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Calcium phosphate phases integrated in silica/collagen nanocomposite xerogels enhance the bioactivity and ultimately manipulate the osteoblast/osteoclast ratio in a human co-culture model

S. Heinemann [a,*], C. Heinemann [a], S. Wenisch [b], V. Alt [c], H. Worch [a], T. Hanke [a]

[a] Max Bergmann Center of Biomaterials and Institute of Materials Science, Technische Universität Dresden, Budapester Str. 27, D-01069 Dresden, Germany
[b] Institute of Veterinary Anatomy, University of Giessen, Frankfurter Str. 98, D-35392 Giessen, Germany
[c] Department of Trauma Surgery, University of Giessen, Rudolf-Buchheim-Str. 7, D-35385 Giessen, Germany

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ABSTRACT

A human co-culture model of osteoblasts and osteoclasts, derived from bone marrow stromal cells and monocytes respectively, was used to characterize the influence of biomaterial modification on the bioactivity and ultimately the ratio of bone-forming to bone-resorbing cells cultivated directly on the surface. Nanocomposites of silica and collagen have been shown to function as skeletal structures in nature and were reproduced in vitro by using a sol–gel approach. The resulting xerogels exhibit a number of features that make it a valuable system for the development of innovative materials for bone substitution applications. In the present study, the incorporation of different calcium phosphate phases in silica/collagen-based gels was demonstrated to enhance the bioactivity of these samples. This ability of the biomaterial to precipitate calcium phosphate on the surface when incubated in simulated body fluids or cell culture medium is generally considered to an advantageous property for bone substitution materials. By co-cultivating human osteoblasts and osteoclasts up to 42 days on the xerogels, we demonstrate that the long-term ratio of these cell types depends on the level of bioactivity of the substrate samples. Biphasic silica/collagen xerogels exhibited comparably low bioactivity but encouraged proliferation of osteoblasts in comparison to osteoclast formation. A balanced ratio of both cell types was detected for moderately bioactive triphasic xerogels with 5% calcium phosphate. However, enhancing the bioactivity of the xerogel samples by increasing the calcium phosphate phase percentage to 20% resulted in a diminished number of osteoblasts in favor of osteoclast formation. Quantitative evaluation was carried out by biochemical methods (calcium, DNA, ALP, TRAP 5b) as well as RT-PCR (ALP, BSP II, OC, RANKL, TRAP, CALCR, VTNR, CTSK), and was supported by confocal laser scanning microscopy (cell nuclei, actin, CD68, TRAP) as well as scanning electron microscopy.

1. Introduction

Bone is a natural composite with outstanding material properties and therefore serves as a model for bone replacement materials. Natural bone mainly consists of collagen fibrils mineralized by hydroxyapatite (HAp)-like calcium phosphate phases (CPP). Therefore, besides the well-established calcium phosphate ceramics, several attempts to develop inorganic/organic composites based on collagen and different calcium phosphate phases have been undertaken [1–3]. Some years ago, silica turned out to be an alternative inorganic phase, mostly combined with CPP [4,5], but also with collagen [6–8]. In particular, the combination of sol–gel silica, fibrillar collagen and CPP was identified as useful for the fabrication of monolithic composite xerogels as novel load-bearing bone substitution materials with adjustable mechanical properties and good biocompatibility towards osteoblasts and osteoclasts [7,9,10]. Furthermore, the first in vivo results of this material implanted into 5 mm full-size defects of rat femurs have been published recently [11].

The addition of CPP to xerogels is not crucial for the xerogel's formation and mechanical properties. This finding facilitates the application of CPP-like HAp predominantly as an agent that controls the bioactivity of the material. High bioactivity, equivalent to the calcium phosphate binding capability, leads to calcium depletion in close vicinity to the biomaterials. Local decrease in calcium ion concentration can affect the viability of osteoblasts [12], whereas an increase in calcium ion concentration stimulates migration, proliferation and differentiation of osteoblasts [13,14]. Increased extracellular calcium ion concentration has repeatedly been reported to trigger the inhibition of bone resorption due to osteoclast dysfunction and apoptosis [15,16].

* Corresponding author. Tel.: +49 351 463 39381; fax: +49 351 463 39401.
E-mail address: sascha.heinemann@tu-dresden.de (S. Heinemann).

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Regarding these calcium ion-mediated cellular functions of bone cells, it can be concluded that extracellular calcium mediates in large part the cellular cross talk between osteoblasts and osteoclasts, and is responsible for the ratio between osteoblasts and osteoclasts localized within a bone multicellular unit. The calcium-sensing receptor has been regarded as a central mediator of cellular functions triggered by varying the extracellular calcium concentration, and is even described as a potential therapeutic target in osteoporosis [17]. In contrast, others favor the role of type L voltage-gated calcium channels in mediating cellular functions in response to extracellular calcium [14]. Regardless of the mechanisms that are involved in mediating the signal pathways of bone cells, the prominent role of calcium ions in regulating remodeling is always obvious.

There is still ambiguity in predicting the in vivo response of composites due to the complexity of interactions between osteoblasts and osteoclasts and their responses to fluctuating local levels of calcium ions caused by highly bioactive CPP as a component of a biomaterial. Hitherto, most of the in vitro biocompatibility testing has relied on mono-culture systems, and the osteoclast response in particular has been ignored completely during in vitro biocompatibility tests. Concerning this issue, we recently reported on a human co-culture model comprising both osteoclasts and osteoblasts which is intended to provide a more accurate picture of what is likely to take place in vivo [18].

The regulation of osteoclastic and osteoblastic activity by means of tailored biomaterials characterized by controlled interaction with extracellular calcium ions offers new therapeutic approaches to treat bone defects and fractures occurring in systemically altered bone, such as osteoporosis. To date, to the best of our knowledge, no biomaterials concepts have been published that would take advantage of these processes.

Based on our previous studies, comprising, on the one hand, xerogels with different CPP contents and different bioactivities and, on the other hand, the human co-culture model, in this study we have investigated the ability of the xerogel to manipulate the ratio of osteoblasts to osteoclasts by controlling the extracellular calcium concentration via its bioactivity. In conclusion, we consider the impact of our findings on the general role of the bioactivity properties of bone substitution materials.

2. Materials and methods

2.1. Preparation of nanocomposites xerogels

Homogeneous suspensions of 30 mg ml^{-1} fibrillar bovine collagen type I (GfN, Germany) were prepared by dialysis of tropocollagen (MWCO 12-14 kDa, Roth, Germany) against deionized water, fibrillation in neutral sodium phosphate buffer solution (30 mM), lyophilization (Christ Alpha 1-4 laboratory freeze-dryer, Germany) and resuspension in 0.1 M Tris–HCl, pH 7.4 (Roth), as described previously [10]. For the preparation of triphasic xerogels, calculated amounts of HAp (No. 102143; Merck, Germany) powder or calcium phosphate cement (CPC) powder (InnoTERE, Germany) were added to the fibrillar collagen suspension. The CPC powder was similar to Biocement D of Khairoun et al. [19], which mainly consists of alpha-tricalcium phosphate and calcium hydrogen phosphate, and finally converts to carbonated hydroxyapatite in a surplus of aqueous liquid within a few days. Silicic acid served as the silica component in the xerogels and was prepared by hydrolysis of tetraethoxysilane (TEOS, 99%, Sigma, Germany; molar ratio TEOS/water = 1/4) under acidic conditions (0.01 M HCl). For the preparation of 800 µl hydrogels, calculated volumes of silicic acid and collagen suspension with or without CPP were mixed in a mold using a vortexer to obtain the final weight ratios listed in Table 1. After gel stabilization for 3 days, gel drying was carried out for 7 days in an Espec SH-221 climate chamber (Japan) at 37 °C and 95% relative humidity. The drying procedure was finished by applying a climate ramp to achieve ambient conditions. The disc-like composite xerogels (diameter: 5 mm, height: 3 mm) obtained were used directly or were gamma-sterilized at 25 kGy for cell culture experiments.

Table 1
Sample labels and xerogel compositions, expressed by final weight ratios of silica, fibrillar bovine collagen (B), HAp (H), and CPC (C).

Label	w_{silica} (wt.%)	$w_{collagen}$ (wt.%)	w_{HAp} (wt.%)	w_{CPC} (wt.%)
B30	70	30	–	–
B30H5	65	30	5	–
B30C5	65	30	–	5
B30H20	50	30	20	–
B30C20	50	30	–	20

2.2. Bioactivity of the xerogels

Disc-like composite xerogel samples were immersed separately at 37 °C in 1× modified simulated body fluid (SBF) according to Oyane et al. [20]. For comparison, similar xerogels were incubated in Dulbecco's modified Eagle's medium (DMEM) with low glucose, supplemented with 10% fetal calf serum (FCS), 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin, in a humidified atmosphere at 37 °C and 7% CO_2 with or without stromal cells cultivated according to Section 2.3. Both solutions were replaced every 3 days. Quantification of the sample's bioactivity was performed by determining the change in calcium concentration in the respective liquid. Therefore 10 µl of the liquid was mixed with 300 µl of a 1:1 mixture of AMP buffer, pH 10.7, and *o*-kresolphthalein complexon, 8-hydroxyquinolin, HCl and Fluitest® CA-CPC detergent (Analyticon, Germany) according to the manufacturer's instructions. After reaction for 10 min in a 96-well tissue culture polystyrene (TCPS) plate, the absorbance at 570 nm was read using a SpectrafluorPlus microplate reader (Tecan, Germany). A calibration line was obtained from graded series of a calcium reference (2.5 mM).

2.3. Cell culture experiments

Human bone marrow stromal cells (hBMSC), isolated from bone marrow aspirate, were kindly provided by Prof. Bornhäuser and coworkers (Medical Clinic I, Dresden University Hospital) [21]. The cells were expanded in DMEM, low glucose, supplemented with 10% FCS, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin in a humidified atmosphere at 37 °C and 7% CO_2. The medium and all supplements were obtained from Biochrom (Berlin, Germany).

Human monocytes (hMc) were isolated from human buffy coats using a modified method based on the OptiPrep™ (ProGen Biotechnik, Heidelberg, Germany) density-gradient medium technique. For this, OptiPrep™ was mixed with alpha-minimal essential medium (Biochrom) to obtain the working solution (WS) as well as 1.078 and 1.068 g ml^{-1} gradient solutions. The target density was calculated and checked using a densimeter (Mettler Toledo Densito 30PX; Mettler Toledo, Greifensee, Switzerland). Buffy coats were centrifuged at 450*g* for 20 min and the leukocyte-rich fraction (LRF) was aspirated. WS was mixed with the LRF to obtain a density of 1.1 g ml^{-1}. The WS/LRF mixture was placed under a layer of 1.078 g ml^{-1} lymphocyte-specific gradient solution. A layer of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (HBS, 1% (w/v) NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM HEPES–NaOH, pH 7.4, containing 0.5% (w/v) bovine serum albumin (BSA)) was placed on top and centrifuged at 700*g* for 20 min. The peripheral blood mononuclear cell (PBMC) fraction