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Silicate Modulates the Cross-Talk Between Osteoblasts (SaOS-2) and Osteoclasts (RAW 264.7 Cells): Inhibition of Osteoclast Growth and Differentiation

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ABSTRACT

It has been shown that inorganic monomeric and polymeric silica/silicate, in the presence of the biomineralization cocktail, increases the expression of osteoprotegerin (*OPG*) in osteogenic SaOS-2 sarcoma cells in vitro. In contrast, silicate does not affect the steady-state gene expression level of the osteoclastogenic ligand receptor activator of NF-κB ligand (*RANKL*). In turn it can be expected that the concentration ratio of the mediators OPG/RANKL increases in the presence of silicate. In addition, silicate enhances the growth potential of SaOS-2 cells in vitro, while it causes no effect on RAW 264.7 cells within a concentration range of 10–100 μM. Applying a co-cultivation assay system, using SaOS-2 cells and RAW 264.7 cells, it is shown that in the presence of 10 μM silicate the number of RAW 264.7 cells in general, and the number of $TRAP^+$ RAW 264.7 cells in particular markedly decreases. The SaOS-2 cells retain their capacity of differential gene expression of OPG and RANKL in favor of OPG after exposure to silicate. It is concluded that after exposure of the cells to silicate a factor(s) is released from SaOS-2 cells that causes a significant inhibition of osteoclastogenesis of RAW 264.7 cells. It is assumed that it is an increased secretion of the cytokine OPG that is primarily involved in the reduction of the osteoclastogenesis of the RAW 264.7 cells. It is proposed that silicate might have the potential to stimulate osteogenesis in vivo and perhaps to ameliorate osteoporotic disorders. J. Cell. Biochem. 113: 3197–3206, 2012.

KEY WORDS: SILICATE; OSTEOBLASTS; OSTEOCLASTS; HYDROXYAPATITE FORMATION; OSTEOBLASTOGENESIS; OSTEOCLASTOGENESIS

The formation of the skeletal system from the earliest metazoans, the sponges (phylum Porifera) [Müller et al., 2009] to the crown taxa, the mammalians [Matsuo and Irie, 2008], and the insects [Moussian, 2010] is dominated by a tuned communication between cells controlling anabolic processes and those executing catabolic reactions. In vertebrates, the influence of osteoblasts on osteoclast proliferation and differentiation is well established. In 1997 the decisive osteoclastogenic ligand receptor activator of NF-κB ligand (RANKL) had been identified as a transmembrane glycoprotein belonging to the tumor necrosis factor (TNF)-α1 superfamily that is produced by stromal cells/osteoblasts [see: Wong et al., 1997]. This membrane-bound ligand is processed

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by metalloproteases to a soluble form [Nakashima et al., 2000] and interacts with receptor activator of NF-κB (RANK) and in turn induces osteoclastogenesis [see: Baud'huin et al., 2007]. This molecular cross-talk that coordinates osteoclastogenesis is controlled by a third component, osteoprotegerin (OPG), an osteotropic effector that acts as a soluble bone protector [see: Feige, 2001]. OPG, as a secreted stromal cell-derived "decoy" receptor, specifically binds RANKL and thereby inhibits osteoclast differentiation [Suda et al., 2001]. This molecular triad, OPG/RANK/RANKL [see: Suda et al., 1999], is not only crucially controlling osteoclast differentiation, but has also been involved in cell differentiation pathways of the immune and vascular systems [see: Baud'huin et al., 2007]. Conversely, recent results also provided experimental evidence that osteoclasts contribute with their cytokines to the fine-tuning of the osteoclast/osteoblast balanced functions [see: Martin and Sims, 2005; Nagase et al., 2009].

Osteoporosis is a systemic skeletal disorder which is characterized by reduced bone compactness that may result in increased fracture risk [Felsenberg and Boonen, 2005]. The fact that osteoporosis is etiologically caused by an imbalance between osteoblastic bone production and osteoclastic bone resorption is known since decades [Parfitt, 1982]. The above mentioned data, OPG-RANKL ratio to be a reliable marker for the differentiation state of osteoblasts and osteoclasts, led to in vivo experiments to clarify if over-expression of *OPG* in vivo causes osteopetrosis, while inactivation of this gene gives rise to osteoporosis. Studies with OPG-knockout mice showed that those animals displayed the phenotype of a severe osteoporosis [Bucay et al., 1998], and on molecular level, that a quantitative imbalance of OPG and RANKL resulted in the manifestation of this bone disease [Raisz, 2005]. As a consequence of this observed imbalance toward a higher activity of the osteoclasts, drugs were designed that should impair/diminish the activity of the osteoclasts. The most widely used drugs for the treatment of osteoporosis are anti-resorptive acting agents, bisphosphonates, estrogen, and calcitonin [Miller, 2008; Reid, 2008]. In order to overcome the observed side effects of those pharmacologically active compounds, especially since those drugs must be taken by patients for a longer period, considerable efforts have been undertaken to develop alternative therapies [Hwang and Putney, 2011].

Following the view that all metazoans originate from one ancestor, to whom the siliceous sponges are most closely related [Müller et al., 2004], it had been postulated that the siliceous skeleton of the sponges shares functional relationship to the Ca-based skeletons of vertebrates [Müller, 2005]. Previous experimental evidence had been elaborated to show that silicate/silicon is an essential trace element in vertebrate nutrition [Carlisle, 1972; Schwarz and Milne, 1972]. Then, tissue level studies revealed that silicate accumulates in mammalians and birds in connective tissue which is the site of bone formation and it has been speculated that silicon is accumulating at the bone growth front [reviewed in: Carlisle, 1986; Jugdaohsingh, 2007]. The positive effect of silicate on HA crystallite formation was first described by Schröder et al. [2005] using osteoblast-like SaOS-2 (sarcoma osteogenic) cells, a non-transformed cell line derived from primary osteosarcoma cells, that has a limited differentiation capacity [Hausser and Brenner, 2005; Kelly et al., 2010]. Recently, it has been demonstrated that in SaOS-2 cells after exposure to poly(silicate) a differential gene expression is seen that strongly up-regulates the steady-state level of *OPG* and leaves the level of *RANKL* almost unchanged [Wiens et al., 2010b]. Based on the observed increase of the OPG/RANKL ratio, it is suggested that silica/silicate has a favorable biomedical potential for the treatment and/or prophylaxis of osteoporotic disorders [Wiens et al., 2010b]. Since silica was found to elicit in vitro an increased [^{3}H]dThd incorporation into DNA and a likewise increased HA formation, an osteogenic potential of silica had been deduced [Wiens et al., 2010a].

In the present study we used the two chamber assay system, originally developed by Boyden [1962] to detect the chemotactic activity in vitro. In the present study we applied this two chamber assay to identify the (potential) effect of diffusible mediators released by SaOS-2 on the functional activity of RAW 264.7 cells. RAW 264.7 cells are monocyte/macrophage precursor cells that are osteoclast-like [Vincent et al., 2009]. During differentiation of RAW 264.7 cells to osteoclasts, they express tartrate-resistant acid phosphatase (TRAP), an enzyme that has been used as a marker for functionally active, multinuclear osteoclasts (TRAP$^+$) [Ballanti et al., 1997]. Following the original suggestion, those cells producing the diffusible substance, here the SaOS-2 cells, were seeded in the lower chamber, while in the upper chamber the target cells, RAW 264.7 cells, were placed. The data obtained show that after exposure to silicate the SaOS-2 cells display a strong potency to form HA crystallites and increasingly express OPG. In the present study we use orthosilicate as a silica source for the in vitro experiments, knowing that under the pH and concentration conditions occurring in the culture assays the "orthosilicate" undergoes partial autocondensation and oligo-/polymerizes to oligo-/polymers [Coradin and Lopez, 2003]. Hence, the inorganic silica sample, found to be active in the present study on RAW 264.7 cells, comprise both monomeric "orthosilicate" and oligo- and perhaps even polymeric silica species. Finally it is shown that after exposure of SaOS-2 cells to silicate the RAW 264.7 cells respond with a lower growth rate and a reduced differentiation capacity.

MATERIALS AND METHODS

CELLS AND INCUBATION CONDITIONS

The human osteogenic sarcoma cells [SaOS-2 cells; Fogh et al., 1977] were cultured in McCoy's medium (Biochrom, Berlin; Germany), containing 15% heat inactivated fetal calf serum (FCS), Na-pyruvate (1 mM), $Ca(NO_3)_2$ (0.5 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) either in 25 cm^3 flasks or in six-well plates (surface area, 9.46 cm^2; Orange Scientifique, Braine-l'Alleud, Belgium) in a humidified incubator at 37°C and 5% CO_2 as described [Schröder et al., 2005; Müller et al., 2007]. Routinely, 1×10^5 cells were added per well (total volume, 3 ml). Different concentrations of Na-orthosilicate (Sigma-Aldrich, Taufkirchen, Germany) were added as described with the respective experiments. A stock solution of 1 M Na-orthosilicate hydrate [$Na_2SiO_2(OH)_2 \bullet 4H_2O$] was prepared and then diluted to 10 mM in a Na-phosphate buffer (pH 7.4); this "silicate" sample was used for the experiments. It might be stressed here that the silica concentration in the in vitro assay system might be slightly higher because of the potential existence of background

levels of silicon from the cell culture and of silicon leaching out from the culture plates.

The murine monocyte/macrophage cell line RAW 264.7 was grown in a humidified atmosphere containing 5% CO_2 at 37°C using the same McCoy's medium/FCS, Na-pyruvate, as used for SaOS-2 cells. 5×10^4 cells were added per well (total volume, 1 ml).

CELL PROLIFERATION/VIABILITY ASSAYS

SaOS-2 cells as well as RAW 264.7 cells were seeded at a density of 5×10^3 cells per well in a 96-multi-well plate (Orange Scientifique) and cultured for 3 days in McCoy's medium/15% FCS. Increasing concentrations of silicate were added to the cells. After incubation, the cell proliferation/growth was determined by the colorimetric method based on the tetrazolium salt XTT (Cell Proliferation Kit II; Roche, Mannheim; Germany) as described [Mori et al., 2007].

To determine the differentiation state of the RAW 264.7 cells, aliquots of cultures were stained by TRAP (using the mouse anti-human TRAP monoclonal antibody; clone 9c5; from BioLegend, St. San Diego), an established osteoclast marker protein [Filgueira, 2004], according to the manufacturer's instructions. Subsequently the cells were cytochemically analyzed [Han et al., 2007].

DIGITAL LIGHT MICROSCOPY

In one series of experiment the number of RAW 264.7 cells was determined optically by counting the cells present in a given area of 40×40 μm. To obtain a suitable contrast the cells were analyzed with a KEYENCE BZ-8000 epifluorescence microscope (KEYENCE, Neu-Isenburg; Germany) using an S-Plan-Fluor 20× lens. The samples were inspected both with white light as well as with fluorescent light using the filter sets ex560 ± 40–630 ± 60 nm/em (for red fluorescence) and ex480 ± 30–535 ± 50 nm/em (for green fluorescence). The images recorded were overlayed and the cells counted on the computer screen.

SCANNING ELECTRON MICROSCOPY

For SEM (scanning electron microscopy), the SU 8000 (Hitachi High-Technologies Europe, Krefeld; Germany) was employed at low voltage (1 kV; suitable for analysis of inorganic morphological structures), as described [Wiens et al., 2010a].

BOYDEN CHAMBER ASSAY

The principle of the two compartments assay system, the Boyden chamber assay, has been outlined and summarized [Chen, 2005]; (Fig. 1). The lower wells were filled with 0.3 ml of medium (McCoy's medium/FCS, Na-pyruvate) and 5×10^4 SaOS-2 cells. The upper chamber with the microporous membrane of a pore size of 3 μm at pore density of $< 1.7 \times 10^6$ pores/cm^2 [#140627 (polycarbonate membrane inserts); Nunc, Roskilde, Denmark], which harbored the RAW 264.7 cells (5×10^3 cells), was inserted into the lower one. No RANKL was added. The total volume of the assay was 500 μl of the McCoy's medium/FCS/Na-pyruvate. After a 3-day pre-incubation period the medium was replaced by fresh McCoy's medium/FCS/Na-pyruvate, and the cells were incubated in the absence or presence of the activation cocktail (10 nM dexamethasone, 50 mM ascorbic acid and 5 mM ß-glycerophosphate) for additional 7 days. Silicate was added to the incubation medium as indicated under "Results".

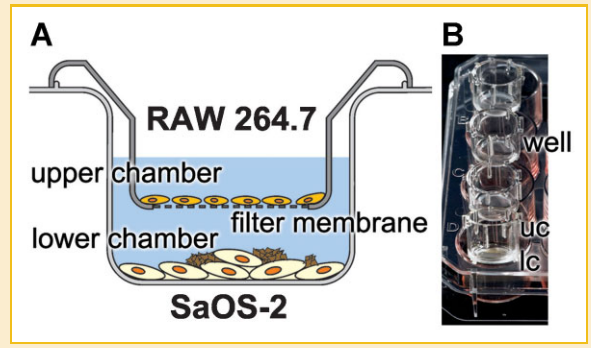

Fig. 1. The Boyden chamber assay system. A: Schematic representation outlining that the upper chamber contains RAW 264.7 cells, while into the lower chamber the SaOS-2 cell were seeded. The two compartments were separated by a microporous membrane filter. B: Picture of one row of a 24-well plate showing the upper four chambers (uc) standing on the lower chambers (lc).

SILICATE INDUCED MINERALIZATION OF SaOS-2 CELLS

The experiments were performed in the Boyden chamber assay, using McCoy's medium/FCS, Na-pyruvate. In this series of experiments the SaOS-2 cells were incubated onto plastic cover slips (Nunc, Rochester, NY), placed into the lower chamber. The upper chamber of the Boyden assay was seeded with RAW 264.7 cells. After an initial pre-incubation period for 3 days, the medium was changed and incubation was continued following the recently published protocol [Müller et al., 2011]. The cells in the Boyden chamber were grown either in the absence or the presence of the activation cocktail. After a 4-day incubation period, the slides were processed and stained with 10% Alizarin Red S (Sigma-Aldrich) for ossification/hydroxyapatite crystal formation test, as described [Young et al., 2000].

Alternatively, the intensity of Alizarin Red S staining was quantitatively assessed using the spectrophotometric assay [Gregory et al., 2004; Müller et al., 2011]. The cells were treated with acetic acid, and after centrifugation the supernatant was neutralized and the optical density was determined at 405 nm. The amount of bound Alizarin Red S is given in moles and the values were normalized to the total DNA in the assays using the PicoGreen method [Schröder et al., 2005].

QUANTITATIVE REAL-TIME RT-PCR ANALYSIS

Quantitative real-time PCR (qRT-PCR) determination of the *OPG*, *RANKL*, and *GAPDH* levels was performed as described [Wiens et al., 2010a; Wiens et al., 2010b]. Briefly, SaOS-2 cells were incubated in the Boyden chamber (lower compartment) together with the RAW 264.7 cells in McCoy's medium/FCS/Na-pyruvate. After the initial 3-day pre-incubation period, the cell medium was exchanged by fresh medium and incubation was continued for additional 1–7 days in the presence of the activation cocktail. In one series of experiments the medium was supplemented with 10 μM silicate. The cells were harvested and total RNA was extracted, freed from possible DNA contamination. Subsequently, first-strand cDNA was synthesized by the M-MLV reverse transcriptase (RT) (Promega, Mannheim; Germany). Each reaction contained approximately 5 μg

of total RNA in the reaction mixture of 40 μl. Finally, qRT-PCR experiments were performed in an iCycler (Bio-Rad, Hercules, CA), using 1/10 serial dilutions in triplicate. The reaction was supplemented with the SYBR Green master mixture (ABgene, Hamburg; Germany) and 5 pmol of each primer pair for the following four transcripts: For the house keeping gene *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase; GenBank accession number NM_002046.3) Fwd: 5′-ACTTTGTGAAGCTCATTTCCTGGTA-3′ [nt_{1019} to nt_{1043}] and Rev: 5′-TTGCTGGGGCTGGTGGTCCA-3′ [nt_{1117} to nt_{1136}] (product size 118 bp); *RANKL* (AF019047) Fwd: 5′-AGAGCGCAGATGGATCCTAA-3′ [nt_{339} to nt_{358}] and Rev: 5′-TTCCTTTTGCACAGCTCCTT-3′ [nt_{499} to nt_{518}] (180 bp); *OPG* (U94332.1) Fwd: 5′-GCAGCGGCACATTGGAC-3′ [nt_{937} to nt_{953}] and Rev: 5′-CCCGGTAAGCTTTCCATCAA-3′ [nt_{986} to nt_{1005}] (69 bp), as well as for *TRAP* [NM_001102405; Cuetara et al., 2006] 5′-AGCAGCCAAGGAGGACTACGTT-3′ [nt_{719} to nt_{740}] and Rev: 5′-TCGTTGATGTCGCACAGAGG-3′ [nt_{927} to nt_{938}] (220 bp). The threshold position was set to 50.0 relative fluorescence units above PCR subtracted baseline for all runs. Expression levels of *OPG* and *RANKL* were normalized to the reference gene *GAPDH*, essentially as described [Wiens et al., 2010a].

STATISTICAL ANALYSIS

The results were statistically evaluated using the paired Student's *t*-test [Sachs, 1984]. Then, the differences were corrected for multiple testing by applying the Bonferroni correction; the analyses were performed using the SAS software (version 9) [SAS/Stat User's Guide, 2003; SAS/STAT 9.2 User's Guide, 2009].

RESULTS

EFFECT OF SILICATE ON CELL PROLIFERATION/VIABILITY OF SaOS-2 AND RAW 264.7 CELLS

Both SaOS-2 cells and RAW 264.7 cells were incubated with increasing concentrations of silicate. The number of viable cells was determined by application of the XTT assay. It was found that increasing concentrations of silicate caused a higher growth rate of SaOS-2 cells. This increase was already significant at a concentration of 3 μM; maximal stimulation was seen at 30 μM (Fig. 2). In contrast, the growth rate of the RAW 264.7 cells was not affected in the concentration range of 3–100 μM silicate (Fig. 2).

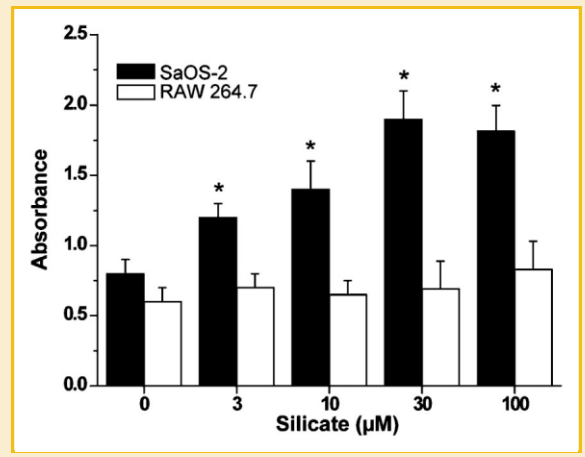

Fig. 2. Effect of silicate on the viability of SaOS-2 cells (black bars) or RAW 264.7 cells (white bars). After an incubation period of 3 days the number of cells was assessed applying the XTT assay. The results are expressed as Mean ± SD (10 experiments each and 2 replicates); * $P < 0.01$.

MINERALIZATION BY SaOS-2 CELLS IN VITRO IN THE PRESENCE RAW 264.7 CELLS AND DIFFERENT CONCENTRATIONS OF SILICATE

In order to verify that the SaOS-2 cells retain the capacity to undergo mineralization in the presence of RAW 264.7 cells, the SaOS-2 cells were seeded into the lower chamber, while the RAW 264.7 cells were grown in the upper one. Under the conditions described under "Materials and Methods" section, the SaOS-2 cells were grown in the presence of McCoy's medium/FCS/Na-pyruvate, onto cover slips. After the initial pre-incubation period of 3 days, the cultures were incubated in the medium in the absence or the presence of the mineralization/activation cocktail (10 nM dexamethasone, 50 mM ascorbic acid, and 5 mM ß-glycerophosphate) for 4 days. In both assays silicate was added after the pre-incubation period at increasing concentrations. After termination, HA formation of SaOS-2 cells was determined both semiquantitatively and quantitatively using the color reagent Alizarin Red S (staining for ossification/hydroxyapatite crystal formation) as described [Young et al., 2000] (Fig. 3). In the absence of any inducers, the extent of biomineralization was low as it is deduced from the color reaction of the cells with the dye (Fig. 3A [minus]). The extent of the mineralization did not substantially increase if silicate concentrations between 1 and 10 μM were added to the assays. Only at the higher concentration (30 μM), a visible increase in staining intensity by the color reagent can be noted. However, adding three components of the cocktail to the SaOS-2 resulted in a more intense red color reaction after 4 days, even in the absence of silicate (Fig. 3A [plus]). Addition of silicate resulted in a concentration-dependent increase in biomineralization, as can be deduced by the increase in red color staining caused by the color reagent; maximal stimulation was seen at 10 μM. In order to quantify this visual perception, the color reaction with Alizarin Red S was quantified spectrophotometrically. In the absence of the activation cocktail, the optical density values after the 4-day incubation period changed non-significantly in the presence of silicate from 0.05 ± 0.01 nmoles bound Alizarin Red μg^{-1} DNA to 0.04 ± 0.01 nmoles μg^{-1} DNA at 10 μM silicate and to 0.07 ± 0.02 nmoles μg^{-1} DNA in assays with 100 μM silicate (data not shown). However, after addition of the activation cocktail, silicate caused a significant concentration-dependent increase of mineralization, from 0.29 ± 0.04 nmoles μg^{-1} DNA (3 μM silicate) to 0.48 ± 0.08 nmoles μg^{-1} DNA (10 μM silicate); Figure 3B. At higher concentrations of silicate the SaOS-2 cells did not react with a further increase in Alizarin Red S binding (Fig. 3B).

In the control series it was ensured that the extent of mineralization of SaOS-2 cells was not significantly influenced by the co-existence of the RAW 264.7 cells during the incubation in the Boyden chamber (data not shown).

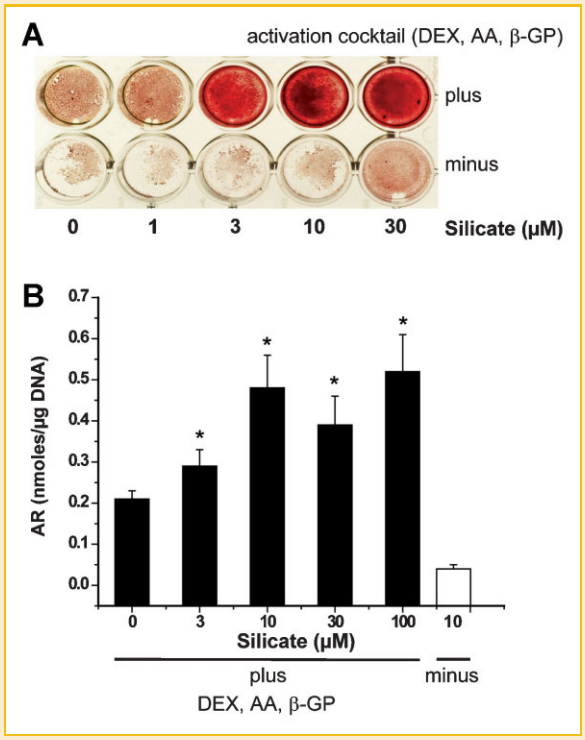

Fig. 3. Silicate-induced mineralization in SaOS-2 cells in the absence (minus) or presence (plus) of the activation cocktail [dexamethasone (DEX), ascorbic acid (AA), and ß-glycerophosphate (ß-GP)]. A: After a pre-incubation period of 3 days cultivation of the cells was continued for 4 days in the absence or presence of the cocktail. Silicate was added at increasing concentrations (1–30 μM). Then the cells were stained with Alizarin Red S for a semiquantitative assessment of HA crystal formation. B: Quantitative photometric determination of HA binding to Alizarin Red S in cells that had been induced with the cocktail (plus) in the presence of 3–100 μM silicate. As a control, the value of dye binding to cells treated with 10 μM silicate in the absence of the activation cocktail (minus) is shown. The results are expressed as Mean ± SD (five experiments each and two replicates); * $P < 0.01$.

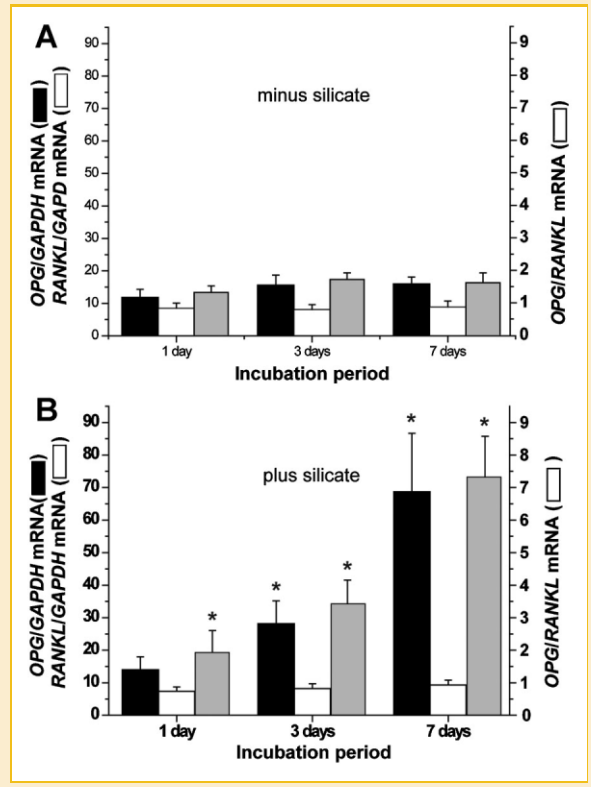

Fig. 4. Silicate-induced *OPG* expression of SaOS-2 cells. SaOS-2 cells were incubated in the Boyden chamber together with RAW 264.7 cells. After the pre-incubation period (3 days), the medium was replaced by fresh medium supplemented with the mineralization activation cocktail and incubation was continued up to 7 days. A: In one series the cultures were not treated with silicate, while (B) in the second series the cells were exposed to 10 μM silicate. Then the cells were removed and RNA was extracted from them. By application of qRT-PCR the expression levels of *OPG*, and *RANKL* as well as of *GAPDH* (house-keeping reference gene) was determined. The expression levels of *OPG* (filled bars) and of *RANKL* (white bars) were correlated/normalized to the transcript level of *GAPDH*. Finally, the *OPG/RANKL* ratio of expression was calculated. The results are expressed as Means ± SEM (five experiments each and two replicates); * $P < 0.05$.

EXPRESSION OF OPG AND RANKL IN SaOS-2 CELLS IN THE PRESENCE OF RAW 264.7 CELLS

Considering the finding that maximal HA formation by SaOS-2 cells in the Boyden chamber in the presence of the inducers (dexamethasone, ascorbic acid, ß-glycerophosphate) was seen at 10 μM silicate, this concentration was chosen for the quantification of the expression level of *OPG* and also of *RANKL*. After a 3-day pre-incubation period silicate was added and the steady-state expression levels were determined by qRT-PCR after 1, 3, and 7 days of incubation. The SaOS-2 cells were collected, RNA was prepared and the steady-state expression levels were determined. The data show that in the absence of silicate no significant change in the transcript level is measured (Fig. 4A). However, if the cultures in the presence of the inducers were exposed to 10 μM silicate a strong upregulation of the steady-state expression of the *OPG* transcripts was seen. The expression level of this gene after 1 day was 14.2 ± 4.8 (normalized to the *GAPDH* level) and increased significantly already after a 3-day incubation period (28.2 ± 7.1), and increased further during the 7-day incubation to 68.7 ± 18.6. In contrast, the level of *RANKL* remained unchanged. In consequence, the expression ratios between *OPG* and *RANKL* increased from 1.9 ± 0.7 (day 1) to 3.4 ± 0.7 (day 3) and finally to 7.3 ± 1.3 (day 7); Figure 4B.

GROWTH RATES OF RAW 264.7 CELLS IN THE PRESENCE OF SaOS-2 CELLS: MICROSCOPIC ANALYSIS

The Boyden chamber assay was used for the co-incubation studies. In an exemplary microscopic study SaOS-2 cells were seeded in the lower chamber on cover slips, while the RAW 264.7 cells were grown in the upper chamber (Fig. 5). The cultures were pre-incubated for 3 days and, after exchange of the medium, incubated for additional 7 days. During this incubation period the cultures remained either non-activated (Fig. 5A,C) or were exposed to the activation cocktail (Fig. 5B,D). After termination of the incubation the SaOS-2 cells were reacted with Alizarin Red S and the intensity of the dye was assessed light microscopically (Fig. 5 column a). In parallel the SaOS-2 cells were analyzed by SEM (Fig. 5 column b). Finally, the

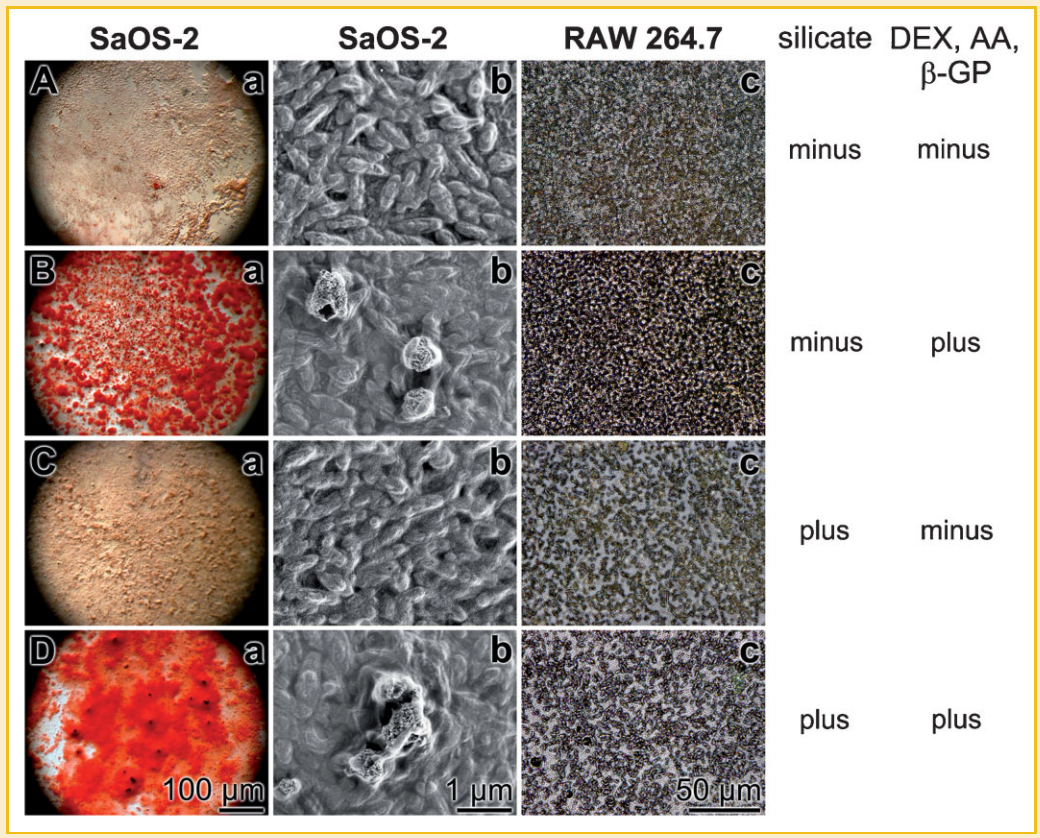

Fig. 5. Effect of silicate on non-activated and activated (with DEX, AA, ß-GP) SaOS-2 cells and on RAW 264.7 cells in the Boyden chamber assay. As indicated, the medium, McCoy's medium/FCS, Na-pyruvate, remained free of silicate (minus; row A, row B), or was supplemented with 10 μM silicate (plus; row C, row D). Furthermore the cultures remained non-activated (minus; row A, row C), or were exposed to the activation cocktail (plus; row B, row D). After an incubation period of 7 days, the SaOS-2 cells were stained with Alizarin Red S (column a) or inspected by SEM (column **b**) while the cell density of the RAW 264.7 cells was assessed by light microscopy (column c).

density of the RAW 264.7 cells present onto the filter membrane was visualized light microscopically (Fig. 5 column c). As expected the SaOS-2 cells showed, after exposure to the activation cocktail, a substantially more intense Alizarin Red S staining (Fig. 5Ba) than the non-activated cultures (Fig. 5Aa). This is also reflected by SEM images showing well developed HA crystallites on the surface of the cells (Fig. 5Bb), while there were no crystallites on the non-activated cells (Fig. 5Ab). Addition of silicate to the cultures resulted in a slightly more intense basal staining level in the absence of the activation cocktail (Fig. 5Ca) but a very strong color reaction if silicate had been added together with the activation cocktail (Fig. 5Da). In order to rule out a potential interference from the staining reaction of 5 mM ß-glycerophosphate with the Alizarin Red S, the experiments were performed with 5 mM inorganic phosphate instead of 5 mM ß-glycerophosphate, as outlined before [Müller et al., 2011]. Basically the same results were determined irrespectively if ß-glycerophosphate or inorganic phosphate was used in the assays as phosphate donor (not shown here). Recently, we also found that the HA crystallites/nodules, visualized by the SEM technique, are rich in calcium and phosphate, corroborating the conclusion that they are composed of HA or at least of Ca-phosphate crystals [Müller et al., 2011].

Very apparent was the reduction of the RAW 264.7 cell concentration in the Boyden chamber assay if the cultures were exposed to silicate. While the cell density was almost identical in assays in the absence of silicate with 28–32 cells per selected area in the activated assays and 30–37 cells/area in the assay incubated with the activation cocktail (Fig. 5Ac,Bc), the cell density was substantially lower in the silicate-treated assays. The cell counts in the non-activated assays varied between 4 to 9/area (Fig. 5Cc), while about 8 to 12/area in the activated assays (Fig. 5Dc).

DIFFERENTIATION STATE OF RAW 264.7 CELLS

Based on the data shown in the preceding paragraph it was suggestive to attribute the reduced cell growth to a reduced level of biologically active RANKL due to the observed higher expression level of *OPG*, in response to silicate exposure. It had been described that RANKL is required for the differentiation and proliferation of RAW 264.7 cells [Lång et al., 2009; Witte, 2010]. In order to determine the differentiation state of the RAW 264.7 cells, the established osteoclast marker protein TRAP was analyzed. The RAW 264.7 cells in the upper compartment of the Boyden chamber were stained for TRAP. Subsequently, the number of cells, reacting positively for TRAP (mono- or poly-nuclear $TRAP^+$ cells [Fujikawa et al., 1996]) were determined light microscopically. The data showed that within a given counting area the number of $TRAP^+$ cells decreased strongly and significantly after incubation of the cultures with 10 μM silicate from 201 ± 14/area to 73 ± 8/area

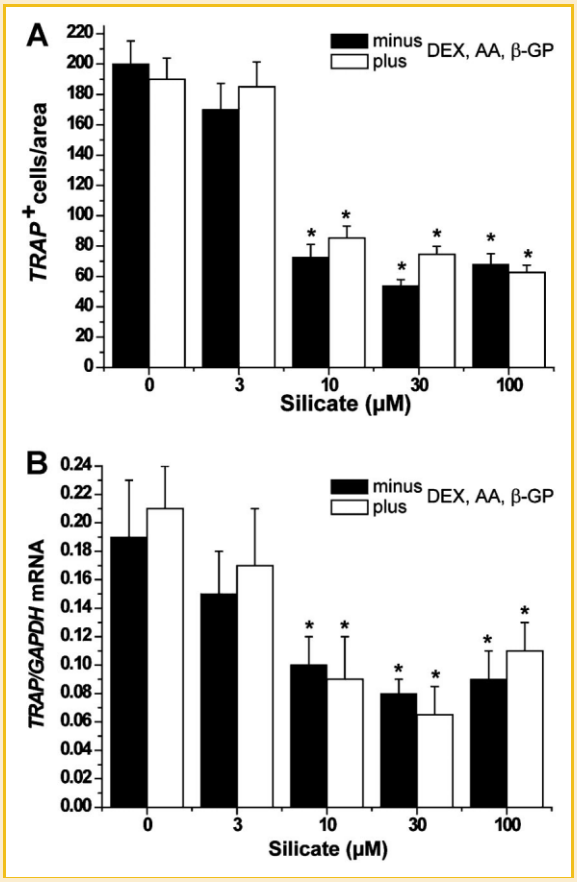

Fig. 6. Inhibition of growth/differentiation of RAW 264.7 cells after co-cultivation with SaOS-2 in the Boyden chamber. After the 3-day pre-incubation, the cultures remained non-activated (black bars) or had been exposed during the 7-day incubation period to the activation cocktail (white bars). During the incubation period silicate was added at concentrations between 0 and 100 μM to the assays. A: At the end of the incubation period the cells were reacted with antibodies and the percentage of $TRAP^+$ cells was determined. B: Expression of *TRAP* in RAW 264.7 cells after co-incubation with SaOS-2 cells in the presence of silicate. Standard errors of the means are shown (five experiments each and three replicates); $^*P < 0.05$

during the 7-day incubation period; higher silicate concentrations did not result in a further reduction (Fig. 6A). The reduction of the number of $TRAP^+$ cells was almost identical in cultures that remained non-activated or were exposed to the activation cocktail.

To substantiate this cytochemical observation, gene expression studies were performed for *TRAP* using the technique of qRT-PCR. These data showed that at silicate concentrations above 10 μM it caused an approximately 50% reduction of the steady-state expression for *TRAP* (Fig. 6B).

DISCUSSION

Major bone diseases, if focusing on HA formation, are the result of pathophysiological interactions between both functional osteoclasts and functional osteoblasts. Increase in the number and also in the size of the osteoclasts, as seen, e.g., during human breast cancer [Clohisy et al., 1996], results in a pronounced osteolysis. It is not only the number of the cells but also the activity of the osteoclasts that cause a dissolution of the HA bone material, e.g., increased H^+ release or enhanced activity of secreted proteolytic enzymes (cysteine proteinase cathepsin K) [Clohisy et al., 1996; Henriksen et al., 2008]. The prime dominator of the differentiation pathway of precursor cells to osteoclasts and also for the activation of osteoclasts, is the osteoclast differentiation factor RANKL [Schramek et al., 2010]. The expression of this mediator is physiologically under the control of 1,25 dihydroxycholecalciferol ($1,25(OH)_2D_3$) and parathyroid hormone (PTH) [see: Lu et al., 1997] and depends on the activation of the element mRL-D5 within the promoter region upstream of *RANKL* [Bishop et al., 2011]. In addition to that it is OPG that regulates the functional state of RANKL (see "Introduction" section).

Several xenobiotics have been found to modulate the functional activity of RANKL within the OPG/RANK/RANKL triad system, e.g., nonsteroidal anti-inflammatory drug (NSAID), PGE2 (prostaglandin) [Moreno-Rubio et al., 2010], and in the broad sense also estrogen and selective estrogen receptor modulators, bisphosphonates, Ca^{2+} flux regulators, and monoclonal antibodies, e.g., denosumab [Masuyama et al., 2008]. Recently, inorganic compounds, first and foremost silicon/silicate-based components [Jugdaohsingh, 2007; Wiens et al., 2010b] and then also polymeric phosphate, complexed with Ca^{2+} [Müller et al., 2011] have been described as promising, potential antiosteoporotic agents. Unanticipated came the finding that silica, in the form of bio-silica, causes a differential gene expression and enhances *OPG* expression while, simultaneously, *RANKL* expression remains unchanged [Wiens et al., 2010b]. This finding, obtained on molecular level, was confirmed by a quantification of the cytokines RANKL and the decoy receptor OPG in the supernatant of SaOS-2 cells, using ELISA assay systems [Wiens et al., 2010b].

From the data mentioned, the important open question remained, if RANKL produced in (human-derived) SaOS-2 cells also affects osteoclasts in in vitro system. Following this line, we used murine-derived RAW 264.7 cells which had previously been found to respond to heterologous RANKL [Tanaka et al., 2006] to determine such an effect and the Boyden two chamber assay system was applied. In this assay, the target cells, RAW 264.7, were seeded into the upper chamber. To determine the effect of defined concentrations of silicate, we applied orthosilicate for the assays, which undergoes partial oligomerization under the culture conditions used, instead of polymeric silica in the experiments as used previously [Wiens et al., 2010b]. In the first series of experiments, described here, it was shown that silicate did not affect the growth of RAW 264.7 cells, while, within the concentration range of 3–100 μM, silicate caused a growth stimulatory effect on SaOS-2 cells. The first highly significant effect was seen in the presence of the activation cocktail at 10 μM silicate, a concentration that has been subsequently used for the additional experiments. Exposure under such conditions resulted, as expected, in an increased steady-state expression of *OPG* while no change was seen for the level of *RANKL*. Based on this finding on gene expression level, and in view of the previous transcriptional as well as translational analyses [Wiens et al., 2010b], it was obvious that SaOS-2 cells produce in the

presence of silicate an increased level of OPG while the production of RANKL remains simultaneously unchanged. On this ground the crucial experiment was performed by determining the effect of silicate in the co-cultures of SaOS-2 cells with RAW 264.7 cells; both cell types were separated by a porous filter, only allowing solute material to pass through. In this system it was quantitatively determined that after exposure of SaOS-2 cells to silicate one (OPG) or perhaps more (until now unknown) factors are released from the osteosarcoma cells that cause a reduced growth/differentiation of RAW 264.7 cells. This effect on RAW 264.7 cells might be attributed alone to the increased level of OPG that is synthesized by SaOS-2 cells after exposure to silicate. This endogenous decoy receptor binds to RANKL and results in an abolishment of the binding activity to RANK (as shown by others; see ''Introduction'' section and reviewed in Wang et al. [2012]). We found that the number of $TRAP^+$ cells in the co-cultivation assay, after treatment with different silica concentrations, was approximately equal in cultures that remained non-activated and in cultures that had been exposed to the activation cocktail. Since we also showed that the HA biomineralization process in SaOS-2 cells in response to silicate is enhanced in the presence of the activation cocktail, we expected a stronger reduction of $TRAP^+$ cells in assays with the activation cocktail. Therefore, we postulate that an additional factor, other than OPG, might act on the growth/differentiation state of the RAW 264.7 cells. One potential candidate is osteocalcin which is produced in SaOS-2 cells [Pautke et al., 2004] and assumed to impair the (recruitment and) differentiation of bone-resorbing cells [Glowacki et al., 1991]. It remains to be studied if in SaOS-2 cells, cultured in the absence of the activation cocktail, a differential expression of OPG and RANKL occurs as well. The answer to this important research issue might open an additional important aspect in the potential application of silica in the therapy of human bone diseases.

Silicate has one unique characteristic that it is a physiological inorganic compound, required for the physiological growth of bone material [reviewed in: Jugdaohsingh, 2007]. Even more, in view of the in vitro data presented here and previously [Wiens et al., 2010b], it has the potential to compensate for the loss of bone material in osteoporotic disorders. The mode of action by which silicate displays its effect on gene expression, and in turn on the differential increase in the steady-state transcript level of OPG remains unclear. In lower metazoans, in siliceous sponges, silica is taken up by a special $Na^+/HCO_3^-[Si(OH)_4]$ (NBCSA)-related transporter [Schröder et al., 2004]. The uptake mechanism of silica in higher metazoans is not known. In view of the existing data it appears to be likely that simple uncharged silica species, e.g., orthosilicic acid, is easier to permeate cell membranes than polymeric/colloidal species of silica [reviewed in: Jugdaohsingh, 2007]. Focusing on the gene transcription machinery, no silica-responsible transcription factors or promoter elements have been described till now.

CONCLUSION

The data presented here underscore that silica/silicate displays a significant increase of the ratio between the steady-state expression of the genes encoding *OPG* and *RANKL* and a distinct reduction of the growth/differentiation potential of RAW 264.7 macrophage cells that differentiate into osteoclast-like cells in vitro. Hence, these data contribute to a further profiling of silicate as a useful/necessary additive to the human diet in general [Spector et al., 2008] and as a potential ameliorating medical component for treatment in osteoporosis in particular [Jugdaohsingh, 2007]. Silicate acts in vitro via two arms, first by inducing osteoblasts to a state allowing an increased secretion of OPG and second, through an inhibition of the differentiation of osteoclast precursor cells. Now, experiments are ongoing to prove that RANKL is the major effector molecule from SaOS-2 cells that influences both the growth kinetics and the stages of osteoclastic differentiation of RAW 264.7 cells in the two chamber assay system. Besides RANKL a potential extracellular signal mediator of osteoclast differentiation might be extracellular reactive oxygen species (ROS). ROS had been suspected to affect angiotensin II which in turn impairs the synthesis of DNA and collagen as well as of the release of alkaline phosphatase in bone cells [Lamparter et al., 1998]. Until now only the generation of intracellular ROS had been identified to be a signaling factor during the RANKL/RANK controlled activation of the serine-threonine kinase Akt, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and mitogen-activated protein kinases (MAPKs) signaling pathways [Ha et al., 2004]. In the course of these planned experiments, the influence of 1,25(OH)2D3 and PTH on SaOS-2 cells, cultivated together with RAW 264.7 cells, will also be investigated to clarify if those agents can further stimulate the silicate-mediated up-regulation of the *OPG* expression—and in turn—cause an increased OPG/RANKL ratio [Takayanagi, 2007]. Taken together, the data presented here corroborate earlier suggestions that silica/silicate might have a considerable biomedical potential for the treatment and prophylaxis of osteoporotic disorders [Wang et al., 2012].

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