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Strontium substitution in apatitic CaP cements effectively attenuates osteoclastic resorption but does not inhibit osteoclastogenesis

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ABSTRACT

Strontium ions were discovered to exert a dual effect on bone turnover, namely an inhibition of cell-driven bone resorption and a simultaneous stimulation of new bone tissue formation. A variety of strontium containing calcium phosphate bone cements (SrCPC) have been developed to benefit from both effects to locally support the healing of osteoporotic bone defects. While the stimulating effect of strontium modification on bone forming cells has been demonstrated in a number of studies, this study focuses on the inhibition and/or reduction of osteoclastogenesis and osteoclastic resorption by a strontium substituted calcium phosphate bone cement (SrCPC). Human peripheral blood mononuclear cells (PBMC) were differentiated into osteoclasts in the presence of different Sr^{2+}-concentrations as well as on the surface of SrCPC disks. Osteoclastogenesis of PBMC was shown to be merely unaffected by medium Sr^{2+}-concentrations comparable to those released from SrCPC *in vitro* (0.05–0.15 mM). However, an altering effect of 0.1 mM strontium on the cytoskeleton of osteoclast-like cells was shown. In direct contact to SrCPC disks, these cells exhibited typical morphological features and osteoclast markers on both RNA and protein level were formed. However, calcium phosphate resorption was significantly decreased on strontium-containing cements in comparison to a strontium-free control. This was accompanied by an intracellular accumulation of strontium that increased with substrate strontium content as demonstrated by Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS). This study illustrates that SrCPC do not inhibit osteoclastogenesis but significantly attenuate osteoclastic substrate resorption *in vitro*.

Statement of Significance

Strontium ions have been shown to promote bone formation and inhibit bone resorption. Therefore strontium is successfully used in the treatment of osteoporosis and also inspired the development of strontium-containing strontium/calcium phosphate bone cements (SrCPC). Studies have shown the positive effects of SrCPC on bone formation, however, the inhibiting effect of strontium on bone resorption in the context of such cements has not been shown so far. We found that the formation of bone-resorbing osteoclasts is not inhibited, but that their resorption activity is decreased in contact to SrCPC. The former is important since those cells play an important role in the bone cell signaling. The latter is a key requirement in osteoporosis therapy, which addresses excess bone resorption.

1. Introduction

While autologous bone transplantation is still regarded as gold-standard in bone defect regeneration that requires defect filling, a variety of bone substitute materials have been developed and investigated in the last decades, including metals, polymers, ceramics and glasses/glass ceramics [1–3]. For defects of small, however critical size defects in non-load bearing locations calcium phosphate bone cements (CPC) have become a widely used bone substitute material [4,5]. This is without doubt justified by their excellent biocompatibility and osteoconductivity, as well as their resorbability that allows implant degradation upon the formation of new bone tissue *in vivo* [5]. Degradation of CPC by means of

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physico-chemical dissolution has been subject of many studies and a variety of solutions and experimental setups have been used as discussed recently by Ito et al. [6]. In contrast to brushite-forming cements that possess a high solubility, apatite forming cements are known to exhibit very low degradation rates in neutral, aqueous solutions [5]. However, under *in vivo* conditions cell-mediated resorption by osteoclasts, often referred to as *biodegradation*, adds up to the physico-chemical degradation, allowing the remodeling of the material into bone tissue [5,7]. Osteoclasts (OC) are multi-nucleated cells originating from monocyte lineage that can resorb mineralized tissues. Proliferation and differentiation of these precursors into mature, resorbing OC is regulated via two distinct cytokines, the macrophage colony-stimulating growth factor (M-CSF) and the receptor activator nuclear factor κB ligand (RANKL) [8]. After attaching to the substrate, OC form a tight bond to the substrate surface, creating a sealing zone between the cell and the material where resorption takes place. Inorganic calcium phosphate is dissolved by acidification of the resorption zone while organic components are degraded enzymatically [8,9].

Strontium ions (Sr^{2+}) have been used in osteoporosis therapy in the form of strontium ranelate because they exhibit a stimulating effect on new bone formation by osteoblasts [10–12]. However, the mechanism how strontium also affects osteoclasts, or rather their resorption activity, is still not yet fully understood. In a study using chicken bone marrow derived OC like cells, Baron et al. demonstrated an inhibitory effect of strontium on the expression of carbonic anhydrase II (CAII), a key enzyme for bone resorption, that allows the degradation of bone mineral via the acidification of the resorption area [13]. In the same study, strontium was also shown to affect the formation of the OC ruffled boarder via inhibition of vitronectin receptor (integrin $a_v\beta_3$) expression, both resulting in a decrease of resorption activity. Interestingly, neither osteoclastogenesis nor cell attachment to the substrate surface was affected by the presence of strontium [13,14]. A possible target for Sr^{2+} affecting OC is the calcium sensing receptor (CaSR) which can be found in most osteoclasts and that is known to interact also with other divalent ions. In cultures of primary mature rabbit OC, activation of the CaSR by strontium ions stimulates apoptosis in a dose-dependent manner. Interestingly, the same study suggests that this effect derives from a potentiating effect of strontium on the induction of mature OC apoptosis by calcium through parallel and converging pathways [15]. The CaSR was also shown to reduce RANKL-induced osteoclastogenesis in murine cell lines as well as in cultures of human peripheral blood monocytes (PBMC) [16]. Another mechanism how strontium affects OC was proposed by Bakker et al., who demonstrated that Sr^{2+} can affect the paracrine signaling of osteocytes towards OC [17]. Albeit a number of approaches towards the integration of strontium ions into CPC have been described [18], to the best knowledge of the authors no data on *in vitro* osteoclastogenesis and substrate resorption under the influence of SrCPC have been published yet. Only one study by Yang et al. demonstrated that osteoclastic activity, by means of resorption pit area, decreased significantly with increasing strontium content of mineralized films prepared by precipitation from calcium and strontium containing liquids, a material resembling a set SrCPC [19]. An α-TCP-based cement system (CPC) comprising anhydrous calcium monohydrogen phosphate (Monetite, $CaHPO_4$), calcium carbonate ($CaCO_3$) and hydroxyapatite ($Ca_5(PO_4)_3(OH)$) served as control and was used as starting material for strontium-modified cements. SrCPC were obtained by partial or complete substitution of $CaCO_3$ by $SrCO_3$ in the cement precursor (S50 and S100, respectively). This cement system has been extensively studied by us regarding its material characteristics, effect on osteogenic cells *in vitro* and *in vivo* bone formation. Besides its improved mechanical characteristics [20], in particular an increased compressive strength, the *in vitro* study using primary human mesenchymal stem cells revealed an enhanced cell proliferation and a stimulating effect of both S50 and S100 on the osteogenic differentiation that could be attributed to Sr^{2+} released from the cements and altered calcium absorption behavior [21]. Furthermore, an increased new bone formation was shown 6 weeks post-implantation of S100 in critical size metaphyseal defects in an osteoporotic rat model [22]. Aim of the present work was to study the effect of these SrCPC on osteoclastogenesis and osteoclast-mediated material resorption *in vitro* to further understand the complex effects of SrCPC on the bone remodeling process comprising both osteogenesis and osteoclastogenesis. In a first approach, strontium chloride ($SrCl_2$) in concentrations resembling those released from the SrCPC was added to the culture medium of PBMC which were stimulated to form functional osteoclasts with M-CSF and RANKL. In a second set of experiments, cells were directly cultivated on two SrCPC and one Sr-free CPC serving as a control. Light and confocal laser scanning microscopy, gene expression analysis of osteoclast-related markers, quantification of osteoclast-specific enzyme activities as well as a SEM-based resorption assay were used to quantify both osteoclastogenesis and resorption activity. The results were correlated to intracellular calcium and strontium level measurements performed by ToF-SIMS.

2. Materials and methods

2.1. Cement preparation

SrCPC was obtained by substitution of $CaCO_3$ by $SrCO_3$ in an α-TCP-based cement precursor as described previously [20] and was provided by InnoTERE GmbH. Cements denoted as S50 and S100 contained 1.10 and 2.21 at% strontium, respectively, and Sr-free cement (CPC) was used as control. A cement paste was prepared from the cement powder by mixing with 4 wt.% disodium hydrogen phosphate (Na_2HPO_4, Sigma-Aldrich) at a liquid-to-powder ratio of 400 µl/g. Cement disks designed to fit into standard 48-well tissue culture plates (diameter 10 mm, 1 mm thickness) were prepared using silicone molds. Cement was allowed to set in water-saturated atmosphere at 37 °C for 4 days to avoid ion leaching as described previously [21]. Subsequently samples were dried at air and sterilized by gamma irradiation (25 kGy). Before start of cell culture, cement disks were immersed overnight in α-MEM containing 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin und 2 mM glutamine to medium equilibration and adsorption of proteins.

2.2. PBMC isolation

Human monocytes were isolated from buffy coats (German Red Cross) of 5 healthy adult donors as described previously [23]. In brief, buffy coats (75–91 ml, each obtained from 500 ml whole blood) were diluted 1:2 with PBS (Gibco) with 2 mM EDTA (Sigma-Aldrich) and 0.5% BSA (bovine serum albumin, Sigma-Aldrich) and centrifuged for 10 min at 1000 *g* over Ficoll® Paque Plus (GE Healthcare) in Leucosep tubes (Greiner) without brake. The obtained peripheral blood mononuclear cell (PBMC) fraction was re-suspended in ice-cold PBS with EDTA and BSA and centrifuged at 250*g* and 4 °C for 15 min. Subsequently, remaining erythrocytes were lysed by re-suspension in 2 ml deionized water for 30 s. An additional washing step was performed by adding 48 ml ice-cold PBS with EDTA and BSA and centrifugation at 250*g*. Cells were then re-suspended in α-MEM containing 10% heat-inactivated FCS 100 U/ml penicillin, 100 µg/ml streptomycin