



Effects of extracellular magnesium extract on the proliferation and differentiation of human osteoblasts and osteoclasts in coculture



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ARTICLE INFO

Article history:

Received 30 March 2015

Received in revised form 24 August 2015

Accepted 25 August 2015

Available online 28 August 2015

Keywords:

Biodegradation

Magnesium

Coculture

Osteoblast

Osteoclast

ABSTRACT

Coculture of osteoblasts and osteoclasts is a subject of interest in the understanding of how magnesium (Mg)-based implants influence the bone metabolism and remodeling upon degradation. Human telomerase reverse transcriptase (*hTERT*) transduced mesenchymal stem cells (SCP-1) were first differentiated into osteoblasts with osteogenic supplements and then further cocultured with peripheral blood mononucleated cells (PBMC) without the addition of osteoclastogenesis promoting factors. Concomitantly, the cultures were exposed to variable Mg extract dilutions (0, 30×, 10×, 5×, 3×, 2× and 1×). Phenotype characterization documented that while 2× dilution of Mg extract was extremely toxic to osteoclast monoculture, monocytes in coculture with osteoblasts exhibited a greater tolerance to higher Mg extract concentration. The dense growth of osteoblasts in cultures with 1× dilution of Mg extract suggested that high concentration of Mg extract promoted osteoblast proliferation/differentiation behavior. The results of intracellular alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) activities as well as protein and gene expressions of receptor activator of nuclear factor kappa-B ligand (RANKL), macrophage colony-stimulating factor (M-CSF), and osteoclast-associated receptor (OSCAR) revealed significantly enhanced formation of osteoblasts whereas decreased osteoclastogenesis in the cultures with high concentrations of Mg extract (2× and 1× dilutions). In conclusion, while an increased osteoinductivity has been demonstrated, the impact of potentially decreased osteoclastogenesis around the Mg-based implants should be also taken into account. Cocultures containing both bone-forming osteoblasts and bone-resorbing osteoclasts should be preferentially performed for *in vitro* cytocompatibility assessment of Mg-based implants as they more closely mimic the *in vivo* environment.

Statement of Significance

An attractive human osteoblasts and osteoclasts cocultivation regime was developed as an *in vitro* cytocompatibility model for magnesium implants. Parameters in terms of cellular proliferation and differentiation behaviors were investigated and we conclude that high concentration of magnesium extract could lead to a promotion in osteoblastogenesis but an inhibition in osteoclastogenesis. It could contribute to the repeated observations of enhanced bone growth adjacent to degradable magnesium alloys. More interestingly, it demonstrates that compared to monoculture, osteoclasts in cocultures with osteoblasts exhibited higher tolerance to the culture environment with high magnesium extract. It might attribute to the neutralization process of the alkaline medium by acid generated by increased amount of osteoblasts in the condition with high concentration of Mg extract. The submitted work could be of significant importance to other researchers working in the related field(s), thus appealing to the readership of Acta Biomaterialia.

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1. Introduction

Magnesium and its alloys have been documented as biodegradable metallic materials suitable for orthopedic implants [1] and cardiovascular stents [2]. They have been shown to have

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advantages over traditional metallic materials, ceramics and biodegradable polymers. Their mechanical properties correspond to a natural bone to a greater extent than other materials, which reduces the likelihood of stress shielding and prevents bone resorption [1]. Moreover, their biocompatibility [3] and inherent biodegradability *in situ* [4,5] eliminate the need for a second surgery for implant removal and could thus lead to a decrease in both morbidity rate of the patients and health care costs due to shorter hospitalization [1].

In vivo studies carried out in small animals, i.e. rats, guinea pigs and rabbits have indicated that Mg or Mg alloys degrade in a safe way without causing significant harm to the neighboring tissues and could promote growth of new bone tissue and bone remodeling [4–10]. *In vitro* investigations of bone cell response to pure Mg metal have demonstrated the beneficial effects of Mg incorporation into bone substitutes on osteoblast adhesion to the functionally graded carbonate apatite containing Mg (FGMgCO₃Ap)–collagen composite [11], the expression of type I collagen extracellular matrix protein [12], and the adhesion and proliferation of human osteoblast-like SaOs2 cells [13]. More recently, two studies conducted by Cai et al. and Park et al. using Mg incorporated in fluoridated HA and Ti-based alloys, respectively, report similar beneficial effects of Mg-enriched materials on bone cell attachment, proliferation and late differentiation [14,15]. Similarly, external addition of Mg ions into the cell culture media results in a significant increase in the proliferation of human osteoblast-like MG63 cells [16].

Most of the *in vitro* assessments have been performed with osteoblast or its precursors. However, bone is a dynamic tissue that is being continuously remodeled through a precisely coordinated process consisting of resorption and synthesis of skeletal tissue [17,18]. Indeed, we could show recently, that the effect of Mg on bone resorption plays an additional important role for bone physiology around Mg-based implants [19]. It is well known that there is a crosstalk between osteoblasts and osteoclasts for the regulation of remodeling [17]. The interaction between osteoblasts and osteoclasts or even the role of osteoclasts on the longevity of the implants is underestimated. This is mainly due to two characteristics of these cells. First, osteoblasts were frequently considered to be the most dominating cells for osseointegration of implants since they are not only solely responsible for the generation of bone extracellular matrix but also for the regulation of osteoclasts differentiation and activity [20,21]. Second, due to the rather low number of osteoclasts under physiological conditions and their difficult isolation procedures *in vitro* investigations based on osteoclasts are extremely limited [22]. The success or longevity of an implant is also determined by the bone resorbing action of osteoclasts and the cross-talk between osteoblasts and osteoclasts [23]. Therefore cocultures consisting of osteoblasts and osteoclasts have gained more and more attention as they could give further insight into the effect on these cells and cell-to-cell interactions. A considerable amount of such coculture systems have been reported mainly on the cell combinations of primary osteoblasts with isolated monocytes of murine or human origin [20–24]. However, although maintaining *in vitro* the potential to differentiate into a variety of mesenchymal tissues [25], due to the senescence-associated growth arrest during the culturing of human mesenchymal stem cells (hMSCs), only limited cell numbers can be generated [26]. It has been also estimated that bone marrow stromal cells (BMSC) had a markedly diminished proliferation rate and gradually lost their multiple differentiation potential after the first passage [27]. SCP-1 is a well-established hMSCs model cell line that was immortalized using constitutive overexpression of *hTERT* and has been further demonstrated to be comparable to primary hMSCs due to their potential to undergo osteogenic and adipogenic differentiation [28]. To the best of our knowledge, studies on human osteoblasts-osteoclasts cocultures derived by hMSCs line SCP-1

and human monocytes for biomaterial testing have not been published until now. The aim of our present study is therefore to develop an appropriate coculture system (derived by SCP-1 and PBMC) which allows the elucidation of possible effects of Mg extract on intercellular signaling between bone-building and bone-resorbing cells and thus reflects more accurately the physiological microenvironment of bone around Mg implants.

2. Materials and methods

2.1. Mg extract preparation

Cuboid specimens with dimensions of 1 cm × 1 cm × 0.5 cm were cut from a Mg ingot with a purity of 99.98 wt% (Hydro Magnesium, Norway) via electrical discharge machining. After sterilization via sonication for 20 min in 100% isopropanol (2-propanol, Merck, Darmstadt, Germany), the Mg extract was prepared with an extraction medium to specimen weight ratio of 0.2 g/mL for the 1× extract preparation according to EN ISO standards 10993:5 [24] and 10993:12 [25]. Mg specimens were immersed in Eagle's minimum essential medium, Alpha modification (α-MEM; Life Technologies GmbH, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories GmbH, Linz, Austria) and incubated for 72 h under the cell culture conditions (95% humidity, 5% CO₂, 20% O₂ 37 °C). To observe a dose–response relationship, less concentrated extracts were serially diluted into 1:2, 1:3, 1:5, 1:10 and 1:30 concentrations afterwards using cell culture medium (referred to as 2×, 3×, 5×, 10×, and 30× dilution of Mg extract, respectively in this manuscript). Pure cell culture medium without Mg extract was used as control. The osmolality, pH values as well as the contents of Mg and calcium (Ca) in the seven dilutions of Mg extract were further determined. Gonotec O30-D cryoscopic osmometer (Gonotec, Berlin, Germany) and an ArgusX pH Meter (Sentron Europe BV, Roden, the Netherlands) were used for the measurement of osmolality and pH quantification, respectively. Mg contents were measured with the metallochromic dye xylydyl blue followed by a colorimetric method. 10 μL of Mg extract, extraction medium, or different concentrations of magnesium chloride (MgCl₂, standard curve) was mixed with 1.5 mL of the xylydyl blue solution (200 mmol/L trishydroxymethylaminomethane buffer, pH 12, containing 0.12 mmol/L xylydyl blue II, 0.05 mmol/L ethylene glycol tetraacetic acid (Titriplex), 69 mmol/L potassium carbonate, 2.1 mol/L ethanol and 0.05% sodium azide (all chemicals were obtained from Sigma-Aldrich Chemie GmbH, Munich, Germany)). After incubation at room temperature (RT) for 10 min, the absorbances were then measured with a 520 nm wavelength using an enzyme-linked immunosorbent assays (ELISA) reader (Tecan Sunrise; TECAN Deutschland GmbH, Crailsheim, Germany). Unknown concentrations of Mg content were determined by the standard curve. Moreover, Calcium O-cresolphthalein kit (Futura system Srl, Rome, Italy) and a calcium chloride (CaCl₂; Sigma-Aldrich Chemie GmbH, Munich, Germany) standard curve were used for the quantification of Ca content. An aliquot of 50 μL of the samples were mixed with 2 mL of reagent and incubated for 5 min at RT, the absorbances of the mixtures were measured at 580 nm wavelength with a Tecan Sunrise microplate reader (Tecan Sunrise; TECAN Deutschland GmbH, Crailsheim, Germany). Unknown concentrations were then determined by the standard curve.

2.2. PBMC isolation and coculture with osteoblasts

PBMC were freshly isolated from buffy coats from healthy donors (purchased from the Institute for Clinical Transfusion Medicine and Immunogenetics Ulm, Ulm, Germany). The isolation

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