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Bone tissue formation with human mesenchymal stem cells and biphasic calcium phosphate ceramics: The local implication of osteoclasts and macrophages

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ABSTRACT

Human mesenchymal stem cells (hMSC) have immunomodulative properties and, associated with calcium phosphate (CaP) ceramics, induce bone tissue repair. However, the mechanisms of osteoinduction by hMSC with CaP are not clearly established, in particular the role of osteoclasts and macrophages. Biphasic calcium phosphate (BCP) particles were implanted with or without hMSC in the paratibial muscles of nude mice. hMSC increased osteoblastic gene expression at 1 week, the presence of macrophages at 2 and 4 weeks, osteoclastogenesis at 4 and 8 weeks, and osteogenesis at 4 and 8 weeks. hMSC disappeared from the implantation site after 2 weeks, indicating that hMSC were inducers rather than effectors of bone formation. Induced blockage of osteoclastogenesis by anti-Rankl treatment significantly impaired bone formation, revealing the pivotal role of osteoclasts in bone formation. In summary, hMSC positively influence the body foreign reaction by attracting circulating haematopoietic stem cells and inducing their differentiation into macrophages M1 and osteoclasts, thus favouring bone formation.
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1. Introduction

Autologous bone grafts are considered the gold standard in bone regeneration because of their osteogenicity, osteoinductivity, osteoconduction and osteointegration characteristics [1]. However, the bone harvesting procedure requires a second surgical site, at which complications have been reported, and the quantity of bone available for grafting is limited.

Synthetic bone substitutes, particularly calcium phosphate (CaP) ceramics, have been proven safe and biocompatible and are widely used to fill bone defects in clinical indications such as dental, maxillofacial and orthopaedic augmentation [2,3]. Despite having a similar chemical composition to bone mineral [1] and possessing osteoconductive properties, CaP ceramics lack the osteogenicity needed to support bone healing in critical size defects [4] thus limiting their clinical use to small bone defects where osteoconduction is sufficient. The use of bone marrow derived mesenchymal stem cells (MSC) in unison with synthetic biomaterial scaffolds may overcome the challenges of autologous bone grafting for the regeneration of large defects. Human MSC (hMSC) are easily isolated, expanded in culture and have the ability to differentiate into multiple lineages such as osteoblasts, chondrocytes and adipocytes [5]. *In vitro*, hMSC cultured on CaP differentiated into osteoblasts even without osteogenic supplements [6] and *in vivo* bone formation is achieved by the use of MSC with ceramic scaffolds in ectopic sites [7] and critical sized defects [8]. In addition to their regenerative properties, MSC are also known to have unique immunoregulatory properties [8,9]. It has been demonstrated that transplanted hMSC promote wound healing by recruitment of host MSC to the wound site [10] and Song and colleagues elegantly showed the homing of bone marrow MSC to ectopic sites for bone formation by using a sex-mismatched dog model [11].

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An inflammatory reaction is expected following implantation of a biomaterial such as CaP due to the host response to a foreign body. During the innate inflammatory reaction, monocytes which circulate in the blood become tissue macrophages [12]. There is evidence that MSC can regulate the macrophage M1/M2 balance to the M2, IL-10 producing anti-inflammatory phenotype [13], by secreting some M2 inducers such as interleukine-4 (IL-4), IL-13 [13], IL-6 [14], and prostaglandin E2 [15]. Bone is essential for a functioning immune system since immune cells originate in the bone marrow; however the significance of immune cells to bone tissue formation is less understood. Nonetheless, disordered bone formation occurs in autoimmune diseases and recently it was shown that ablation of macrophages inhibits intramembranous bone healing [16]. The contribution of macrophages to tissue engineered bone formation is unclear however.

While osteoclast activity is generally associated with bone resorption, it has been demonstrated *in vitro* that osteoclasts secrete mediators which induce the migration and osteogenic differentiation of MSC [17]. In addition, the Wnt/BMP signalling pathway and sphingosine-1-phosphate cytokines, secreted by osteoclasts, seem to be implicated in osteoblast precursor recruitment [18]. Furthermore, osteoclast ablation was found to inhibit ectopic bone formation by β-TCP [19]. However, the influence of transplanted MSC on osteoclast activity during *in vivo* bone formation is unknown.

In this study, the hypothesis that transplanted hMSC, associated with BCP granules, can mobilize circulating monocytes thereby favouring bone formation is tested. Specifically, we investigated the interactions of transplanted hMSC, macrophages and osteoclasts in ectopic ossification in nude mice. To further elucidate the contribution of osteoclasts to bone formation, we used an anti-mouse RANKL antibody depleting osteoclast in mice [20].

2. Materials and methods

2.1. Biomaterials

Micro-porous biphasic calcium phosphate (BCP) granules of 0.5–1 mm in size and composed of HA/β-TCP in a ratio of 20/80 in weight were used as received by the supplier (MBCP+®, Biomatlante, Vigneux de Bretagne, France). Briefly, BCP granules were prepared by mixing calcium deficient apatite with organic pore makers, followed by compaction and sintering at 1050 °C. The overall porosity (% vol) was 75 ± 5%, with a pore size distribution of 70% (0–10 μm), 20% (10–100 μm) and 10% (100–300 μm). Aliquots of 40 mg of BCP granules were prepared in Eppendorf centrifuge tubes sterilized by autoclaving at 121 °C for 20 min.

2.2. hMSC isolation and culture

The human bone marrow mesenchymal stem cells (hMSC) were kindly provided by Prof. Markus Rojewski, Institute for Clinical Transfusion Medicine, Ulm, Germany. After receiving informed consent from adult donors, a bone marrow aspiration was performed under local anaesthesia by haematologists. The cells were seeded at low density on treated culture polystyrene flasks (Corning) and cultured in α-modified Eagle's medium (αMEM, LONZA), supplemented with 10% foetal bovine serum (FBS, Lonza), 100 UI/ml penicillin, 100 μg/ml streptomycin. hMSC were isolated by plastic adherence, amplified in culture until passage 4 in a humidified atmosphere at 37 °C and 5% CO_2. hMSC were characterized by flow cytometry as being positive for CD73, CD90, CD105 and negative for CD45. Multipotency was demonstrated by culturing cells in adipogenic, chondrocytic and osteogenic conditions, as previously described [21]. For the preparation of cell-loaded implants, 1.4×10^6 viable hMSC suspended in 70 μL PBS were added to 40 mg sterile BCP particles. The average time between cell seeding and surgery was approximately 1 h.

2.3. In vivo experimental design

All animal handling and surgical procedures were conducted according to European Community Guidelines (2010/63/EU) for the care and use of laboratory animals. An animal experimentation protocol was prepared, submitted and approved by the local Ethic Committee (CEEA.2012.27).

In order to evaluate the influence of transplanted hMSC on bone formation, *Adult nude NMRI Nu/Nu* female mice (4 weeks old, body weight 20 g) were purchased from a professional stock breeder (Janvier Labs, France). The nude mice were kept in Hepa filtered closets with water and food *ad libitum* and were quarantined for a minimum of 10 days prior to surgery. The mice were randomly and equally divided into 2 groups: BCP particles alone (40 mg) and BCP + hMSC (40 mg and 1.4×10^6 viable cells) that were bilaterally implanted in paratibial muscles. Five mice from each treatment group ($n = 5$) were followed for 1, 2, 4 or 8 weeks after surgery. Animals were placed under general anaesthesia by inhalation of isoflurane (2.5% Flucka, 1 L/min) and an intramuscular injection of the analgesic Buprenorphine (Buprecare 60 μL/kg, Axience) was performed 30 min before surgery. A skin incision of 0.5 cm was made for exposing the muscle, and fibres were taken away to create a pocket where BCP granules were inserted. Skin incisions were closed with sutures (Filapeau 4/0, Peters). The mice were euthanized under general anaesthesia by cervical dislocation.

To determine the impact of osteoclastogenesis on bone formation initiated by hMSC, 8 weeks experiments were reproduced in nude mice which received injections of mAb anti-RANKL. RANKL is a key factor for osteoclast differentiation and activation. Anti-RANKL is known to drastically inhibit the formation of TRAP positive multinucleated cells *in vitro* and *in vivo* [20]. *Adult nude NMRI Nu/Nu* female mice ($n = 4$) were implanted with BCP alone (40 mg on left leg) and BCP + hMSC (40 mg and 1.4×10^6 viable cells, on right leg) and treated with a subcutaneous injection (50 μg dose) of antibody anti-mouse RANKL, kindly provided by Prof. Hideo Yagita, Department of Immunology, Juntendo University School of Medicine, Tokyo, three days before implantation. This same injection regime was repeated twice a week and animals were sacrificed after 8 weeks under general anaesthesia by cervical dislocation. Implants were sectioned after euthanasia to perform histology and RNA extraction.

2.4. Histological analysis

The muscles were excised, fixed in 4% paraformaldehyde, decalcified in a PBS solution with 0.5% paraformaldehyde 4.13% EDTA in a microwave decalcifying automat (KOS Histostation) at 46 °C. Samples were then embedded in paraffin and 3 μm thick sections were cut. Three sections through each implant were attained for histological analysis. The sections were stained with Masson's Trichrome and qualitative/quantitative histological evaluations of soft-tissue in-growth and bone formation were performed. Masson's trichrome technique combines hematoxylin for cell nuclei (blue/black), fuchsine for cytoplasm, muscle and erythrocytes (red) and light green solution for collagen (green). The quantity of new bone was measured in separate sections with the software ImageJ. The bone formation was evaluated according to:

Newly formed bone = (bone surface) * 100/[(total implant surface) − (BCP granules surface)]

Tartrate resistant acide phosphatase (TRAP) is highly expressed by osteoclasts and therefore can be used to stain these cells in histological sections. The sections were stained using a commercial TRAP staining kit (Acid Phosphatase Leukocyte Staining Kit, Sigma) following the manufacturer's instructions. Briefly, the staining solution was prepared with Fast Red TR salt (3.9 mM), naphthol AS-TR phosphate disodium salt (2.3 mM), N–N dimethylformamide (68 μM), and L(+)-tartaric acid (100 mM) all diluted in sodium acetate buffer (0.1 M, pH 5.2). Deparaffinised sections were incubated in the solution for 90 min at 37 °C and then counterstained with Mayer's hematoxylin. TRAP-positive stained cells appeared red. Qualitative/quantitative histological evaluations of osteoclasts were then performed.

2.5. In situ hybridization

In situ hybridization using the human-specific repetitive Alu sequence, which comprises approximately 5% of the total human genome was performed for identification of human cells. Sections were deparaffinized and rehydrated in graded series of ethanol and washed with tris buffered saline (TBS) tween 0.05% pH 7.6 three times for 5 min each under gently agitation. Slides were treated with 3% H_2O_2 for 15 min at room temperature (RT) to block endogenous peroxidase activity, followed by three washes with TBS tween 0.05%. Sections were then treated with 10 μg/mL proteinase K (P2308, Sigma Aldrich, France) for 10 min at 37 °C. After a further three washes for 5 min each in TBS tween 0.05%, sections were treated with 0.25% acetic acid in 0.1 M triethanolamine (TEA) pH 8.0 for 20 min at RT under agitation. Pre-hybridization was performed for 3 h at 56 °C in a hybridization buffer containing 4 × SSC (S6639, Sigma Aldrich France), 50% deionized formamide, 1 × Denhardt's solution, 5% dextran sulphate and 100 μg/mL salmon sperm DNA and molecular grade H_2O. Hybridization buffer was replaced by fresh hybridization buffer containing 70 nM DIG-labeled human locked nucleic acid (LNA) Alu probe 5DigN/5′-TCTCGATCTCCTGACCTCATGA-3′/3DigN (Exiqon, Vedbaek, Denmark) and then target DNA and the probe were denatured for 5 min at 95 °C. Hybridization was carried out for 40 h at 56 °C in a wet chamber. Sections were washed twice in 2 × SSC, then twice in 0.5 × SSC at 56 °C for 10 min each time, followed by three washes in TBS tween 0.05%. Finally, the hybridized probe was detected by immunohistochemistry using biotin-SP-conjugated IgG fraction monoclonal mouse anti-digoxin (Jackson Immunoresearch, Baltimore, USA) diluted 1/200 in PBS for 45 min at 37 °C. After three washes in TBS tween 0.05% at RT, streptaperoxidase was added (1/200 in TBS tween 0.05%) for 45 min at 37 °C before diaminobenzidine (DAB) substrate addition (Dako). Sections were counterstained with Gill-2 hematoxylin (Thermo Shandon Ltd, Runcorn, UK) dehydrated and mounted using Pertex (HistoLab Products AB, Sweden).

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