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A new insight into the dissociating effect of strontium on bone resorption and formation

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ABSTRACT

Calcium phosphates are widely used as biomaterials and strontium (Sr) is known to have the ability to modify the bone balance towards osteosynthesis. In the present study we investigated the capacity of Sr-substituted sol–gel calcium phosphate to modify the expression of genes and proteins involved in extracellular matrix synthesis by primary bone cells. We first determined the most effective concentration of strontium using human primary bone cells. Sol–gel biphasic calcium phosphate (BCP) powders were then synthesised to obtain release of the optimal concentration of strontium. Finally, human osteoblasts obtained from explant cultures were cultured in the presence of sol–gel BCP, Sr-substituted BCP (5% Sr-substituted BCP, corresponding to a release of 5×10^{-5} M [Sr^{2+}] under the culture conditions ($BCP_{5\%}$)) and medium containing strontium chloride ($SrCl_2$). Viability, proliferation, cell morphology, protein production and protein activity were studied. We demonstrated that 5×10^{-5} M $SrCl_2$ and $BCP_{5\%}$ increased the expression of type I collagen and SERPINH1 mRNA and reduced the production of matrix metalloproteinases (MMP-1 and MMP-2) without modifying the levels of the tissue inhibitors of MMPs (TIMPs). Thus strontium has a positive effect on bone formation.

1. Introduction

Despite significant advances in orthopaedic and dental surgery, no perfect bone filling material has yet been clinically established. Autografts, which are still considered to be the gold standard, require two surgical procedures (one to harvest bone graft material from the patient and one for the graft itself), which can be painful and could expose the patient to side-effects inherent to surgical procedures. Moreover, autografts are limited by the quantity of harvested material, which may not be sufficient to fill large defects. According to MacArthur et al. [1] the "underlying supposition of tissue engineering is that the employment of natural biology of the system will allow for greater success in developing therapeutic strategies aimed at the replacement, repair, maintenance, and/or enhancement of tissue function". It appears that optimised tissue reparation requires active scaffolds which can enhance tissue regeneration. Recent scaffold developments have associated an organic matrix, calcium phosphate and cells. Calcium phosphates, particularly biphasic calcium phosphate (BCP), are widely used as biomaterials thanks to their similarity to the inorganic phase of bone. They are widely used as bone filling materials and for orthopaedic implant coatings. Despite their good biocompatibility and their ability to increase the integration of coated implants [2–5], they have a relatively low activity in terms of modifying bone physiology. They also have a tendency to fragment and to generate wear particles which could increase implants failure [6]. So it is important to develop biomaterials which generate particles having the ability to modify the balance of bone formation toward osteosynthesis. Thus we developed BCP ceramics which have intrinsic osteoinductive properties in order to enhance the osseointegration of the material itself or the implants it coats [7].

Strontium has interesting properties. It modifies the bone balance towards osteogenesis and is widely used as a treatment for osteoporosis [8]. Development of the use of strontium ranelate in the treatment of osteoporosis has provided large amounts of data

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on the mechanism of action of strontium in bone cells. In vitro data have demonstrated that strontium acts in part by decreasing bone resorption [9]. Strontium reduces osteoclast activity and bone resorption in vitro, an effect that is not observed with calcium [9,10]. Strontium ranelate decreases functional osteoclast marker expression [10] and disrupts the cytoskeleton, essential for the resorption activity of osteoclasts [11], and increases osteoclast apoptosis [12]. The mechanisms are not clear but strontium is known to increase the osteoclastogenesis inhibitory factor/receptor activator of nuclear factor κ-B ligand ratio, suggesting an effect of strontium ranelate on bone formation and resorption [12]. The calcium-sensing receptor (CaSR) may also be implicated [13]. Strontium ranelate also has the ability to induce positive effects on osteoblastogenesis and osteoblast activity. Strontium ranelate enhances the replication of pre-osteoblastic cells [14–17] and increases many osteoblast markers, in particular type-1 collagen, in murine bone marrow-derived mesenchymal osteoprogenitor cells and immature osteoblasts [9,15–17]. The same results were found when Sr-containing bioactive ceramics were cultured with human bone-derived cells [18] or with a human osteosarcoma cell line [19]. In that way, strontium treatment could significantly increase osteoblast-related gene and alkaline phosphatase (ALP) expression of osteogenic differentiating bone marrow mesenchymal stem cells (BMSCs) [20]. Sr-containing ceramic scaffolds could also induce the attachment and differentiation of human bone-derived cells (HOB) [21].

Studies on primary human osteoblasts indicate that strontium also promotes the ultimate differentiation of osteoblasts into osteocytes [17]. It is therefore likely that multiple mechanisms may mediate bone balance modification by strontium ranelate in vitro and that they remain to be determined [8]. As well as enhanced osteogenic differentiation, increased phosphorylation of mitogen-activated protein kinase (MAPK) ERK1/2 and p38 was detected in strontium-treated MSCs [20]. Furthermore, selective inhibitors of ERK1/2 kinase and p38 attenuated the effect of strontium on osteogenesis. It was also demonstrated that rat sarcoma viral oncogene homolog (RAS), an upstream regulator of ERK1/2 and p38, was activated by strontium treatment and siRNA-mediated Ras knockdown inhibited strontium-stimulated expression of osteogenic markers [20].

In vivo studies in a large animal model (goats) indicated that Sr–Ca co-administration considerably increased bone mineral apposition rate. The expression of insulin-like growth factor (IGF)-1 and runt-related transcription factor 2 (Runx2) was significantly up-regulated within the treated group; tumour necrosis factor expression was also significantly down-regulated in that group [22]. Small animal models (rats) have demonstrated that BMSCs produced larger osteogenic colonies but smaller adipogenic colonies in Sr-treated OVX rats. The mRNA level of osteogenic genes was higher, while the mRNA level of adipogenic genes was lower in BMSCs from Sr-treated rats [23].

So, we hypothesised that strontium included in BCP may be used to have positively affect the physiology of primary human bone cells. To that end we have modified BCP [24] by the addition of strontium so that the particles released from the bulk material have the ability to modify the bone balance. Strontium-substituted calcium phosphate ($BCP_{5\%}$) powders were synthesised and incorporated into human primary bone cell cultures.

First, we determined the most efficient Sr^{2+} concentration in our culture model. This allowed us to select the powder that released strontium at the optimum concentration. Then we compared the effects of this substituted powder with those of non-substituted BCP particles (without strontium supplementation) and to a $SrCl_2$-supplemented medium on cell parameters such as proliferation and the expression of genes implicated in type 1 collagen expression (COL1A1 and SERPINH1), cell morphology and matrix metalloprotease (MMP-1 and MMP-2) expression and synthesis.

Since BCP is known to show good biocompatibility and strontium is known to have a positive effect on bone balance, we expect that our $BCP_{5\%}$ particles, when released, will also induce such an effect.

2. Materials and methods

2.1. Reagents

Dulbecco's phosphate-buffered saline 1× (DPBS), trypsin, Trypan blue, ethylenediamine tetraacetic acid (EDTA), penicillin, streptomycin, Dulbecco's modified Eagle's medium (DMEM) + Glutamax, collagenase II and fetal calf serum (FCS) were purchased from Invitrogen/Gibco (Cergy Pontoise, France). $SrCl_2$ and bovine gelatine were from Sigma–Aldrich (Saint-Quentin Fallavier, France). $Sr(NO_3)_2{\cdot}4H_2O$, $Ca(NO_3)_2{\cdot}4H_2O$ (Aldrich) and P_2O_5 (Avacado Research Chemicals) for the hydroxyapatite (HA) powder were from Aldrich (USA) and Avocado Research Chemicals (Heysham, UK), respectively. The Quantikine® ELISA sets for pro-MMP-1 and total MMP-2, the DuoSet® ELISA sets for TIMP-1 and TIMP-2 and recombinant human pro-MMP-1, pro-MMP-2, TIMP-1 and TIMP-2 were from R&D systems (Lille, France). The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay was from Promega. RNeasy Micro Kits and the RNase-Free DNase Set were from Qiagen. Power SyBR® Green PCR Master Mix and the high capacity reverse transcription kit were from Applied Biosystems. Glutaraldehyde and 1,1,1,3,3,3-hexamethyldisilazane (HMDS) were from VWR (Fontenay sous Bois, France) and Agar Scientific (Stansted, UK), respectively.

2.2. Sol–gel preparation of strontium-substituted BCP

To produce 2 g of BCP powder, 4.7 g of $Ca(NO_3)_2{\cdot}4H_2O$ and 0.84 g of P_2O_5 were dissolved in pure anhydrous ethanol (99.5%) under stirring and under reflux at 85 °C for 24 h. Then this solution was cooled and maintained at 55 °C for 24 h, to obtain a self-consistent white gel, which was further heated at 80 °C for 10 h to obtain a white powder. Finally, the powder was heated at 1100 °C for 15 h. Use of such a high temperature allows the synthesis of well-crystallised BCP. To prepare Sr-substituted BCP 0.21 g of $Sr(NO_3)_2{\cdot}4H_2O$ was added to the solution. To prepare Sr-substituted HA, the required amount of $Sr(NO_3)_2$ was added to the solution. Addition of strontium modified the calcium phosphate obtained by the sol–gel method: we obtained more whitlockite but that is the price to be paid to incorporate Sr in the materials. It is impossible to modify the lattice of HA without changing the amount of other calcium phosphate present. Moreover, BCP (HA + tricalcium phosphate) has been demonstrated to be a better material than pure HA [7]. The decision to study cell interactions with powders enabled us to investigate the response of bone cells to particles of from 1 to 100 μm (the mean particle size as determined by laser granulometry was around 30 μm, with significant polydispersity) and may give data about their reaction if wear debris were generated or if used in a bone filling approach.

2.3. Surface area ratio method

In the present work the surface area ratio (SAR = surface area of material/surface area of cell) method was used [25]. Using this method, a non-uniform challenge may result if particles of varying sizes and surface areas are being compared. Since osteoblasts interact with the surfaces of nanoscale to microscale materials particles [26] it is more logical to challenge cells based on their surface areas. Although some authors have used weight percentage, volume percentage or number of particles in their studies, our previous studies using the SAR approach have produced good results

ID	Title	Pages
1367	A new insight into the dissociating effect of strontium on bone resorption and formation	11