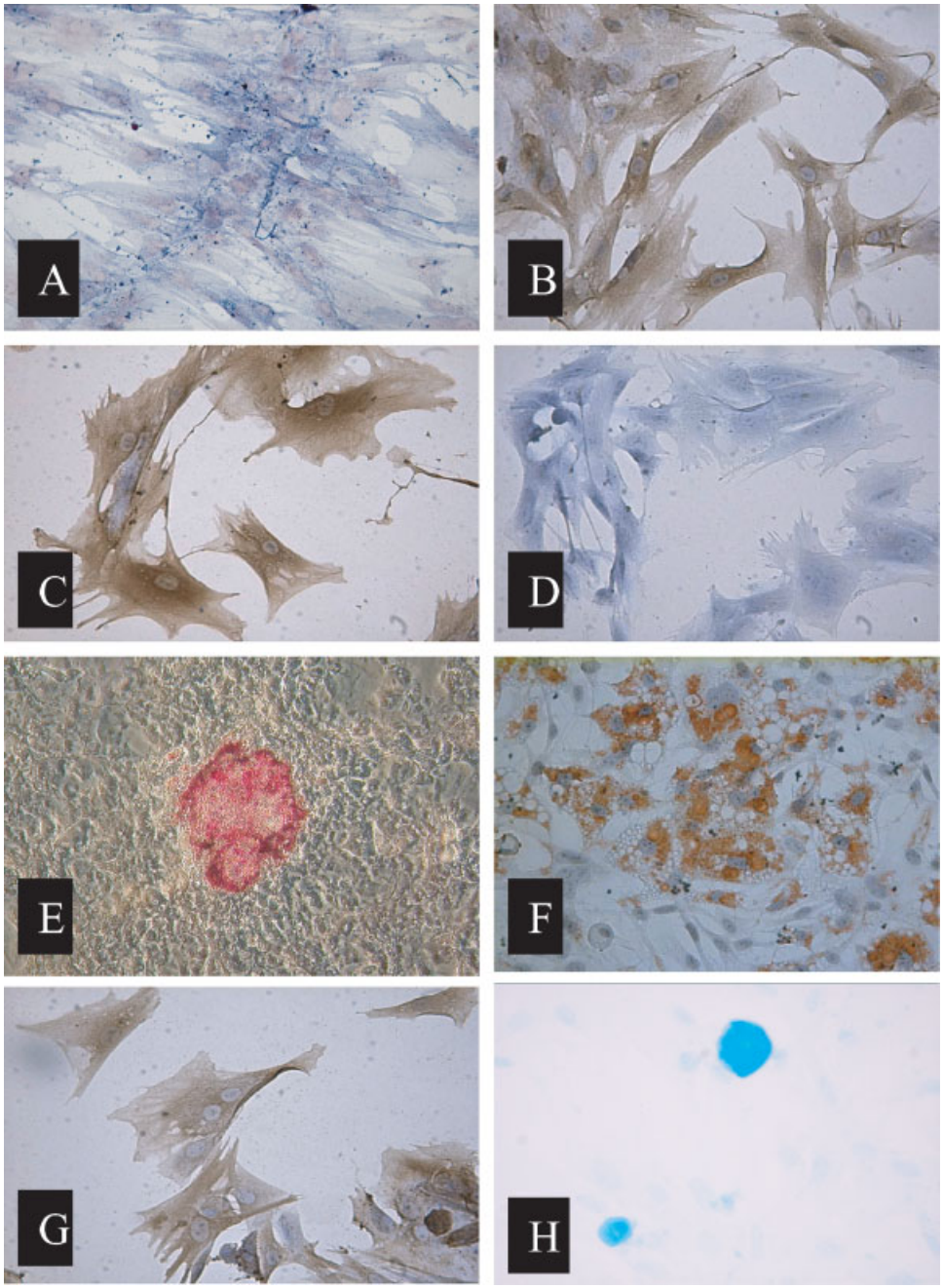

Figure 1. Rabbit PBMSCs possessed multidifferentiation potential when cultured in specific inductive media. Under osteogenic inductive conditions, these cells expressed osteoblastic markers including ALP (A), collagen type I (B), osteocalcin (C), and positive Alizarin Red staining for mineralization (E). Oil Red-O positive lipid droplets appeared under adipogenic inductive conditions (F). PBMSCs underwent chondrogenic differentiation in chondrogenic inductive conditions, with collagen type II expression (G), and Alcian blue positive nodules formation (H). No positive staining was seen in the nonprimary antibody control (D). (A–D), (F–H), original magnification, ×200; (E) original magnification, ×100.

were performed by using statistical package SPSS11.0. The one-way analysis of variance (ANOVA) was first used, followed by nonparametric LSD tests, significant level was considered at $p < 0.05$.

RESULTS

Characteristics and Multipotent Differentiation of the PB MSCs

MSCs derived from rabbit peripheral blood were demonstrated to possess similar cell morphology and differentiation potential in vitro as the marrow derived MSCs did.[23] On day 7, adherent, spindle-shaped fibroblastic cells formed colonies appeared in both of PB and BM mononuclear cell cultures. With time lapsing, the PB derived colonies further expanded, containing two distinct morphology of cells, the spindle, and the polygonal ones. The colony forming efficiency (CFE) of PBMSCs ranges from 1.2 to 13 per million PBMNCs; whereas the CFE of BMMSCs were 20 to 35 per million MNCs.

The PBMSCs possessed multidifferentiation potential when cultured under specific induction media. Under the OIC conditions, these cells underwent osteoblastic differentiation with specific phenotype markers including positive staining for ALP activity [Fig. 1(A)], collagen type I [Fig. 1(B)], osteocalcin [Fig. 1(C)], and Alizarin Red S [Fig. 1(E)]. Oil red-O positive lipid droplets accumulated under AIC culture conditions

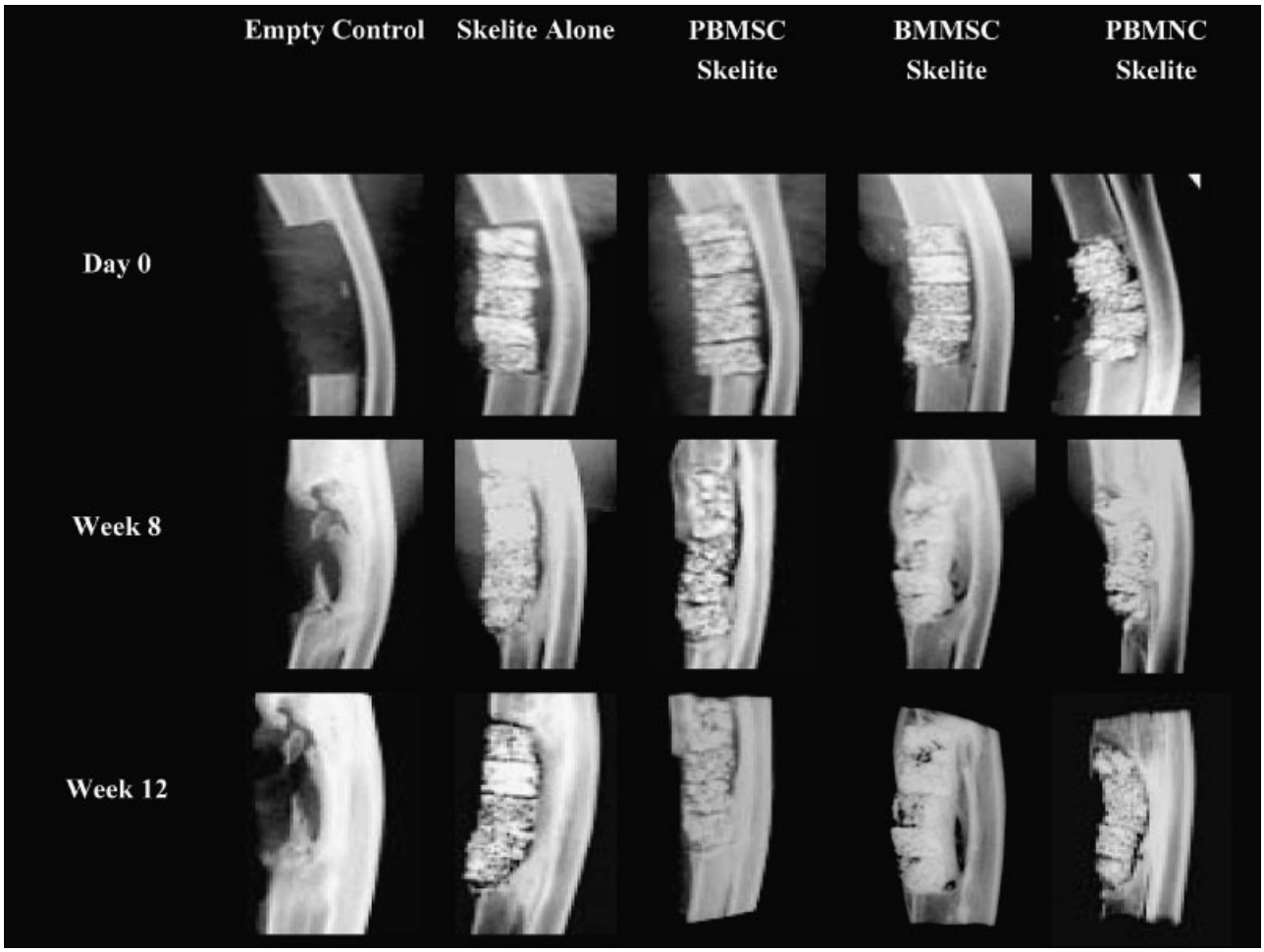

Figure 2. Representative radiographs of all experimental groups at day 0, 8, and 12 weeks postsurgery. The pores of PBMSCs/Skelite and BMMSCs/Skelite implants became more radiopaque at 8 and 12 weeks, indicating mineralization occurring in the pores of the Skelite. Callus was formed around the periphery of the implants as well as along the adjacent host bone. There was a significant enhancement of bone regeneration in the PBMSCs/Skelite and BMMSCs/Skelite implants treated groups compared to those of the PBMNCs/Skelite, Skelite alone and empty control groups at 12 weeks postsurgery. The segmental defect in the empty control group was not healed at 12 weeks.

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[Fig. 1(F)], suggesting adipocytic differentiation. The cells were directed to chondrogenic differentiation under the CIC conditions, showing collagen type II expression [Fig. 1(G)], and formation of Alcian blue positive nodules [Fig. 1(H)].

Radiographic and pQCT Analysis

Radiographs demonstrated that segmental defects treated with porous calcium phosphate resorbable substitute (Skelite) containing allogeneic PBMSCs or BMMSCs united at the proximal and distal host bone–implant interfaces at 12 weeks postsurgery (Fig. 2). The porous appearance of the implants at day 1 of surgery, gradually changed to a more radiopaque appearance at 8 and 12 weeks postsurgery, indicating that mineralization took place in the pores of the Skelite. Callus was also formed around the periphery of the implants as well as along the adjacent host bone surfaces. There is a significant enhancement of bone regeneration in the groups of PBMSCs/Skelite and BMMSCs/Skelite compared to the groups of the PBMNCs/Skelite and Skelite alone at 12 weeks postsurgery, according to the X-ray score [$p < 0.05$; Fig. 3(A)]. The segmental defect in the empty control group was not healed, and non-union was apparent at 12 weeks in this group. pQCT data showed that there were higher total bone mineral density (BMD) in the PBMSCs/Skelite and BMMSCs/Skelite groups than those of the PBMNCs/Skelite and Skelite groups [$p < 0.05$; Fig. 3(B)].

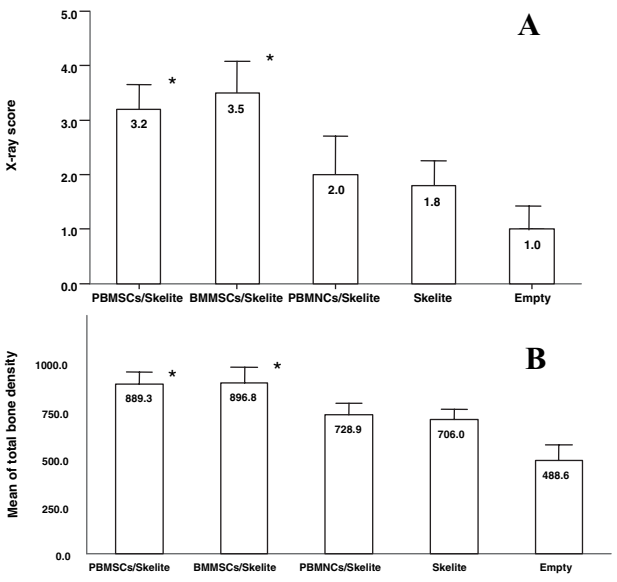

Figure 3. (A) Mean scores of X-rays at 12 weeks postsurgery. The scores were significantly higher in the PBMSCs/Skelite and BMMSCs/Skelite groups, compared to PBMNCs/Skelite, Skelite alone and empty control groups (*$p < 0.05$, LSD test). Means and SDs were plotted. (B) pQCT data showing that total bone mineral density was higher in the PBMSCs/Skelite and BMMSCs/Skelite groups than those of the PBMNCs/Skelite, Skelite alone, and empty control groups (*$p < 0.05$, LSD test). Means and SDs were plotted.

Histological and Immunohistological Study

For defects treated with allogeneic PBMSCs or BMMSCs seeded implants, no rejective reaction such as lymphocytic infiltration was seen in any of the specimens. After 12 weeks implantation in the rabbit ulna critical-sized segmental bone defect, typical lamellar bone formed at the host bone–implant interface and within the porous spaces of the PBMSCs/Skelite implants [Fig. 4(A)], as well as in the BMMSCs/Skelite implants [Fig. 4(B)]. Small amount of new bone were also formed in PBMNCs/Skelite implants [Fig. 4(C)] and Skelite alone group [Fig. 4(D)], only loose connective tissue were seen in the empty control group [Fig. 4(E)].

DISCUSSION

The role of reparative cells in the peripheral blood involved in tissue repair and regeneration after trauma, burns, inflammation, or metabolic diseases remain unclear because of the lack of information on specific cell surface markers, their origin and progeny evolution. Previous studies have shown that peripheral blood fibrocytes participate in the regeneration of damaged tissues.[24–27] Chesney and colleagues demonstrated that a novel population of peripheral blood cells, named fibrocytes, which possessed both mesenchymal and hematopoietic features including collagen type I+, vimentin+, CD34+, CD45+, and CD13+, contributed to scar formation and tissue repair.[25,26] Zhao and colleagues characterized an adult pluripotent stem cells (PSC) from a subset of human peripheral blood monocytes, resembling fibroblasts in appearance, which could be induced to differentiate into multilineages such as macrophages, lymphocytes, epithelial cells, endothelial cells, and neuronal cells, suggesting their possible involvement in various tissue regeneration.[28] Though these so-called fibrocytes or fibroblastic cells display an adherent, spindle-shaped morphology, and possessed multidifferentiation potential, they were different with the PBMSCs. Several studies have isolated and characterized the PBMSCs in various mammalian species, though the results are debated.[6–16] It was expected that the PBMSCs might serve as a new cell source for tissue repair or regeneration.

The pathophysiological functions of PBMSCs and their relationship with BMMSCs are yet not fully elucidated. It was demonstrated that the PBMSCs were distinct from the BMMSCs, for example, they were negative for some of the marrow stromal markers, Stro-1, ALP, endoglin, and Muc-18.[16] A serial studies suggested that systemic delivered BMMSCs homed to the bone marrow or fracture site and functioned as bone forming cells. Labeled stromal cells were found localize to fracture sites, especially along the margins of woven bone, following intravenous injection, in murine femoral fracture models.[29,30] This was consistent with another murine model that identified transplanted stromal cells localizing to the endosteal surfaces of a nonfractured femur.[31] Also, systemically administered MSCs transduced with IGF-1 localized to a fracture site in mice and enhanced healing compared to the controls.[30] When wild-type stromal cells were infused into transgenic mice with a phenotype of osteogenesis imperfecta, the collagen and bone mineral content were significantly increased after a month.[32] Following an allogenic mesenchymal cell infusion, children with severe osteogenesis imperfecta had an accelerated growth velocity in 6 months.[33,34] The above findings indicated that systemic delivered MSCs or

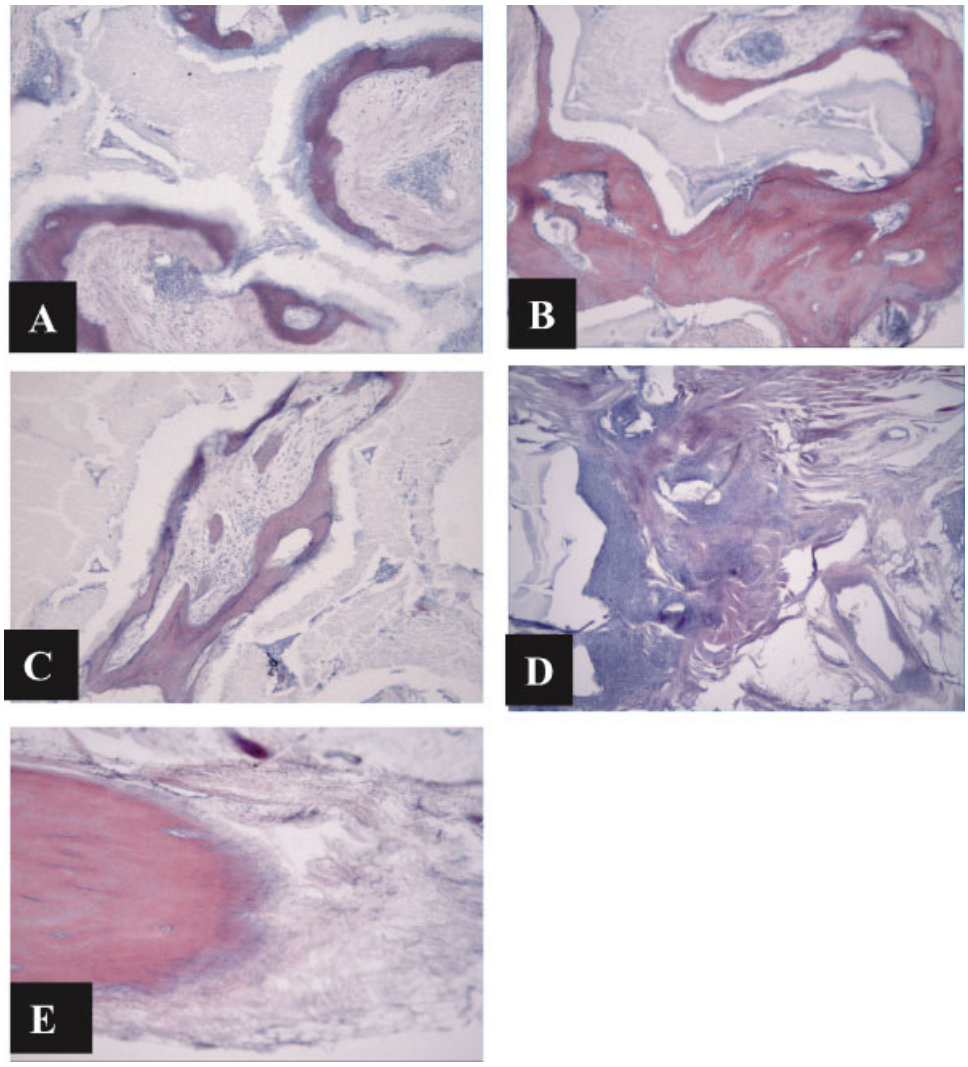

Figure 4. Histology of the rabbit ulna critical-sized segmental bone defect at 12 weeks postsurgery. Typical lamellar bone was formed in the PBMSCs/Skelite implants (A), and the BMMSCs/Skelite implants (B). Small amount of newly formed bone were also seen in the PBMNCs/Skelite group (C) and Skelite alone implants (D). Only loose connective tissues were found in the defect gaps of the empty control group (E). (A–E) H&E staining, original magnification, ×50.

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PBMSCs may serve as a new concept for bone repair or regeneration.

In clinical trauma and orthopaedics, large bone defects often occur as a result of trauma, bone tumors resection, and metabolic diseases. These defects result in reconstructive surgery in more than one million cases annually.[35] Autograft bone is considered as the standard implant for bone defect reconstruction, however it is limited by insufficient amount of tissue that can be harvested.[36,37] Allograft bone is an alternative and more readily available, but associated with a risk of infection, disease transmission, and poorly remodeling of the implants.[38–41] The BMMSCs have been considered as an alternative for autografts or allografts. However, the requirements of bone marrow aspiration from the patient will cause pain and morbidity of the donor sites. The existence of the PBMSCs in circulation suggests that it may be an alternative cell source for BMMSCs.

In the present study, the rabbit PBMSCs were isolated and expanded, demonstrated having similar cell morphology and differentiation potential with that of the BMMSCs in vitro. The PBMSCs possessed multidifferentiation potential when cultured under specific inductive media. Radiographs demonstrated that critical-sized segmental defects treated with allogeneic PBMSCs or BMMSCs seeded porous calcium phosphate biomaterials healed by 12 weeks, while the animals in the control groups did not heal. Histologically, typical lamellar bone formed at the host bone–implant interface and within the porous spaces of the PBMSCs/Skelite and BMMSCs/Skelite implants, only loose connective tissues were found in the defects in empty control groups.

The colony forming efficiency of BMMSCs is much higher (about 20-fold) than that of PBMSCs, indicating that there are more MSCs in the bone marrow than that in the peripheral circulation. However, once isolated the in vivo osteogenic potentials are similar among the two cell sources when similar amounts of cells were used for implantation. This suggests that PBMSCs may be an alternative cell source for BMMSCs, as the harvesting of PBMSCs from peripheral blood is less traumatic than bone marrow inspirations. Methods of enriching PBMSCs in peripheral circulation and better culture conditions for PBMSCs need further careful investigations.

The use of allogenic MSCs for tissue engineering purposes has wider clinical implications. Allogenic BMMSCs has been shown to be able to form new bone at the fracture sites when translated[29] and there was no known complication associated with the use of allogenic MSCs for transplantations in animals and humans.[29,33,34] In our preliminary experiments, we found that bone formation efficiency was similar between allogenic and autologous PBMSCs as well as BMMSCs. But allogenic MSCs preparations were less laborious and more practical for clinical application. The difference of species between human and rabbit has also to be considered. PBMSCs were found with ease in lower species, such as adult mouse, rat, and guinea pig,[10,16] but the number of PBMSCs was much lower in adult humans.[14,16] However, in certain disease conditions, such as trauma or systemic inflammation, the increased number of PBMSCs has been found.[6,42] Some drugs, hormones, or cytokines may also regulate the release the MSCs from bone marrow into peripheral circulation, and hence increase the number of PBMSCs.[6] More basic research work in this field is needed to address these practical issues before PBMSCs can be used as a reliable source to replace BMMSCs.

In conclusion, the present study demonstrated that the rabbit PBMSCs possessed multidifferentiation potential, allogeneic PBMSCs loaded onto porous calcium phosphate resorbable substitute enhanced bone regeneration in a rabbit critical-sized segmental defect model. Hence, allogenic PBMSCs may serve as a new source of osteogenic stem cells for bone regeneration, cell therapy, and tissue engineering.

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