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The role of biosilica in the osteoprotegerin/RANKL ratio in human osteoblast-like cells

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ABSTRACT

Earlier studies have demonstrated that biosilica, synthesized by the enzyme silicatein, induces hydroxyapatite formation in osteoblast-like SaOS-2 cells. Here we study the effect of biosilica on the expressions of *osteoprotegerin* [*OPG*] and the *receptor activator for NF-κB ligand* [*RANKL*] in the SaOS-2 cell model. We show that during growth of SaOS-2 cells on biosiliceous matrices hydroxyapatite formation is induced, while syntheses of cartilaginous proteoglycans and sulfated glycosaminoglycans are down-regulated. Furthermore, quantitative real-time RT-PCR analysis revealed a strong time-depended increase in expression of *OPG* in biosilica exposed SaOS-2 cells while the steady-state expression level of *RANKL* remained unchanged. These results have been corroborated on the protein level by ELISA assays. Therefore, we propose that biosilica stimulated OPG synthesis in osteoblast-like cells counteracts those pathways that control RANKL expression and function (e.g. maturation of pre-osteoclasts and activation of osteoclasts). Hence, the data obtained in the present study reveal the considerable biomedical potential of biosilica for treatment and prophylaxis of osteoporotic disorders.

1. Introduction

The non-metallic element silicon (Si), with 28 wt% the second most abundant element in Earth's crust, served as the first inorganic component of the animal skeletal system during the Proterozoic (800 mya; reviewed in [1]). Elemental Si is only very rarely found. However, in its oxidized form, as silicate/silica, it is among the most common minerals on our planet. Si comprises almost exclusively a tetrahedral coordination, with 4 oxygen atoms that surround the central Si atom, and occurs as SiO_2 in the thermodynamically most stable crystalline form; the amorphous form of silica is less abundant [2]. In the living world SiO_2 occurs, with only one exception, i.e. the nanorods of sponge spicules [3], as amorphous biosilica, in particular in plants, algae, and sponges [1]. In a pH range of 7–9 and at concentrations below 2 mM, Si exists predominantly as soluble silicic acid [$Si(OH)_4$]. The monomeric form of silica, orthosilicate is water-soluble and has the pK_a of a weak acid, 9.6. At neutral pH, the tetrahedral silica is uncharged but has the tendency to polymerize to poly(silicate), particularly at silica concentrations above 2–3 mM [4]. At these concentrations, poly(silicate) initially forms linear polymers, followed by small colloidal species, which ultimately turn into amorphous precipitates that are gels at neutral pH. Basically, silicic acid is considered to be inert. Yet, it is taken up by cells of plants, algae, and sponges [5] and provides the basis for intricate biosiliceous exoskeletons [1]. Moreover, controlled interaction of silicic acid with proteins and polysaccharides has been suggested [6]. In this context, interaction of silicic acid with alkyl diols of saccharides, under formation of five- and six-coordinated Si complexes, has been described [7].

Below pH 9, Si exists physiologically predominately as $Si(OH)_4$ as the most stable species. The physiological concentration of Si/Si $(OH)_4$ in the vertebrate/human body is lower, with 1–2 g totally, than the trace elements iron and zinc [8]. Nevertheless, Si deficiency causes defects in connective skeletal tissue [9,10]. Si is taken up via gastrointestinal absorption and becomes metabolically

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available [11]. After circulation through the blood, where it remains unbound [12], $Si/Si(OH)_4$ is filtered by renal glomeruli and eliminated via the kidney into the urine [13]. The plasma level of Si is low and measures between 0.2 and 4 mg/l [7–142 μM] [12], well below the concentration required for poly(silicate) formation.

Following dietary intake, highest silicon concentrations have been found in connective tissue, bone, and blood vessels [14]. Interestingly, $Si/Si(OH)_4$ is present in particular in active calcification sites during bone formation [9]. Epidemiological studies provided evidence that in human the highest silica intake rate is positively associated with bone mineral density [BMD] at the hip site of men and pre-menopausal women, but not in post-menopausal women [15]. A similar positive correlation has been established for post-menopausal women during hormone treatment [16]. Based on these findings, it has been proposed that the level of silicon is associated with higher BMD, bone strength, and likely also with estrogen status [8].

In addition to *in vivo* observations also *in vitro* experiments revealed the beneficial effects of silica on bone and collagen metabolism, e.g. a stimulation of the prolyl hydroxylase, an enzyme involved in collagen synthesis [17]. Moreover, silicic acid has been shown to promote osteoblast proliferation, extracellular matrix differentiation, increase in enzyme activity [e.g. alkaline phosphatase] and gene expression [18]. Consequently, application of Si-containing implant materials and ceramics has been thoroughly studied. Especially Si-substituted hydroxyapatite and bioglass have attracted great interest as promising bone and tooth substitutes [19–21]. Furthermore, the Si-containing compound Zeolite-A was found (i) to affect human osteoblast-like cells mitogenically, by stimulating proliferation and differentiation, (ii) to enhance the differentiation of mesenchymal cells, and (iii) to promote osteoblast activity [reviewed in [22]]. Moreover, silica was found to augment gene expression of collagen type-I [23] and bone morphogenetic protein-2 [BMP-2] [24]. A recent study demonstrated that Si/silica of seawater causes differential expression of several murine genes, such as *BMP-2 runt-related transcription factor 2* [*Runx*-2], *collagen type-I* [*COL*1], *osteoprotegerin* [*OPG*], and *receptor activator of NF-κB ligand* [*RANKL*] [25]. Especially interesting in this study was the finding that the ratio of *OPG*/*RANKL* increased at higher Si concentrations.

These results prompted us to study the effect of biosilica on the expression of *OPG*/*RANKL* in cell culture experiments, using the osteoblast-like SaOS-2 cell model. *OPG* and *RANKL* have been identified to be one of the most crucial signaling systems that mediate and modulate bone resorption [reviewed in [26]]. It is the cellular and soluble factor-mediated crosstalk between osteoblasts that controls the differentiation of pre-osteoclast to mature osteoclast [27]. On the cellular level the following three members of the tumor necrosis factor (TNF) and TNF receptor superfamily are involved, (i) the receptor activator of NF-κB (RANK) that is expressed on hematopoietic cells and controls osteoclastogenesis/regulation of bone mass and calcium metabolism, (ii) OPG that is secreted by osteoblasts and inhibits osteoclastogenesis, and (iii) membrane-bound RANKL, a protein found on osteoblasts, which activates osteoclasts through interaction with RANK on osteoclast precursor cells [reviewed in [28]]. Studies with transgenic mice revealed that overexpression of OPG results in osteopetrosis (marble bone disease)-like symptoms, while OPG-knockout mice showed the phenotype of severe osteoporosis [29]. Based on these findings, it is now well-established that RANKL/RANK interaction is crucial for differentiation and maintenance of osteoclast activity, and thus is fundamentally involved in the manifestation of osteoporosis [30].

Since the discovery of the poriferan enzyme silicatein, a fundamental molecular tool is available to harness the biogenous synthesis of poly(silicate), biosilica. Biosilica is the inorganic scaffold that forms the siliceous skeleton in sponges [reviewed in [31]]. In sponges, biosilica revealed morphogenetic potential by stimulating the expression of collagen [32]. Moreover, in the SaOS-2 cell model, biosilica promotes hydroxyapatite formation [33] and causes altered expression of amelogenin, ameloblastin, and enamelin [34]. First prototypic bioactive implant materials that comprise the enzyme silicatein and its silicate substrate have successfully been tested [35]. In addition, silicatein has been bioengineered to obtain hydroxyapatite-binding affinity, consequently facilitating application to osseous and dental scaffolds [36].

To evaluate a future application of biosilica for osteoporosis treatment and prophylaxis, the present study investigates its effects on SaOS-2 cells, which previously have been shown to express RANK, OPG, and RANKL [37,38].

2. Materials and methods

2.1. Materials

The following materials were obtained from Fluka/Sigma–Aldrich (Taufkirchen; Germany), TEOS [tetraethoxysilane], gentamicin, Alizarin Red S, Alcian Blue, and Rhodamine 123. McCoy's medium was purchased from Biochrom (Berlin; Germany); fetal calf serum (FCS) from GIBCO (Grand Island, NY; USA); UltraCruz Mounting Medium from Santa Cruz Biotechnology (Santa Cruz, CA; USA); Silica Test Colorimetric Assay Kit (1.14794) and silicon standard (1.09947) from Merck (Darmstadt; Germany); and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT) from Carl Roth (Karlsruhe; Germany).

2.2. Recombinant silicatein

The preparation of recombinant silicatein was described previously [35,36]; Supplementary Methods 1.

2.3. Cells and incubation conditions

SaOS-2 cells (human osteogenic sarcoma cells [39]) were cultured in McCoy's medium, supplemented with 5% heat-inactivated FCS [fetal calf serum], 2 mM L-glutamine, and gentamicin (50 μg/ml) in 25 cm^2 flasks or in multi-well plates (Orange Scientifique, Braine-l'Alleud; Belgium) in a humidified incubator at 37 °C and 5% CO_2. Routinely, 3×10^5 cells were added per well (3 ml total volume). For the experiments the cells were cultivated either on untreated substrates or on substrates that had been coated with silicatein/biosilica (see below).

To avoid potential effects of fresh serum on gene expression, medium with 5% FCS was used 2 days before expression analyses were initiated [34,37].

2.4. Cell proliferation/viability assays

Orthosilicate was prepared from prehydrolyzed TEOS as described [40]. In short, a stock solution of 5 mM TEOS was mixed in a 1:3 M ratio with 1 mM HCl for 30 min and subsequently neutralized. The orthosilicate formed was added to the cells at 10–1000 μM. SaOS-2 cells were seeded at a density of 5×10^3 cells per well of a 96-multi-well plate (Orange Scientifique) and cultured for 72 h in McCoy's medium, supplemented with 5% FCS in the absence or presence of orthosilicate. Then, cell proliferation was determined by a colorimetric method based on the tetrazolium salt XTT (Cell Proliferation Kit II; Roche, Mannheim; Germany), following the recommendations of the supplier and as described [37]. In a second approach, cell viability was quantified by the trypan-blue exclusion assay [41]. For this experiment, SaOS-2 cells were seeded at a density of 15×10^3 cells per well of a 24-multi-well plate (Orange Scientifique) and cultured in McCoy's medium with 5% FCS in the absence or presence of orthosilicate. The number of viable and dead cells was determined microscopically after 72 h.

2.5. Silicatein-coating of culture wells

To coat 6-well plates with recombinant silicatein, the plates were incubated with 200 μl/cm^2 of a solution containing 5 μg/ml of the recombinant enzyme in 50 mM Tris–HCl (pH 7.4) and 150 mM NaCl. After incubation for 3 h at 20 °C, the plates were washed once with the Tris–HCl buffer. Then, to evaluate immobilization of silicatein, the wells were washed with phosphate-buffered-saline [PBS; pH 7.4] and blocked with 3% bovine serum albumin [BSA] for 1 h at room temperature. Afterwards, the wells were incubated consecutively with polyclonal rabbit anti-silicatein antibodies (PoAb–aSilic; 1:5000 dilution in 0.3% BSA [42]) and anti-rabbit IgG (alkaline phosphatase [AP]–conjugated secondary antibodies; 1:2000; Sigma).