Materials Science and Engineering C 52 (2015) 212–218

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Resorption of monetite calcium phosphate cement by mouse bone marrow derived osteoclasts

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ARTICLE INFO

Article history:
Received 25 September 2014
Received in revised form 10 February 2015
Accepted 22 March 2015
Available online 24 March 2015

Keywords:
Bone filler
Acidic calcium phosphate cement
Bone marrow macrophages
Osteoclastic resorption

ABSTRACT

Recently the interest for monetite based biomaterials as bone grafts has increased; since *in vivo* studies have demonstrated that they are degradable, osteoconductive and improve bone healing. So far osteoclastic resorption of monetite has received little attention. The current study focuses on the osteoclastic resorption of monetite cement using primary mouse bone marrow macrophages, which have the potential to differentiate into resorbing osteoclasts when treated with receptor activator NF-κB ligand (RANKL). The osteoclast viability and differentiation were analysed on monetite cement and compared to cortical bovine bone discs. After seven days live/dead stain results showed no significant difference in viability between the two materials. However, the differentiation was significantly higher on the bone discs, as shown by tartrate resistant acid phosphatase (TRAP) activity and Cathepsin K gene expression. Moreover monetite samples with differentiated osteoclasts had a 1.4 fold elevated calcium ion concentration in their culture media compared to monetite samples with undifferentiated cells. This indicates active resorption of monetite in the presence of osteoclasts. In conclusion, this study suggests that osteoclasts have a crucial role in the resorption of monetite based biomaterials. It also provides a useful model for studying *in vitro* resorption of acidic calcium phosphate cements by primary murine cells.

1. Introduction

Bone has the ability to repair itself following skeletal fracture or surgery; however, in cases where the defect exceeds a critical size it needs to be filled with a bone graft, to prevent fibrous tissue ingrowth [1]. The gold standard today is to use autologous bone grafts, where bone tissue is harvested from another part of the patient's body and placed into the defect. This alternative has several drawbacks such as the need for additional surgery, limited quantities available and donor site morbidity [2,3]. Among the synthetic bone grafts available, calcium phosphate cements have attracted considerable interest since their discovery in the 1980s. Calcium phosphate cements typically consist of one or more calcium orthophosphate powders, which upon mixing with an aqueous liquid form a paste that within minutes hardens to either hydroxyapatite or acidic cement phases. The end product that is formed is dependent on the initial powders and the pH of the paste used. The advantage of acidic cements is their fast degradation, which allows for rapid bone replacement [4]. The acidic cements can be further divided into brushite (dicalcium phosphate dihydrate, DCPD) and monetite (dicalcium phosphate anhydrous, DCPA) [5]. Recently, special attention has been paid to the monetite based materials. Several *in vivo* and *in vitro* studies have confirmed their osteoconductive properties and some even claim that monetite has superior properties over brushite [6–9]. Although monetite is the more stable of the two phases, brushite is normally formed during cement reactions [10]. Monetite can, however, be obtained by controlling the conditions during cement setting. Conditions favouring monetite formation include high acidity, low amounts of the aqueous reactant during setting and elevated temperatures [5,11]. To achieve a milieu favouring monetite formation, premixed cements have been tested, indeed showing a high content of monetite [11,12]. In the pre-mixed cement used in this study, the aqueous part is exchanged with glycerol, hence resulting in a paste that will not start the setting reaction until immersed in an aqueous solution, such as water or body fluids. Upon immersion, the glycerol content will be replaced by the aqueous solution through diffusion and the setting reaction can start [12,13]. The pre-mixed cement described has thus monetite as the final phase and a paste with extended working time compared to the conventional cements.

The degradation of monetite has been suggested to follow three different routes, namely, chemical dissolution, macrophages engulfing cement debris and osteoclast mediated resorption [5,14]. So far osteoclastic resorption of monetite has received little attention and to our knowledge only one *in vitro* study exists, concerning the resorption of monetite by the monocyte/macrophage cell line RAW 264.7 [14]. This cell line is known to have limited resorption capabilities [15], thus, perhaps underestimating the osteoclastic resorption of monetite. There are also *in vivo* studies by Tamimi et al. [7,8] that demonstrate signs of

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http://dx.doi.org/10.1016/j.msec.2015.03.038
0928-4931/

"pitting resorption", which might be an indication of osteoclastic activity although it is not clear. The current study therefore aims to focus on osteoclastic resorption of monetite cement, using primary murine cells.

Osteoclasts are multinucleated cells, derived from the monocyte/macrophage cell lineage, that have the ability to resorb mineralized tissue. The mature osteoclast is tightly attached to the bone surface and resorbs the material beneath by dissolving the inorganic calcium phosphate, through the acidic milieu created between the cell and the bone matrix, followed by enzymatic degradation of proteins [16]. There are two essential signalling factors, cytokines, needed for the proliferation and differentiation of precursor cells to mature osteoclasts, the macrophage colony-stimulating factor (M-CSF) and the receptor activator NF-κB ligand (RANKL). While M-CSF is an important factor for the proliferation of osteoclast precursor cells, the RANKL is the signalling molecule that starts the differentiation [17,18].

In this study primary osteoclasts derived from mouse bone marrow macrophages were used to evaluate their proliferation and differentiation on monetite samples. Bovine bone discs were included for comparison. Furthermore, osteoclastic resorption was analysed by measuring calcium ion level in culture media and images of cells were taken by scanning electron microscopy.

2. Materials and methods

2.1. Preparation of cement samples

A pre-mixed calcium phosphate cement that sets into monetite was used in this study, as previously described by Aberg et al. [12]. In brief, the powder phase of the cement consists of β-tricalcium phosphate (β-TCP; Sigma Aldrich, Steinheim, Germany) and monocalcium phosphate anhydrous (MCPA), with a particle size of >100 μm. MCPA was obtained by heating monocalcium phosphate hydrate (Alfa Aesar, Karlsruhe, Germany) for 24 h at 100 °C and then sieving the powder in order to remove particles smaller than 100 μm. Both powders were sterilised by heat treatment prior to use, β-TCP at 160 °C for 2 h and MCPA at 120 °C for 24 h. After sterilisation all procedures were performed in a sterile milieu. First the β-TCP was mixed with glycerol (American Bioanalytical, Massachusetts, USA) in a vacuum mixer (Renfert, Hilzingen, Germany) at 400 rpm for 40 s. Subsequently the MCPA powder was added and the mixing procedure was repeated. The powders were mixed with a molar ratio of 1:1 and the amount of glycerol was such that the powder to liquid ratio was 4 g/ml. The paste obtained was moulded into silicon moulds, 6 mm in diameter and 1 mm thick which were set at 37 °C in 80% relative humidity for 72 h. In this milieu the water vapour reacts with the cement paste which sets into monetite. The solidified cement discs were removed from moulds, placed in 96 well plates and washed with phosphate buffered saline (PBS; Sigma Aldrich, Steinheim, Germany) for 72 h, changing the PBS every 24th hour, to remove unreacted MCPA and to neutralise the pH. The bovine bone discs (fixed bovine bone cut into discs and stored in ethanol) were purchased from Immunodiagnostic Systems Nordic, Denmark.

2.2. Phase composition

X-ray diffraction patterns of the monetite cement samples were obtained with a diffractometer (Siemens D5000, Germany) using Cu-Kα at an angular sweeping rate of 0.02°/s. X-ray diffraction patterns were recorded for both samples that were washed in PBS for 72 h and for samples that had been incubated in cell media for over two weeks.

2.3. Isolation and culture of mouse bone marrow cells

This study was carried out in strict accordance with the recommendations in the guide for "Care and Use of Laboratory Animals of Sweden". The protocol was approved by the Committee of the Ethics of Animal Experiments at Uppsala University (Permit Number: C346/11). The bone marrow macrophages were isolated from mice and used as precursors for primary osteoclast *in vitro* differentiation [19,20]. In brief, femur and tibia from 8 day old C57/B6 mice were dissected free of adhering tissue. Bone ends were removed and the marrow cavity was flushed with Minimum Essential Medium (α-MEM; Hyclone, Utah, USA) with a sterile needle, 23 G. After gentle centrifugation for 5 min (300 ×*g*) the red blood cells were lysed with RBC lysis solution (Miltenyi Biotech, Bergisch Gladbach, Germany). The remaining cells were resuspended in complete α-MEM supplemented with 1% Pen/Strep (PAA, Pasching, Austria) and 10% heat-inactivated FBS (Hyclone, Northumberland, UK) and seeded in non-treated Petri dishes (BD Biosciences, San Jose, USA) at a density of 2×10^5 cells/cm^2. Cells were grown in 100 ng/ml M-CSF (R&D systems, Minneapolis, USA) for 3 days before non-adherent cells were washed away with PBS and discarded. The remaining, adherent cells were detached with ice-cold 0.02% EDTA/PBS for 15 min before seeding onto bovine bone and monetite discs in 96-well plates. Prior to seeding, bone and monetite discs were pre-incubated with complete media for 30 min. Cells were then seeded with a density of approximately 10^5 cells/cm^2 and incubated in complete media without cytokines for 1 h. The media were subsequently changed to remove cells that did not attach [21] and replaced by media with cytokines, 100 ng/ml M-CSF with or without 50 ng/ml RANKL (R&D Systems, Minneapolis, USA). Cells treated only with M-CSF were used as a negative control for osteoclast differentiation. Media were changed twice a week and collected for TRAP and calcium concentration measurements.

2.4. Cell viability

The viability of cells cultured on bone and monetite discs, was analysed after 1 day and 7 days using a live/dead staining kit (Life Technologies, USA). Briefly, the specimens were rinsed twice in PBS followed by 20 min of incubation in PBS containing 2 μM Calcein-AM and 1 μM Propidium Iodide. The cells were then visualised using a fluorescent microscope (Eclipse TE2000-U, Nikon, Japan). Three images from different areas of each sample were selected and the number of viable cells in each area was quantified using the free software ImageJ version 1.45 s.

2.5. Tartrate-resistant acid phosphatase (TRAP) staining and activity

TRAP staining was used as a marker to identify multinucleated, differentiated osteoclasts. The cell media from cultures on bone and monetite discs were collected and stored at −20 °C, for later use. Cells were rinsed with PBS and fixed in 4% formaldehyde at 37 °C for a few minutes. The discs were rinsed again with PBS and then stained for TRAP using a leukocyte acid phosphatase kit (Sigma Aldrich, Runnymede Malthouse, UK). To visualise multinucleated cells, the samples were also counterstained with 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI), which stains the cell nuclei (Sigma Aldrich, St. Louis, USA). A portable Dino-lite microscope (AD7013MZT, Dino-Lite microscope, USA) was used to take the overview pictures and a Nikon microscope (Eclipse TE2000-U, Nikon, Japan) to obtain the higher magnification images. For quantification of TRAP activity in the medium, an assay was performed as previously described by Ek-Rylander et al. [22]. Briefly, medium samples were mixed with TRAP activity mix in each well of a 96-well plate and incubated for 1 h at 37 °C. The reaction was stopped with 0.3 M NaOH and absorbance was read at 405 nm. To ensure that the measured activity was from TRAP, molybdate was used to block TRAP activity.

2.6. Quantitative RT-PCR analyses

Total RNA was extracted using TRI-Reagent (Sigma Aldrich, St. Louis, USA). Five hundred nanograms of total RNA was transcribed to cDNA using the TaqMan system (Applied Biosystems, USA). Quantitative real