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Osteogenic potential of rat mesenchymal stem cells after several passages

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Abstract

Osteogenic potential of serially passaged rat bone marrow derived mesenchymal stem cells (BMCs) was evaluated for clinical feasibility. Osteogenic differentiation in vitro was evaluated by means of the concentration and mRNA expression of alkaline phosphatase and osteocalcin. For in vivo osteogenesis, BMCs in various degrees of differentiation were implanted into the athymic mice. Although elevated levels of osteogenic markers were prominent in the less passaged BMCs continuously cultured with osteogenic supplements (OS group), they decreased with passaging. Similar to the in vitro experiments, abundant bone and cartilage formations inside the membrane were observed in the P0 through P2 cells of the OS group. In the P3 cells, however, the chambers were filled with fibrous tissues showing the failure of osteogenesis. Establishment of the culture conditions that permit the rapid expansion of BMCs while retaining their potential for differentiation will be required for future clinical applications.

Keywords: Mesenchymal stem cell; Osteogenic differentiation; Passage; Bone marrow

The regeneration of bone tissue is an ambitious goal in orthopaedic surgery for healing large bone defects secondary to tumor or trauma, and for treating delayed unions or non-unions. Autologous or allogenic bone grafting has been applied for these pathologic conditions. However, a lack of sufficient materials precludes the use of autologous bone while the use of allogenic bone for transplantation carries potential risks of immune responses. Tissue engineering, that is small biopsy specimens from the relatively uninvolved sites can be obtained from the patient and cells can be isolated, grown, and expanded in culture in large numbers, may overcome these problems. Bone tissue engineering has emerged as a possible alternative strategy to regenerate bone. It depends on the combination of osteogenic cells with appropriate carrier materials.

Bone marrow derived mesenchymal stem cells (BMCs) have the potential to form a variety of mesenchyme tissues including bone, cartilage, tendon, ligament, muscle, and fat [1–7]. Bone marrow is considered to be a suitable donor tissue for bone tissue engineering since it can be obtained from a comparatively small amount of iliac crest biopsy and contains multipotent mesenchymal stem cells (MSCs). BMCs can be directed towards the osteogenic lineage in vitro when they were cultured in the presence of dexamethasone, β-glycerophosphate, and ascorbic acid [2,8–14]. Osteogenic differentiation of BMCs was generally characterized by appearance of osteoblastic cell morphology and increased synthesis of osteogenic marker proteins such as alkaline phosphatase (ALP) and osteocalcin (OC).

Although osteocompetent cells could be found in bone marrow, their proportion was rather low and decreased with age [15–17]. The total number of BMCs generated in primary culture often seems to be limited in clinical feasibility for bone regeneration. Expanding the cell population by several passages would be necessary to obtain a sufficient number of cells. There is a problem, because serially passaged BMCs have been shown to lose their capacity to differentiate into osteoblasts [18–20]. In this study, osteogenic potential of rat BMCs, cultured with or without osteogenic supplements

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within the culture medium, was analyzed during several passages. Osteogenic differentiation in vitro was evaluated in terms of the ALP activity and the OC concentration in the supernatants and the expression of ALP, OC mRNA in the cultured cells. In addition, these BMCs in various degrees of differentiation were subcutaneously implanted into the athymic mice, and in vivo bone formation was analyzed to determine the optimal cell condition for clinical applications of BMCs.

Materials and methods

Cell isolation and culture. BMCs were isolated from Sprague–Dawley (SD) rats using the method described by Maniatopoulos and Sodek [8]. Cells were cultured in two different kinds of culture medium; the control medium and the osteogenic medium. The control medium consisted of minimum essential medium alpha medium (α-MEM; Gibco-BRL, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Dainippon Pharmaceutical, Osaka, Japan) and 100 μl/ml penicillin–streptomycin (Gibco-BRL, Life Technologies, Grand Island, NY, USA). The osteogenic medium consisted of the control medium supplemented with 0.2 mM ascorbic acid (Sigma, St. Louis, MO, USA), 10 mM Na-β-glycerophosphate (Sigma, St. Louis, MO, USA), and 10^{-8} M dexamethasone (Sigma, St. Louis, MO, USA). The bone marrow suspensions were cultured in polystyrene six-well dish and non-adherent cells were removed from the cultures after 2 days by a series of phosphate-buffered saline (PBS) washes and subsequent medium changes. Adherent cells were expanded as monolayer cultures in a 5% CO_2/95% air atmosphere at 37 °C with medium changes every 3 days. These primary cells were referred to as P0. The confluent cells were dissociated with trypsin and subcultured in new six-well culture dishes at a plating density of 6×10^4 cells/dish. These handlings were repeated four times and these cultures were referred to as P1, P2, P3, and P4. To assess osteogenic differentiation of the cells, the BMCs were divided into three groups according to the culture media. The cells continuously cultured in the osteogenic medium or control medium were included in the OS and the CM groups, respectively. The OS/CM group represented the cells cultured in the control medium and subsequent change into the osteogenic medium.

In vitro osteogenic differentiation. The culture media from the OS, OS/CM, and CM groups were obtained, respectively, every 3 days during all passages. The ALP activity and the OC concentration in the supernatants were measured using Alkaline phospha-B-test wako (Wako, Japan) and Rat Osteocalcin EIA Kit (Biomedical Technologies, USA), according to the instruction manual.

Total RNA was isolated from individual cell layers after 7 days subcultured using Trizol reagent (Gibco-BRL, Life Technologies, Grand Island, NY, USA). One microgram of total RNA was used as a template for reverse transcription into cDNA by Reverse Transcription System (Perkin–Elmer Gold Taq, Roche, USA). cDNA was amplified by PCR with oligonucleotide primers (forward primer 5′-TCC ATGGTGGATTATGCTCA-3′, and reverse primer 5′-TTCTGTTCC TGCTCGAGGTT-3′) for ALP, (forward primer 5′-AGGACCCTCT CTCTGCTCAC-3′ and reverse primer 5′-AACGGTGGTGCCATAG ATGC-3′) for OC [13], and (forward primer 5′-TGAACGGGAAGC TCACTGG-3′, and reverse primer 5′-TCCACCACCCTGTTGCTGT A-3′) for GAPDH (LightCycler primer/probe Set, Roche). DNA amplifications were performed that an initial denaturation at 95 °C for 8 min, followed by 28 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. The final cycle included 7 min for extension at 72 °C. PCR products from each sample were analyzed by electrophoresis on 2.0% agarose gel and visualized by staining with ethidium bromide.

Quantitative real-time PCR was carried out using a LightCycler instrument (Roche Molecular Biochemicals) according to the instruction manual. The oligonucleotide primers used in the amplification reaction were same as above. Amplification was carried out in a 20 μl final volume containing 3 mM $MgCl_2$, 0.5 μM of each primer, 1 μl of the sample, and 2 μl of LightCycler-Fast Start DNA Master SYBR Green I (Roche Molecular Biochemicals). The amplification program included an initial denaturation step at 99 °C for 10 min for Fast Start *Taq* DNA polymerase activation, followed by 40 cycles of denaturation at 99 °C, 5 s annealing at 60 °C, and 15 s extension at 72 °C. Ramping times were at 20 °C/s. Cycle-to-cycle fluorescence readings were plotted on the computer screen for continuous monitoring of PCR product. At the end of each run, fluorescence data were analyzed by setting a noise band to remove background fluorescence. Values obtained for external standards (Rat GAPDH Standard, LightCycler primer set, Roche) were employed to construct a reference curve and copy numbers for the sample were calculated by interpolation in the obtained reference curve. Calculated values of GAPDH, ALP, and OC were analyzed.

In vivo osteogenic differentiation. The individual cultured BMCs during all passages were trypsinized and suspended in 100 μl α-MEM, and mixed with 100 μl of 0.5% type I collagen gel (KOKENCELLGEN I-PC, Japan) at the concentration of 3.0×10^5 cells/200 μl. These cell suspensions were then loaded into the diffusion chambers (0.45 μm pore size; Millipore, Bedford, MA, USA). The chambers were subcutaneously implanted into the dorsal side of 8-week-old male athymic mice in pockets formed by blunt dissection. The mice were sacrificed at 4 weeks after implantation and the newly formed tissues in the chambers were analyzed for osteogenesis radiographically and histologically. The removed chambers were immediately radiographed with soft X rays for 1.5 min at 45 kV and 1.6 mA. After radiographic examinations, specimens were fixed in 10% formalin and embedded in paraffin. Serial sections cut by 6 μm were stained with hematoxylin and eosin (HE) and Alcian blue for histological examinations.

Results

In vitro osteogenic differentiation

In the P1 through P3 cells, the ALP activity in the supernatant was the highest in the OS group that showed statistical significance among the three groups. Significantly higher ALP activity was observed in the OS/CM group than in the CM group in the P1 cells (Fig. 1). Similarly the expression of ALP and OC mRNA was noted in the P1 through P3 cells of the OS group while lower expression was observed in the OS/CM and CM groups (Fig. 2).

Within the OS group, both ALP activity and the OC concentration were prominent in the P0 and P1 cells, but they decreased with passages (Fig. 3). The expression of ALP and OC mRNA was demonstrated in the P0 through P3 cells (Fig. 4A), but different expression levels of the two genes within the passaged cells were ascertained by quantitative real time RT-PCR analysis (Fig. 4B).

In vivo bone formation

Radiological examinations of the diffusion chamber at four weeks after implantation demonstrated marked

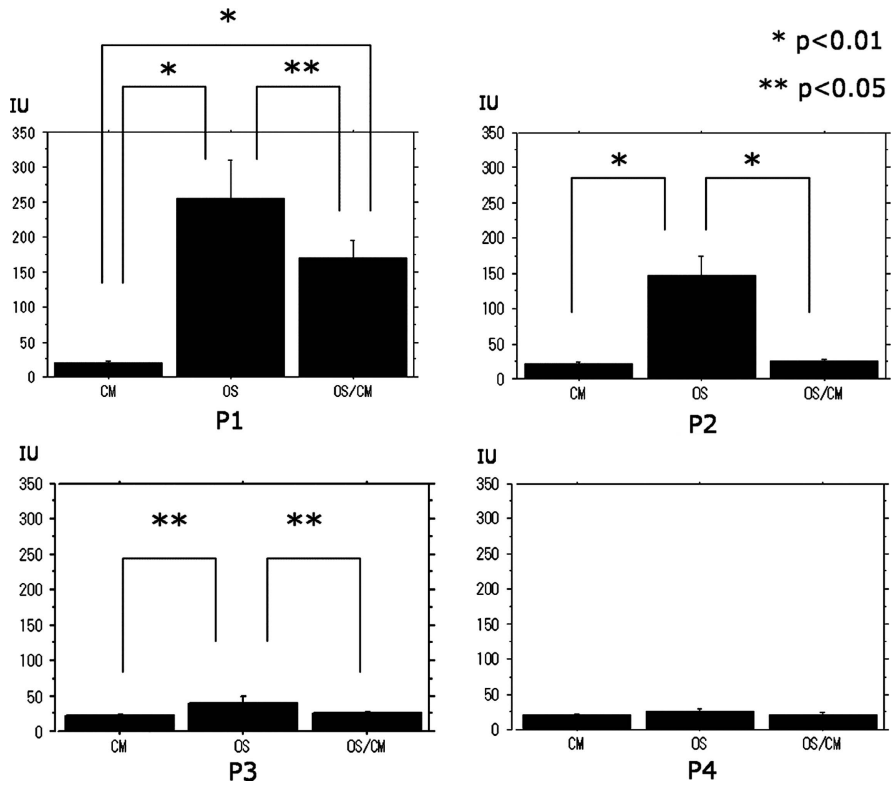

Fig. 1. ALP activity in the supernatants in the P1 through P4 cells of the OS, OS/CM, and CM groups. Data are means ± SEM ($n = 6$). *$p < 0.01$, **$p < 0.05$.

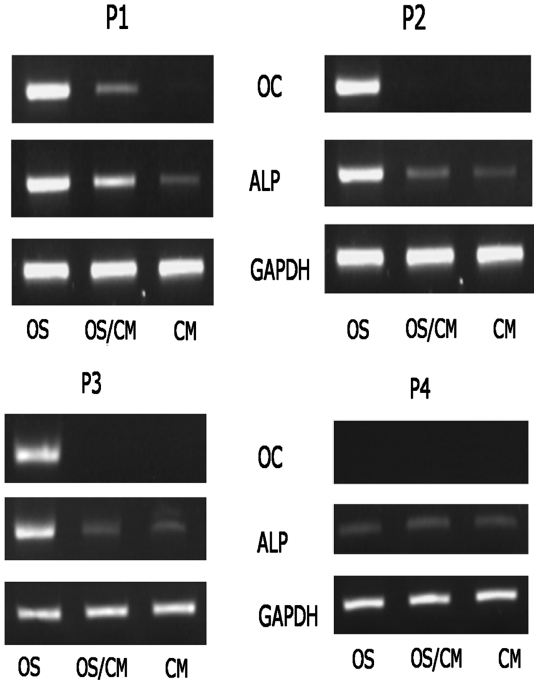

Fig. 2. mRNA expression of ALP and OC in the P1 through P4 cells of the OS, OS/CM, and CM groups.

calcified tissue formations in the P0 and P1 cells of the OS group and sparse calcifications in the P2 cells of the OS group and the P1 and P2 cells of the OS/CM group. On the other hand, few calcifications were seen in the P3 cells of all groups (Fig. 5).

Histological examinations demonstrated that massive bone formations inside the membrane (Figs. 6A–C) and abundant cartilaginous matrices together with chondrocyte-like cells (Figs. 7A–C) were remarkable in the P0 through P2 cells of the OS group. In the P3 cells, however, most of the newly formed tissues were filled with fibroblastic cells with little evidence of osteochondrogenesis (Figs. 6D and 7D).

Discussion

Bone marrow contains a population of multipotent MSCs [1] that generate the progenitors for osteogenic [2], chondrogenic [3,4], adipogenic [5,6], and myogenic cells [7]. Differentiation into the osteogenic lineage was induced by the addition of dexamethasone that was thought to increase the number of osteoprogenitor cells. Several authors reported an increased expression of osteogenic markers such as ALP and OC in dexamethasone-treated BMCs [2,8–14]. In agreement with previous studies of in vitro BMC differentiation, our BMC cultures supplemented with dexamethasone (OS and OS/CM groups) showed elevated expression of differentiation markers especially in the less passaged cells.

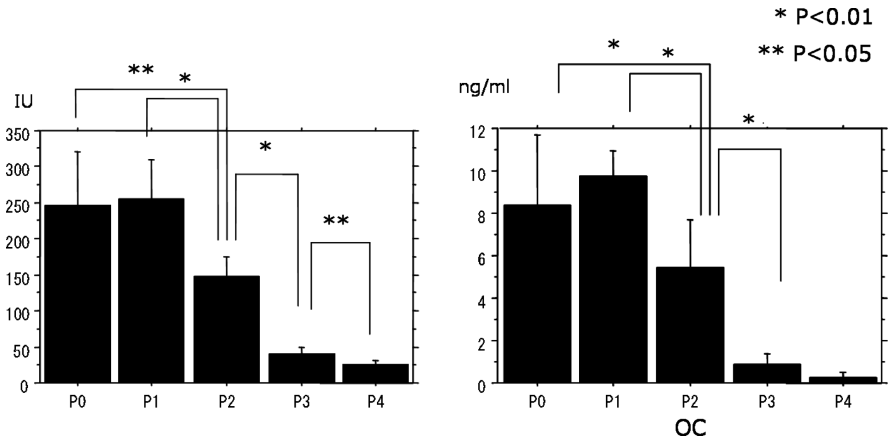

Fig. 3. ALP activity and the OC concentration in the supernatants in the P1 through P4 cells of the OS groups. Data are means ± SEM ($n = 6$). *$p < 0.01$, **$p < 0.05$.

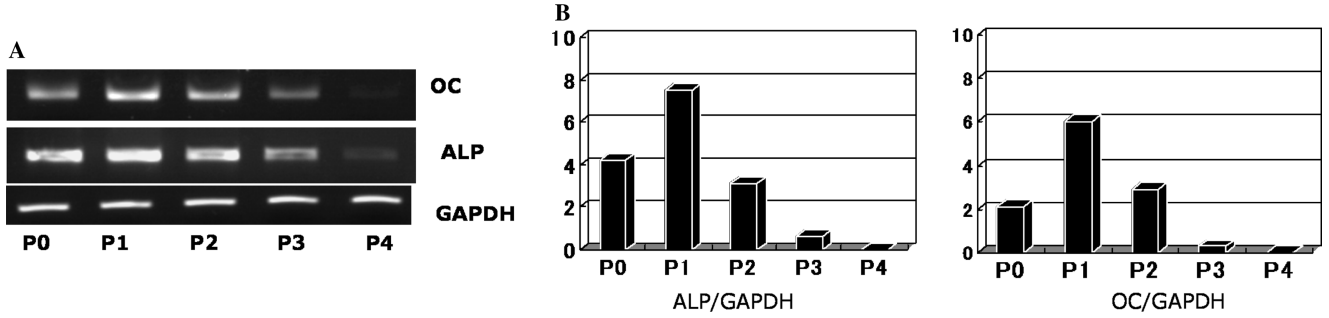

Fig. 4. mRNA expression of ALP and OC in the P1 through P4 cells of the OS group (A). Quantitative mRNA analysis for expression ratios of ALP and OC mRNA in the P1 through the P4 cells of the OS group (B).

Between the two groups cultured supplemented with dexamethasone, the OS group resulted in higher expression of the osteogenic markers compared with the OS/CM group. Dexamethasone may not only induce the BMCs into the osteogenic cells but also recruit the cells with osteogenic potential in primary culture, or multipotential MSCs may lose their ability of differentiation into osteoblasts when they are cultured without dexamethasone.

Quantitative mRNA analysis demonstrated that the expression of differentiation markers increased between the P0 cells and the P1 cells within the OS group. This may be due to the low percentage of the adherent cells during the first medium change that resulted in lower seeding density in the primary culture rather than the first passage cells. Osteogenic differentiation of BMCs could depend on the seeding density of the cells. Even in the BMCs continuously cultured in the OS medium, expression of osteogenic markers decreased with passages. Ter Brugge and Jansen [18] reported that BMCs continuously cultured in the presence of dexamethasone initially showed high ALP expression and abundant mineralization, but no ALP activity and calcification were found in the passaged cells three times. The seeded cells with passages will be a combination of differentiated and undifferentiated cells. Since the differentiated cells may have a limited capacity for proliferation, loss of differentiation may be due to decreased percentage of osteogenic cells in culture with passages.

Although in vivo bone formation by implantation of a limited number of passaged BMCs has previously been demonstrated [19–28], detailed documentation of several passaged BMCs with regard to in vivo osteogenic potential has not been delineated. Similar to the in vitro experiment, in vivo osteogenic potential to form chondro-osseous tissues tended to decrease with passages within the OS group. Massive bone and cartilage formation could be constituted by transplanted differentiated BMCs since the host cells did not penetrate into the diffusion chamber. Although the transplanted cells showed increased expression of osteogenic markers, abundant cartilage formation associated with positive Alcian blue staining was characteristic in addition to intramembranous ossification adjacent to the membrane. The cartilage-like tissues, bordering on the ossification regions with no evidence of vascularization, seemed to be suggestive of the so-called transchondroid

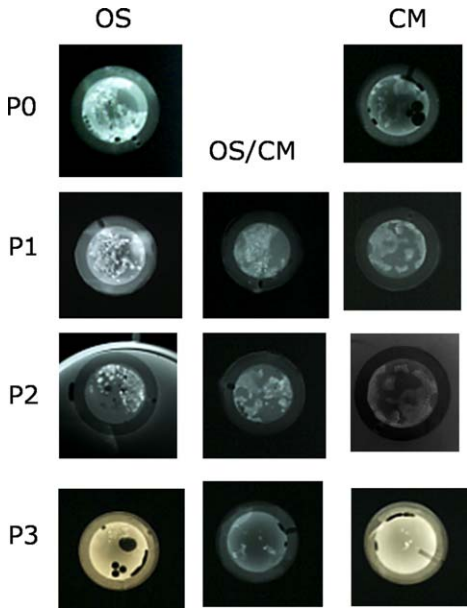

Fig. 5. Soft X-ray radiographs of the diffusion chamber of the OS, OS/CM, and CM groups in the P0 through P3 cells at 4 weeks after subcutaneous implantation. Radio-opaque configurations that indicate mineralized structures were observed in the P0 through P2 cells. Few calcifications were noted in the P3 cells irrespective of the groups.

ossification that was observed specifically in distraction osteogenesis. The exact reason for in vivo chondrogenesis is still unknown, but MSCs with chondrogenic potential may be included in the transplanted cells or the differentiated cells may be influenced by microenvironment within the chamber leading to the unique chondro-osseous tissue formation.

Since BMCs are relatively easily isolated from the bone marrow and can be manipulated in vitro, they could be good candidates for the development of various therapeutic modalities aimed to regenerate mesenchymal tissues. Better understanding of the molecular mechanism directing the differentiation of BMCs will eventually allow us to properly manipulate BMCs both in vivo and ex vivo for regeneration of complex tissues and organs. Less passaged BMCs cultured with dexamethasone proved to possess in vivo osteogenic potential. This can be applicable to use cell therapy in the repair of bone defects. In clinical feasibility, however, large cell numbers with osteogenic potential will be required. The failure of in vivo osteogenesis in the implanted P3 cells was a dilemma for autologous cell therapy for bone regeneration. Further investigations are needed to establish the culture conditions that

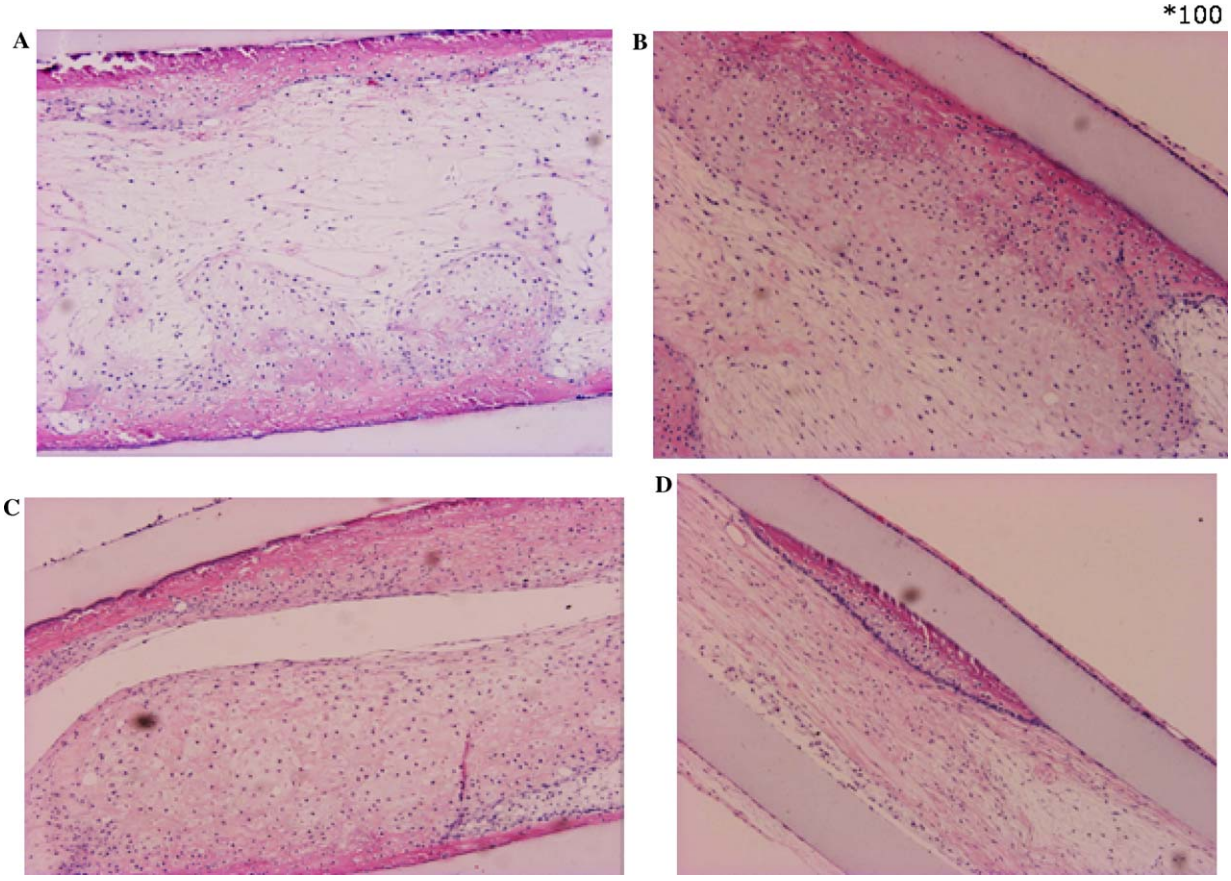

Fig. 6. Photomicrographs of the histologic section of the diffusion chamber of the OS group in the P0 (A), P1 (B), P2 (C), and P3 (D) at 4 weeks after implantation. Bone-like tissues were observed adjacent to the membrane in the P0 through the P2 cells. Fibrous tissues were filled in the P3 cells. Hematoxylin and eosin staining; original magnification 100×.

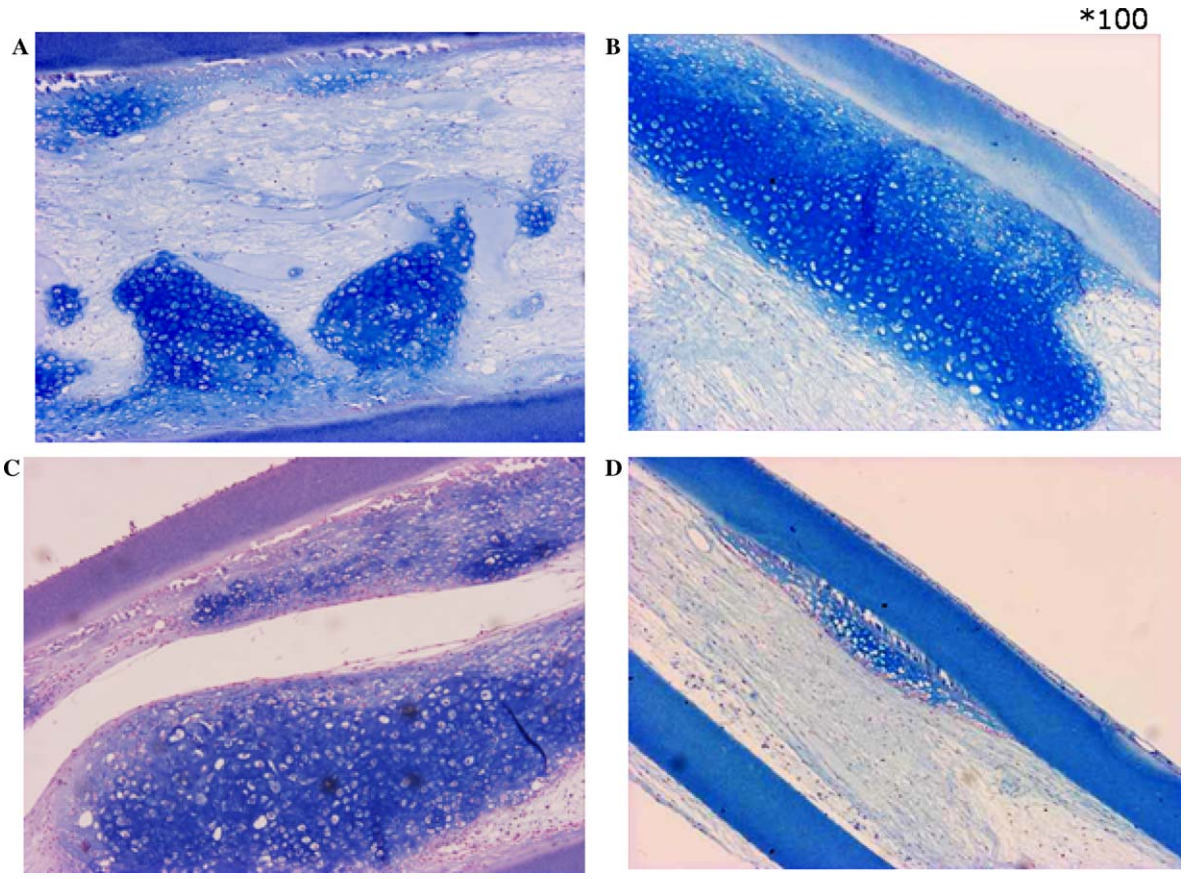

Fig. 7. Photomicrographs of the histologic section of the diffusion chamber of the OS group in P0 (A), P1 (B), P2 (C), and P3 (D) at 4 weeks after implantation. Cartilage tissues strained with Alcian blue were prominent in the P0 through P2 cells. Alcian blue staining; original magnification 100×.

permit the rapid expansion of MSCs while retaining their potential for differentiation.

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