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A comparison of osteocyte bioactivity in fine particulate bone powder grafts vs larger bone grafts in a rat bone repair model

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ABSTRACT

The osteogenic potential for bone grafts is based on numbers and activities of cells that survive transplantation. In this study, we compared the bioactivity of osteocytes in 300–500 μm fine particulate bone powder grafts to 2 mm larger bone grafts in a rat radial defect model. Expression levels of bone morphogenetic protein-2 (BMP-2), transforming growth factor-beta 1 (TGF-β1), alkaline phosphatase (ALP), and collagen I were semi-quantified by both immunohistochemistry and RT-PCR at days 1 and 4, as well as weeks 1, 2, 4, 6 and 10 post-transplantation. Within two weeks post-transplantation, more cells stained positively for BMP-2, TGF-β1, ALP, and collagen I within the bone grafts and in the surrounding tissues in the group transplanted with the fine particulate bone powder grafts than in those with larger bone grafts ($P < 0.05$). The mRNA levels of all four markers in the group transplanted with fine particulate bone powder graft peaked earlier and were expressed more highly than in the larger bone graft group, suggesting that fine particulate bone powder grafts provide more viable and active osteocytes to accelerate bone defect healing than larger bone grafts.

Introduction

Severe bone defect repair is a significant clinical challenge in spine, bone fracture, and joint arthroplasty surgeries (Hollister and Murphy, 2011; Qiu et al., 2011). Autogenous bone graft is the most effective means to repair a defect, because it elicits no immunological rejection and has the best osteoinductive, osteoconductive and osteogenic properties (Nandi et al., 2010). Autograft bone can be implanted as blocks or small particles. Block bone graft offers better structural support for implanted devices and is incorporated into the surrounding bone through slow creeping substitution (Goldberg and Stevenson, 1993). Although particulate bone graft does not provide substantial structural support, the surface topography of particulate bone graft permits better cellular attachment, cell migration, nutrient diffusion, and blood vessel in-growth during the bone repair process (Aloy-Prosper et al., 2011).

Autografts usually contain viable osteogenic cells, bone matrix proteins, and osteoinductive factors. It is widely believed that viable cells from bone grafts can directly transform into osteoblasts or contribute to graft consolidation by releasing growth factors (Khan et al., 2005). However, the grafted bone can also become necrotic and be replaced by the recipient bone (Volkmann et al., 2007; Phemister, 2008). There are only limited data comparing cell viability and bioactivity of block bone grafts to particulate bone grafts for bone defect treatment. In our previous study, we demonstrated that more osteocytes in fine particulate bone powder grafts (300–500 μm) appeared to survive after surgery, and fine particulate bone powder grafts demonstrated better bone formation than larger bone grafts (2 mm) in a rat radial defect model (Wang et al., 2012). In this study, we evaluated the bioactivity of osteocytes in fine particulate bone powder grafts vs larger bone grafts by examining the expression levels of bone morphogenetic protein-2 (BMP-2), transforming growth factor-beta 1 (TGF-β1), alkaline phosphatase (ALP), and collagen I. Our results showed that fine particulate bone powder grafts resulted in faster and better bone repair because more bioactive osteocytes survived transplantation

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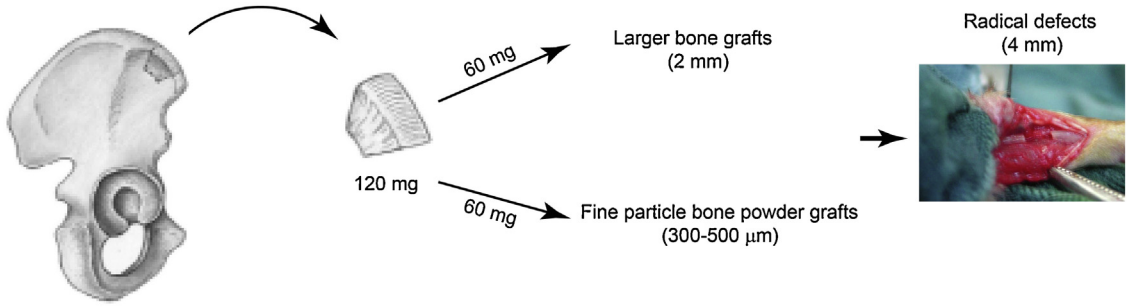

Fig. 1. Schematic diagram depicting use of bone grafts and the radial defect model.

and the osteoinductive growth factors were expressed sooner and at higher levels than in larger grafts.

Materials and methods

Animal model

One hundred and twelve male syngeneic, inbred DA (Dark Agouti) rats (body weight 180–200 g) were supplied by the Medical Experimental Animal Center of Harbin Medical University, China. Animals were housed at 22 ± 2 °C on a 12-h/12-h light–dark cycle, and fed a standard pellet lab chow. The study was approved by the ethical committee of Harbin Medical University and was performed in accordance with the Guidelines for Animal Research of Harbin Medical University.

A rat radial defect model was established bilaterally as previously described (Turgeman et al., 2001; Wang et al., 2012). Briefly, animals were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (40 mg/kg). As shown in Fig. 1, autogenous iliac bone (120 mg) was harvested from each rat. Half of the iliac bone of each animal with the periosteum and cartilage removed was made into 2 mm diameter bone grafts (larger bone graft). The remaining halves of the bones, from which the periosteum, cartilage, endosteum and bone marrow were removed, were ground into bone powder grafts of 300–500 μm in diameter, using a spherical grinding drill. Each bone graft was transplanted into a 4 mm defect in the radius. The 112 rats were divided randomly into two groups: the larger bone graft group (Group I) and the fine particulate bone powder graft group (Group II). The larger bone grafts were transplanted into the left radial defects, and the fine particulate bone powder grafts were transplanted into the right radial defects. Implant specimens were harvested at post-transplantation days 1 and 4, as well as at weeks 1, 2, 4, 6 and 10. Immunohistochemical examination and reverse transcription (RT)-PCR were performed at each time point.

Immunohistochemistry

Paraffin wax sections (3 μm) were dewaxed in xylenes and rehydrated in ethanol baths. Antigen retrieval was performed in a high-pressure cooker for 2 min. Endogenous peroxidases were blocked by incubating sections in 3% hydrogen peroxide for 20 min at room temperature. After blocking with 1% BSA, sections were incubated with anti-BMP-2 (1:100; Boster, Wuhan, China), anti-TGF-β1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ALP (1:50; Boster), or anti-collagen I (1:200; Boster) primary antibodies overnight at 4 °C, then incubated with PV6001 secondary antibody (Zhongshan Golden Bridge Biotechnology Co., Beijing, China) for 1 h at room temperature. Positive immunoreactivity was detected using DAB chromogenic substrate (Zhongshan Golden Bridge Biotechnology Co.) according to the manufacturer's instructions. Primary antibody replaced with PBS served as a negative control. Sections were counterstained with hematoxylin for 30 s, followed by rinsing 5 min in running water. Ten fields from each sample were consecutively observed for positively stained cells under a light microscope (400×). Results were semi-quantified using MMD 6.0 software (Dayueweijia, Beijing, China).

RT-PCR analysis

Total RNA was prepared from bone specimens in the defect areas, using Trizol reagent (Life Technologies, Carlsbad, CA, USA). The first cDNA strand was synthesized from 1 μg of total RNA using the SuperScript II Reverse-Transcriptase Kit (Life Technologies) in a final volume of 20 μl. The following sequences of primers were used for PCR: *BMP-2* (100 bp), forward 5′GCTGTGTCCCCACTGAGCTT3′, reverse 5′GCAACCCTCCACAACCATGT3′; *TGF-β1* (298 bp), forward 5′CTT CAGCTCCACAGAGAAGAACTGC3′, reverse 5′CACGATCATGTTGG ACAACTGCTCC3′; *ALP* (326 bp), forward 5′CAGGATTGACCACGGGCACC3′, reverse 5′GCCTGGTAGTTGTTGTGAGC3′; and *collagen I* (399 bp), forward 5′ TACCCGCCAATCGTT TCAAGTTGAG3′, reverse 5′ TAGGGTAGGGTTCCACGAAGCACTAC3′.

PCR amplifications were performed in a final volume of 20 μl containing 1 μl cDNA, 2 μl PCR buffer, 1.8 mM $MgCl_2$, 200 μM of each dNTP, 20 pmol of each primer, and 1 U of *Taq* DNA polymerase (Invitrogen). Cycling conditions (denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, and extension at 68 °C for 90 s) were repeated for 35 cycles. The PCR products were separated on 2% agarose gels and imaged using the Tanon GIS2010 gel imaging system (Tanon Science & Technology Co., Shanghai, China).

Statistical analysis

Quantitative data were presented as the mean ± SD. Differences between groups were analyzed by one-way ANOVA, followed by Bonferroni post hoc analyses as appropriate with SPSS 10.0 (SPSS, IBM, Chicago, IL, USA). A statistically significant difference was considered if P was <0.05.

Results

BMP-2 and TGF-β1 expression as detected by immunohistochemical staining

Group I **(larger bone graft)**

BMP-2 and TGF-β1 expression increased after transplantation, and the highest expression levels were seen at four weeks for both markers (Fig. 2A and B; Tables 1 and 2). From days 1 to 4 post-transplantation, most bone lacunae were empty. BMP-2 and TGF-β1 were weakly expressed in the cytoplasm of adjacent mesenchymal cells, and negatively expressed in the bone matrices. One