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In vivo bone formation by human marrow stromal cells in biodegradable scaffolds that release dexamethasone and ascorbate-2-phosphate ☆

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Abstract

An unsolved problem with stem cell-based engineering of bone tissue is how to provide a microenvironment that promotes the osteogenic differentiation of multipotent stem cells. Previously, we fabricated porous poly(D,L-lactide-co-glycolide) (PLGA) scaffolds that released biologically active dexamethasone (Dex) and ascorbate-2-phosphate (AsP), and that acted as osteogenic scaffolds. To determine whether these osteogenic scaffolds can be used for bone formation in vivo, we seeded multipotent human marrow stromal cells (hMSCs) onto the scaffolds and implanted them subcutaneously into athymic mice. Higher alkaline phosphatase expression was observed in hMSCs in the osteogenic scaffolds compared with that of hMSCs in control scaffolds. Furthermore, there was more calcium deposition and stronger von Kossa staining in the osteogenic scaffolds, which suggested that there was enhanced mineralized bone formation. We failed to detect cartilage in the osteogenic scaffolds (negative Safranin O staining), which implied that there was intramembranous ossification. This is the first study to demonstrate the successful formation of mineralized bone tissue in vivo by hMSCs in PLGA scaffolds that release Dex and AsP.
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The requirement for new bone tissue to restore the function of damaged or lost bone is a major clinical and socioeconomic need [1]. Autologous bone graft is the treatment of choice for bone defects, but grafting is restricted by donor site morbidity and limited availability [2]. Allografts have been used, but they are in limited supply and increase the risk of disease transmission. Calcium phosphates such as tricalcium phosphates and hydroxyapatites have been reported to act as osteoconductive synthetic bone substitutes; however, the clinical applications of these substances are limited by insufficient mechanical properties [3]. Bone-tissue engineering has been studied as an alternative treatment for bone regeneration, and bone marrow stromal cells (MSCs) represent a potential source of multipotent cells for autologous bone-tissue engineering [4–7].

MSCs should undergo osteogenic differentiation to form bone tissue. Dexamethasone (Dex), either alone

☆ *Abbreviations:* PLGA, poly(D,L-lactide-co-glycolide); Dex, dexamethasone; AsP, ascorbate-2-phosphate; BMPs, bone morphogenetic proteins; hMSCs, human marrow stromal cells; ALP, alkaline phosphatase; ISH, in situ hybridization.

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or in combination with ascorbate-2-phosphate (AsP), and bone morphogenetic proteins (BMPs) are osteogenic inducers for MSCs [8–10]. However, BMPs are not as effective in humans as in rodents [11,12], and it is not well known whether BMPs have adverse effects. BMPs have been associated with, and might aggravate, cancer [13–16]. By contrast, Dex and AsP are not adversely associated with cancers. In fact, the results of several studies suggested that Dex is anticarcinogenic [17–20]. Moreover, because Dex has been used clinically for a long time, the side effects of Dex are better known than those of BMPs. Finally, Dex and AsP are cheaper than BMPs.

Most models of bone-tissue engineering are based on seeding MSCs onto three-dimensional scaffolds. Poly(D,L-lactide-co-glycolide) (PLGA) has been used widely for several decades as a biodegradable and biocompatible scaffold material [21]. However, PLGA alone does not provide an osteoinductive environment to mesenchymal stem cells for bone-tissue engineering [22]. In a previous study, we developed porous PLGA scaffolds that release biologically active Dex and AsP for at least one month in vitro to provide an osteogenic environment for seeded rabbit MSCs [23].

The in vitro induction of stem cells alone is a limited approach to bone-tissue engineering. In vitro differentiation is not an entirely physiological process because it lacks many of the elements present in the in vivo environment, such as optimal oxygen and CO_2 pressures, appropriate mechanical stimuli, tissue-specific growth factors, and contacts with peer cells, which might be both bone-specific and necessary for osteogenic differentiation. Therefore, MSCs would probably form bone tissue more successfully if the in vivo milieu could be combined with Dex and AsP. Here, we describe for the first time the successful formation of mineralized bone tissue by human MSCs (hMSCs) in PLGA scaffolds that release Dex and AsP.

Materials and methods

Materials. PLGA 75:25 (inherent viscosity, 0.86 dl/g; in chloroform, 25 °C; 0.5 g/dl) was purchased from Purac (Gorinchem, Netherlands). Dex, AsP, acetone, NaCl, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma Chemical (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Invitrogen (Grand Island, NY). All other chemicals were of the purest analytical grade.

Fabrication of osteogenic scaffolds and study of AsP and Dex release. Osteogenic scaffolds were fabricated as described previously [23] with slight modifications. Briefly, atomized AsP [23] was suspended in acetone at a concentration of 7.5 mg/ml. PLGA (0.8 g) and Dex (0.32 mg) were dissolved in 8 ml of AsP-suspended acetone (60 mg AsP), and 8 g NaCl (particles sieved to 300–450 μm) was added. The dispersion was then cast in a 4 cm diameter Teflon mold. The resulting 1.5 mm thick PLGA/Dex/AsP/NaCl composite matrices were cut into 6 mm diameter disks that were stored in a desiccator under vacuum at −20 °C until use. PLGA scaffolds without AsP and Dex were used as control scaffolds. Before cell seeding, the disks were immersed in distilled deionized water (DW) for 10 h (DW changed every 3 h) with mild stirring to leach out salt. The scaffolds were frozen-sectioned as described [23] just before cell seeding and stained by von Kossa method [24].

An in vitro release study was performed, and the concentrations of Dex and AsP released from the scaffolds were measured by high performance liquid chromatography as described previously [23].

Cell culture. Human bone marrow aspirates were obtained during pelvic osteotomy after obtaining informed consent and the approval of the Institutional Review Board of Yonsei University Medical Center. MSCs were isolated as described previously [25] and were cultured in complete medium consisting of DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 10% FBS. Third- or fourth-passage cells were used for experiments.

Assay of in vivo bone formation. All animal experimental procedures were carried out in accordance with the Guidelines and Regulations for the Use and Care of Animals of the National Institute of Health in Korea. The scaffolds were sterilized by immersion in 100% and 70% ethanol, each for 1 h, before being washed with sterile DW [26]. The scaffolds were coated with serum protein by submerging them in FBS supplemented with 10% antibiotics for 2 h and were then washed with sterile DW. The hMSCs were suspended at a concentration of 4×10^7 cells/ml, and 25 μl of the cell suspension (10^6 cells) was poured onto each scaffold. The cells were allowed to adhere to the scaffolds for 3 h. The cell–scaffold complexes were covered with 2 ml of medium. After 1 h, the complexes were implanted into the dorsal subcutaneous area of athymic mice (BALB/c-nu; Japan SLC, Hamamatsu, Japan). Scaffolds lacking hMSCs were also implanted as control.

At 3 and 9 weeks after implantation, the implanted scaffolds were excised, fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned. The sections were stained by the von Kossa method to observe mineralized tissue [24] and were also stained with Safranin O to detect cartilage [22].

Measurement of alkaline phosphatase expression. At 1, 2, and 5 weeks, the implants were excised and gene expression was assayed by real-time reverse transcription-polymerase chain reaction (RT-PCR). The excised tissues were chopped in TRIzol (Invitrogen, Carlsbad, CA) before being minced using a homogenizer (IKA-WERKE, Germany). Total RNA was isolated according to the manufacturer's instructions. The RT reaction was carried out as described previously [27]. Quantitative real-time PCR was performed using the 7900 HT sequence detection system (Applied Biosystems, Foster City, CA) and SYBR Green master mix (Applied Biosystems). Primer pairs for the real-time PCR were as follows: for alkaline phosphatase (ALP), forward 5′-CTGCCATCCTGTATGGCAATG-3′, reverse 5′-GGCACGAAGGC TCATCATTC-3′; for β-actin (human-specific sequence), forward 5′-GCCCAGTCCTCTCCCAAGTC-3′, reverse 5′-GGCACGAAGGC TCATCATTC-3′. The expression of ALP was normalized to that of human β-actin.

Calcium assay. Calcium deposition was measured as described previously [23] with slight modifications. Briefly, PLGA scaffolds before cell seeding and implants excised at 3 and 9 weeks after implantation were rinsed mildly with calcium-free Dulbecco's phosphate-buffered saline, and 0.2 ml of 1 N HCl per scaffold was added. The tissues were chopped before being placed on an orbital shaker overnight to extract calcium. The mixtures were centrifuged at 10,000*g* for 10 min. The calcium concentration in the supernatant was measured using a commercially available calcium assay kit according to the manufacturer's instructions (Diagnostic Chemical, Charlottetown, Prince Edward Island, Canada).

In situ hybridization for human-specific alu sequence. We performed in situ hybridization (ISH) to detect implanted human cells in the scaffolds [28] using a universal ISH kit (Innogenex, San Ramon, CA). A human-specific repetitive *alu* sequence [29] probe labeled with fluorescein was purchased from Innogenex (San Ramon, CA). Human cells were identified using an anti-fluorescein antibody (Innogenex) labeled with phycoerythrin. The sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; included in the ISH kit).

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