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ORIGINAL ARTICLE

Fate, origin and roles of cells within free bone grafts

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Abstract
Background The efficacy of autologous bone grafting in repairing nonunion fractures, large bone defects and spinal instability is widely accepted. However, the cellular and molecular mechanisms underlying new bone formation in bone grafting have yet to be fully elucidated. The purpose of this study was to clarify the fate, origin and the contribution of the cells within the grafted bone.
Methods This study was designed to investigate the role and fate of cells contained in the grafted bone and their contribution to new bone formation in the graft in an animal model. Middiaphyseal cylindrical bone samples obtained from green fluorescent protein (GFP) transgenic and wild-type rats were transplanted into the back muscle of wild-type and GFP rats, respectively. The transplanted bones were evaluated by immunohistochemistry, in situ hybridization and quantitative reverse transcription polymerase chain reaction.
Results Immunohistochemical analyses showed that all the cells in the newly formed bone originated from the grafted bone, and osteoblasts were gradually replaced by host cells. Conversely, osteoclasts were immediately replaced by host cells 2 weeks after the bone graft. In addition, expression of *bone morphogenetic protein (Bmp)-4*, *Bmp receptors* and *Noggin* in the grafted bone was significantly upregulated before new bone formation occurred, indicating that the grafted cells might contribute to the recruitment of mesenchymal cells into the graft bed.
Conclusion This study revealed the possible molecular mechanisms of the contribution of cells contained in grafted bone to facilitate new bone formation.

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Introduction

Autologous bone grafting is a standard modality to promote local osteogenesis in the treatment of nonunion fractures, spinal instability or bone defects caused by high-energy trauma or bone tumor resection. The clinical outcome of the autograft has been acceptable because it has an excellent capacity to promote local bone formation in spite of limitations that include additional surgery to procure the bone graft, donor site morbidities and limited graft mass [1]. Recently developed molecular biology techniques enable identification of cells by marking them with β-galactosidase (β-gal) or green fluorescent protein (GFP), or by detecting the Y-chromosome using a sex-mismatched model [2–4]. However, the osteogenic mechanisms driven by the autograft at the cellular or molecular level have not been fully elucidated. For instance, the fate or role of the cells

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included in the graft has not been clarified, and there has been controvery about whether the grafted cells actually survive and propagate to form new bone. If the cells do not survive, what cellular reactions work to generate new bone in the bone graft? Among the various possible molecular mechanisms underlying new bone formation and fracture healing, the contribution of bone morphogenetic proteins (BMPs) has been studied [5–7]. Based on this background information, this study was designed to address the issues regarding the fate of the cells in the bone graft, the origin of cells contributing to graft-induced new bone formation and the possible contribution of the BMP signaling system at the bone graft location.

Materials and methods

Animals

Lewis wild-type rats were purchased from Japan SLC (Shizuoka, Japan). GFP transgenic Lewis strain rats were kindly provided by PhoenixBio Co. (Tochigi, Japan) [8]. Eighty wild-type and 80 GFP transgenic male rats at 6 weeks of age were used in this series of experiments. All experiments were performed in strict accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals at Osaka City University.

Surgical procedure

Animals were anesthetized with an intraperitoneal injection of ketamine (30 mg/kg) and xylazine (10 mg/kg). Both diaphyses of the femurs of wild-type rats were surgically harvested under sterile conditions. Soft tissue, including the periosteum, was completely removed from the femurs with a scalpel. Two middiaphyseal cylindrical bone grafts of 5 mm length were cut out with a band saw and transplanted immediately into the bilateral back muscle pouches (one per pouch) of an isogenic GFP transgenic rat (wild-type to GFP series) and vice versa (GFP to wild-type series). Grafts that had been devitalized of cells using liquid nitrogen as previously reported [9, 10] were implanted as controls in the same manner as in the experimental rats.

Schedule for harvesting grafts

Host rats were euthanized at days 1, 4, 7, 14, 21, 32 and 42 after grafting by an overdose of anesthesia, and the grafts were harvested for histology and RNA extraction.

Histological sections

To prepare histological sections, the harvested bone grafts were fixed with 4 % paraformaldehyde (PFA) and demineralized with 10 % ethylenediamine tetraacetic acid (EDTA, pH 7.4) for 6 weeks at 4 °C. Specimens were dehydrated through a graded ethanol series, then embedded in paraffin, sagittally sectioned with 4 μm thickness at the middle of the graft and stained routinely with hematoxylin and eosin (H&E).

Bone histomorphometry

Twenty areas, each of 400 × 250 μm, within the newly formed bone located in both intramedullary ends in each of ten samples from every time point at the same magnification were randomly selected. Analyses were performed using OsteoMeasure (OsteoMetrics, Atlanta, GA, USA) [11].

Immunohistochemistry (IHC) and TRAP staining

After deparaffinization, IHC was performed with the usual protocols using rabbit anti-GFP antibody (1:200, Molecular Probes, Eugene, OR) and mouse monoclonal anti-human underdecarboxylated osteocalcin (OCN) antibody (1:200, Takara Bio Inc., Shiga, Japan) for mature osteoblasts. Subsequently, sections were stained with FITC-conjugated swine anti-rabbit IgG secondary antibody (DAKO, Glostrup, Denmark) and Alexa Fluor 594 goat anti-mouse IgG secondary antibody (Molecular Probes) for 2 h and then counterstained with DAPI. All pictures were taken with a Leica TCS-SP5 confocal laser microscope (Leica Microsystems, Tokyo, Japan). For osteoclasts, TRAP staining was carried out using a staining kit (Cell Garage, Tokyo, Japan) according to the manufacturer's protocol. The ratio was quantitatively calculated in 20 middle-power visual fields randomly selected from both edges of the internal graft.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared from homogenized transplanted grafts using ISOGEN. Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen). Real-time RT-PCR was performed according to the manufacturer's instructions. TaqMan probes for *Bmp-4*, *Bmpr1a*, *Bmpr2*, *Noggin* and *Gapdh* were purchased from Applied Biosystems (Foster City, CA, USA). Normalization to *Gapdh* was performed as described previously [12]. Experiments were performed on three separate test occasions ($n = 4$ for each).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical differences between two groups were analyzed