Acta Biomaterialia 10 (2014) 3254–3263

Contents lists available at ScienceDirect

Acta Biomaterialia

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

The size of surface microstructures as an osteogenic factor in calcium phosphate ceramics

Jingwei Zhang [a,b], Xiaoman Luo [c,d], Davide Barbieri [d], Ana M.C. Barradas [e], Joost D. de Bruijn [c,d,f], Clemens A. van Blitterswijk [a], Huipin Yuan [a,d,*]

[a] Department of Tissue Regeneration, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, P.O. Box 217, 7500AE Enschede, The Netherlands
[b] Key Laboratory of Advanced Technologies of Materials, Ministry of Education, School of Materials Science and Engineering, Southwest Jiaotong University, Chengdu, People's Republic of China
[c] Department of Biomaterials Science and Technology, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, P.O. Box 217, 7500AE Enschede, The Netherlands
[d] Xpand Biotechnology BV, Bilthoven, The Netherlands
[e] Department of Medical Cell Biophysics, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, P.O. Box 217, 7500AE Enschede, The Netherlands
[f] School of Engineering and Materials Science (SEMS), Queen Mary University of London, London E1 4NS, UK

ARTICLE INFO

Article history:
Received 4 November 2013
Received in revised form 13 March 2014
Accepted 20 March 2014
Available online 27 March 2014

Keywords:
Calcium phosphate
Microstructure
Surface topography
Mesenchymal stem cell
Osteogenesis

ABSTRACT

The microporosity of calcium phosphate (CaP) ceramics has been shown to have an essential role in osteoinduction by CaP ceramics after ectopic implantation. Here we show that it is not the microporosity but the size of surface microstructural features that is the most likely osteogenic factor. Two tricalcium phosphate (TCP) ceramics, namely TCP-S and TCP-B, were fabricated with equivalent chemistry and similar microporosity but different sizes of surface microstructural features. TCP-S has a grain size of 0.99 ± 0.20 μm and a micropore size of 0.65 ± 0.25 μm, while TCP-B displays a grain size of 3.08 ± 0.52 μm and a micropore size of 1.58 ± 0.65 μm. In vitro, both cell proliferation and osteogenic differentiation were significantly enhanced when human bone marrow stromal cells were cultured on TCP-S without any osteogenic growth factors, compared to TCP-B ceramic granules. The possible involvement of direct contact between cells and the TCP ceramic surface in osteogenic differentiation is also shown with a trans-well culture model. When the ceramic granules were implanted in paraspinal muscle of dogs for 12 weeks, abundant bone was formed in TCP-S (21 ± 10% bone in the available space), whereas no bone was formed in any of the TCP-B implants. The current in vitro and in vivo data reveal that the readily controllable cue, i.e. the size of the surface microstructure, could be sufficient to induce osteogenic differentiation of mesenchymal stem cells, ultimately leading to ectopic bone formation in calcium phosphate ceramics.

1. Introduction

The need for reconstruction of massive and segmental bone defects frequently arises from skeletal disease, congenital malformations, infection, trauma and post-cancer ablative surgery [1]. Currently, autografts, allografts and synthetic materials are considered for bone regeneration [2–4]. For decades, autogenous bone graft has been the gold standard for bone repair; however, the amount of harvestable autografts is limited. Allografts, which provide much larger amounts of tissue than autografts, may carry the unpredictable risks of immunological reaction and disease transmission [5]. Therefore, increasing attention has been paid to developing synthetic materials with similar performances to autologous bone.

Calcium phosphate (CaP) ceramics are nowadays widely used as bone graft substitutes because their chemical composition resembles that of bone mineral and they have excellent biocompatibility and bioactivity [6–8]. The repair of non-critical-sized bone defects can be achieved with osteoconductive CaP ceramics, which can lead to bone ingrowth from the surrounding host bone bed. However, to repair critical-sized bone defects, osteoinductive materials that could induce mesenchymal stem cells (MSCs) to form bone (i.e. after an ectopic implantation) are necessary. A specific group of CaP ceramics has been reported to be osteoinductive in several animal models after heterotopic/ectopic implantation of

* Corresponding author at: Xpand Biotechnology BV, Professor Bronkhorstlaan 10-d, 3723 MB Bilthoven, The Netherlands. Tel.: +31 30 2297292; fax: +31 30 229 7299.
E-mail address: h.yuan@utwente.nl (H. Yuan).

http://dx.doi.org/10.1016/j.actbio.2014.03.021
1742-7061/

the materials (e.g. subcutaneously or intramuscularly) [9]. It has also been shown that materials with higher osteoinductive potential could heal non-critical-sized defects faster [10,11]. Furthermore, osteoinductive CaP ceramics could perform similarly to autologous bone grafts and recombinant human bone morphogenetic protein 2 in repairing critical-sized bone defects [12].

Osteoinduction of CaP ceramics is material dependent. Of the factors involved in osteoinduction, chemical composition [11–13], macrostructural properties [9,14] and microporosity (i.e. the volume percentage of pores smaller than 10 μm) [15–17], microporosity has been shown to be of great importance. Previous studies have shown that microporous hydroxyapatite (HA) ceramics could induce bone formation after subcutaneous [18] and intramuscular [19] implantations in dogs, while no bone was formed in those materials lacking microporosity. It has been also reported that the osteoinductive potential of CaP ceramics increased with increasing microporosity [12,20].

It has been suggested, but not demonstrated, that the presence of micropores in CaP ceramics increases the surface area, thereby enhancing the local concentration of growth factors, including osteoinductive bone morphogenetic proteins (BMPs), which may induce osteogenic differentiation of MSCs to form bone [12,19–21]. If the surface area, and thus protein adsorption, plays a role in osteoinduction by CaP ceramics, changing the microstructural properties by altering the size of the micropores would change their osteoinductive potential.

Besides the possible role of protein adsorption in osteogenic differentiation of MSCs, an increasing number of studies have shown that MSCs could respond to extracellular matrix signals via topographical cues and tend towards osteogenic differentiation [22–24]. The change of microstructural features' dimensions alters the surface properties, which, at a certain scale and with specific topographies, may initiate the osteogenic differentiation of stem cells [25]. In addition, the possible role of ion release from CaP ceramics in osteogenic differentiation of MSCs cannot be ignored. The resulting change in the surface area will affect ion exchanges (e.g. calcium ion) with the surroundings, ultimately influencing the osteogenic response of the cells [26].

Taking together the possible roles of protein adsorption, surface topography and ion exchange in osteogenic differentiation of MSCs, we hypothesized that altering the surface microstructure size will influence the osteoinduction potential of CaP ceramics. To test this hypothesis, we prepared two tricalcium phosphate ceramics (TCP-S and TCP-B) with similar microporosity but with various micropore sizes and evaluated their osteoinductive potential in vitro with human bone marrow stromal cells (hBMSCs) and in vivo in a canine ectopic model.

2. Materials and methods

2.1. CaP ceramics

2.1.1. Preparation of TCP ceramics with various microstructure dimensions

CaP powders were synthesized by mixing calcium hydroxide (Fluka) and phosphoric acid (Fluka) at a Ca/P ratio of 1.50. TCP powders allowing large (TCP-B) or small (TCP-S) grains in the final ceramics were prepared by controlling the reaction rates. H_3PO_4 solution was directly poured into a $Ca(OH)_2$ suspension to get TCP-B, while H_3PO_4 solution was added dropwise to a $Ca(OH)_2$ suspension to get TCP-S. The powders were then foamed with diluted H_2O_2 (1%) and porogen (i.e. wax particles) to obtain porous green bodies. TCP-S ceramic was obtained by sintering the porous green bodies at 1050 °C for 8 h as the target, while TCP-B porous green bodies were sintered at different temperatures (1050, 1100 and 1150 °C) for 8 h in order to get target TCP-B ceramic having porosities (both total porosity and microporosity) close to those of TCP-S, as measured by mercury intrusion porosimetry (Auto Pore IV 9500, Micromeritics, Monchengladbach, Germany).

TCP-S and TCP-B granules, 1–2 mm in size, were prepared from the sintered porous ceramic bodies and ultrasonically cleaned, first with acetone, then with 70% ethanol and finally with demineralized water. The granules were then dried at 80 °C and finally sterilized with gamma irradiation (25 kGy, Isotron, Netherland BV, Ede, The Netherlands).

2.1.2. Physico-chemical properties of TCP ceramics

The chemical composition and crystal structure of the TCP granules were determined by X-ray diffraction (XRD; Rigaku, Tokyo, Japan). Grain and micropore size were studied with an environmental scanning electron microscope (XL30, ESEMFEG, Philips, Eindhoven, The Netherlands) in the secondary electron mode and quantified with ImageJ (v1.43u, NIH, USA). Grains and micropores were randomly selected from an image and the vertical lengths crossing the centre of each grain and micropore were considered to be the grain and pore size, respectively. Data of total porosity (i.e. the volume percentage of pores), microporosity and micropore size distribution of the porous ceramics were obtained by mercury intrusion. The specific surface area was analyzed with the Brunauer–Emmett–Teller method (BET; ChemBET monopoint Physisorption, QuanTachrome GmbH, Germany).

2.1.3. Protein adsorption of the ceramics

To demonstrate the influence of microstructure size on the protein adsorption, 100 mg of TCP-S and TCP-B granules was immersed in 2 ml of basic culture medium (BM), consisting of minimal essential medium-alpha (α-MEM; Gibco) supplemented with 10% fetal bovine serum (Lonza), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Gibco), 2 mM L-glutamine (Gibco) and 0.2 mM L-ascorbic acid 2-phosphate (Sigma–Aldrich) and incubated at 37 °C in a humid atmosphere with 5% CO_2 for 1 and 4 days. After washing three times with PBS, 0.2 ml of RIPA buffer (Thermo scientific) was added to each sample and kept at 4 °C for 10 min. Thereafter the amount of protein detached from the ceramics into the RIPA buffer was measured with the QuantiPro™ BCA Assay Kit (Sigma, The Netherlands) following the manufacturer's instructions and fluorescence was measured with a spectrophotometer (Anthos Zenyth 3100, Anthos Labtec Instruments GmbH, Salzburg, Austria) at 620 nm. A calibration curve was prepared using standard BSA solutions. Three samples were used per material ($n = 3$) and the protein content was expressed as mean ± SD.

2.1.4. Ion release from the ceramics

To evaluate the influence of microstructure size on the ion release, 100 mg of the TCP granules was immersed in 2 ml of BM and incubated at 37 °C in a humid atmosphere with 5% CO_2 for 1, 4, 7 and 14 days. Calcium concentration in the culture medium was measured with a QuantiChrom™ Calcium assay kit (BioAssay, USA) and compared to the control (i.e. BM without samples) following the manufacturer's guidelines. Absorbance measurements were performed with a spectrophotometer at 620 nm. The calcium concentration was expressed as mean ± SD, measured through standard calibration curves. Three samples were used per material ($n = 3$). Meanwhile the ceramic particles were collected and subjected to XRD analysis and scanning electron microscopy (SEM) evaluation of possible surface changes.

2.2. Cell culture

2.2.1. Isolation and expansion of hBMSCs

Bone marrow aspirates (5–20 ml) were obtained from a donor (69 years old, female) with written informed consent. In brief,