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Morphogenetic Activity of Silica and Bio-silica on the Expression of Genes Controlling Biomineralization Using SaOS-2 Cells

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Abstract In a previous study (Schröder et al., J Biomed Mater Res B Appl Biomater 75:387–392, 2005) we demonstrated that human SaOS-2 cells, when cultivated on bio-silica matrices, respond with an increased hydroxyapatite deposition. In the present contribution we investigate if silica-based components (Na-silicate, tetraethyl orthosilicate [TEOS], silica-nanoparticles) (1) change the extent of biomineralization in vitro (SaOS-2 cells) and (2) cause an alteration of the expression of the genes amelogenin, ameloblastin, and enamelin, which are characteristic for an early stage of osteogenesis. We demonstrate that the viability of SaOS-2 cells was not affected by the silica-based components. If Na-silicate or TEOS was added together with ß-glycerophosphate, an organic phosphate donor, a significant increase in biomineralization was measured. Finally, expression levels of the amelogenin, ameloblastin, and enamelin genes were determined in SaOS-2 cells during exposure to the silica-based components. After exposure for 2 days, expression levels of amelogenin and enamelin strongly increased in response to the silica-based components, while no significant change was seen for ameloblastin. In contrast, exposure of SaOS-2 cells to ß-glycerophosphate resulted in increased expression of all three genes. We conclude that the levels of the structural molecules of the enamel matrix, amelogenin and enamelin, increase in the presence of silica-based components and substantially contribute to the extent of hydroxyapatite crystallite formation. These results demonstrate that silica-based components augment hydroxyapatite deposition in vitro and suggest that enzymatically synthesized bio-silica (via silicatein) might be a promising route for tooth reconstruction in vivo.

Keywords Biomineralization · Crystal structure/crystallinity · Odontoblast · Ameloblast · Hydroxyapatite

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In general, morphogenesis is one of three fundamental aspects in developmental biology; it requires differential gene expression, cell growth, and cellular differentiation. Morphogenesis involves the control of an organized spatial distribution of cells, which gives rise to the characteristic forms of tissues/organs/overall body anatomy. The formation of hard tissue from inorganic components present in the extracellular space requires cellular activities that govern nucleation, growth, morphology, and final location of the mineral deposits [1]. For biomineralization processes in most metazoans, from the phylogenetically oldest metazoan phylum (Porifera) [2] to the vertebrates [3], the

extracellular structural, filamentous collagen net serves as an organic layer around the skeletal elements. Surely, collagen controls the form, shape, and size of the mineralization products; but likewise important are the additional organic matrices which exist around and within the extracellularly mineralized deposits.

During vertebrate tooth formation, enamel, the hardest and most highly mineralized substance of the vertebrate body, is formed on an organic matrix which is produced by the ameloblasts. The enamel matrix proteins can be grouped into (1) amelogenin and (2) nonamelogenin proteins, including tuftelin, ameloblastin, and enamelin [4]. The amelogenins are low-molecular weight proteins (25 kDa) that are found in developing tooth enamel; after release from the cells, amelogenins assemble through their anionic hydrophobic regions and form globular aggregates, nanospheres. These spheres build the lattices that guide the spacing and the orientation of enamel crystallites [5]. Among the nonamelogenin proteins, ameloblastin contributes to 5–10% of all enamel proteins; it controls the elongation of the enamel crystals and serves as a sheet protein to stabilize the Tomes processes to the enamel matrix [reviewed in 4, 6, 7]. Enamelin is a high-molecular weight acidic protein, which undergoes progressive degradation to a 32-kDa polypeptide [8] that binds to enamel crystallites [reviewed in 4, 9]. Hence, these three enamel matrix proteins – amelogenin, ameloblastin, and enamelin – constitute functional significance in controlling crystal formation in the enamel: amelogenin self-assembles to nanospheres, which are directly involved in the initial mineralization process [10]; ameloblastin functions as a modulator of ameloblast cell function and the enamel extracellular matrix [11]; and enamelin is an essential component controlling an ordered biomineralization process [12]. The initiation of tooth formation is understood to a considerable extent on the gene expression level, especially with respect to the heterodimeric transcription factors; e.g., the core-binding factors [13] or homeobox genes, e.g., *HOXA10*, are known to be necessary for embryonic patterning of skeletal elements and perhaps for bone formation [14]. In addition, the direct and indirect involvement/regulation of rare elements during the expression of osteoblast phenotypic genes has been extensively studied [see, e.g., 15, 16]. One direction of bone/tooth restoration is the application of biocompatible glasses, composed, e.g., of CaO, P_2O_5, SiO_2, and Al_2O_3, which have the potency to modulate the expression of genes involved in bone morphogenetic protein (BMP)-induced bone formation [17, 18].

In earlier studies experimental evidence was presented indicating that silicon, an essential trace element in nutrition, is required for bone and cartilage formation [19, 20]. More specifically, it had been documented that prior to ossification (hydroxyapatite formation) silicon deposition occurs, an effect that was found not to depend on the action of vitamin D [21]. Furthermore, silicon causes growth promotion of mammals [22]. This element is a component of glycosaminoglycans and their protein complexes have been implicated in structural functions [23]. Moreover, it was found that silicon accelerates bone mineralization. These finding have been extended by the finding that biogenic silicon is colocalized with hydroxyapatite in vertebrate bone tissue [24]; very likely silicon exists in bone as oligomerized silicic acid. In support of these observations, we recently demonstrated, by in vitro experiments with SaOS-2 cells, that bio-silica stimulates calcium-phosphate deposition [25]. This has been taken as strong evidence for the beneficial property of bio-silica in tissue engineering and/or for applications with medical implants. Bio-silica is – like opal – an amorphous $SiO_2{\cdot}nH_2O$ mineraloid "gel," provided with unusual mechanical properties, combining strength, stiffness, and toughness [26].

Silica (bio-silica), oligo-/polymerized silicic acid, is synthesized in siliceous sponges, more specifically in their skeletal elements, the spicules. On average, the water content of siliceous spicules is approximately 10%, providing them with high stability and simultaneous flexibility [27]. Bio-silica (polymerized/polycondensated from silicic acid) is synthesized enzymatically by silicatein, a protein that has been isolated from several siliceous sponges, e.g., *Tethya aurantium* [28, 29] and *Suberites domuncula* [30–33]. This silica material represents the inorganic material of the spicules of demosponges, which is embedded into an organic matrix [34, reviewed in 35]. The skeletal elements function as the stabilizing scaffold, around which the sponge cells arrange and which allows formation of the characteristic body plan [36]. Previous experiments also revealed that bio-silica is nontoxic for mammalian cells, already suggesting its biocompatibility [25, 34]. Also interesting is the finding that in sponge tissue siliceous spicules are dissolved enzymatically by silicase, an enzyme which is closely related to carbonic anhydrase [2, 37, 38].

In the present study we investigated if silicic acid (and tetraethyl orthosilicate [TEOS], which hydrolyzes in aqueous solution to silicic acid) and bio-silica have an effect on the expression of the genes that control the formation of enamel: amelogenin, ameloblastin, and enamelin. The experiments were performed with human osteosarcoma SaOS-2 cells in vitro. SaOS-2 cells have been demonstrated to contain extractable bone-inducing agents that can induce heterotopic bone formation in mice [39]. Subsequently, these cells were considered to possess osteoblastic properties [40] and had been termed

"osteoblast-like" cells [41]. Finally, SaOS-2 cells had been successfully used for osteoblastic differentiation and the development of biomaterials for surgical implants [42]. To the best of our knowledge, these cells have not yet been used for expression studies of the amelogenin, ameloblastin, and/or enamelin genes.

We used SaOS-2 cells as a model cell line since they are considered as a useful model for in vitro investigation of mechanisms in morphogenesis [43]. The steady-state expression of amelogenin, ameloblastin, and enamelin strongly increases after exposure of SaOS-2 cells to ß-glycerophosphate (as shown in this study), a potent organic phosphate donor [44]. The results summarized here indicate that silicic acid and nanoparticles, obtained from spicules by mechanical disintegration, cause differential gene expression. While expression of amelogenin and enamelin is upregulated, that of ameloblastin remains unchanged.

Materials and Methods

Materials

Na-silicate (orthosilicate), alizarin red-S, tetraethoxysilane (TEOS), ß-glycerophosphate, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO); Dulbecco's modified Eagle medium, McCoy's medium, penicillin, streptomycin, ascorbic acid, and fetal calf serum (FCS) were from GIBCO (Grand Island, NY); bovine serum albumin was from Roth (Karlsruhe; Germany); RPMI 1640 medium was from Biochrom (Berlin, Germany), and PicoGreen dsDNA Quantitation Reagent was from Invitrogen (Karlsruhe, Germany). SaOS-2 cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Bio-silica Nanoparticles

Spicules were isolated from specimens of the marine sponge *S. domuncula* (Porifera, Demospongiae, Hadromerida) that had been collected in the northern Adriatic near Rovinj (Croatia). Clean spicules were obtained by treatment with nitric acid/sulfuric acid (1:4 v/v) [45]. Spicule nanoparticles with an average size of 30 nm were obtained by mechanical disintegration using a homogenizer (Peqlab, Erlangen, Germany). The spicules were suspended in distilled water and broken by treatment for eight cycles (25 seconds each, 6,800 rpm) using the Precellys 24 head. The steel beads had a diameter of 2.8 mm.

Cells and Incubation Conditions

SaOS-2 cells (human osteogenic sarcoma cells [46]) were cultured in RPMI 1640 medium, with 10% heat-inactivated FCS, 2 mM L-glutamine, and 10 U/mL penicillin/streptomycin on plastic surfaces of six-well culture plates (4.5 cm^2; Nunc, Wiesbaden, Germany) at 37°C in 5% CO_2 and 95% air. All experiments were carried out at least in quadruplicate and repeated three times each.

For the mineralization studies, SaOS-2 cells ($10^5/cm^2$) were incubated in McCoy's medium, supplemented with 10% FCS, antibiotics, and 50 µM ascorbic acid in either tissue culture flasks (24 cm^2) or 60-mm Petri dishes for 1 day (expression studies) up to 14 days (mineralization experiments), as described [47, 48]. The cultures either remained untreated or were incubated with 5 mM ß-glycerophosphate, 20 µM Na-orthosilicate, 60 µM TEOS, or 50 µg/mL spicule nanoparticles in different combinations, as indicated with the experiments. The standard inducer of mineralization, ß-glycerophosphate, was added either alone or together with the silica-based component (Na-silicate, TEOS, or spicule nanoparticles).

Cell Viability

To determine the viability of SaOS-2 cells, the procedure described by Mosmann [49] was used, which is based on the MTT colorimetric assay. SaOS-2 cells were seeded in 96-well cluster plates (Nunc) at a concentration of 10^3/well and incubated in McCoy's medium (supplemented with FCS, antibiotics, and ascorbic acid) together with ß-glycerophosphate, Na-silicate, TEOS, or spicule nanoparticles for 1–3 days. MTT solution was added (4 hours at 37°C), the dye was eluted from the wells with acidified isopropanol, and absorbance was measured at 570 nm. Wells without cells but with medium were used as negative controls.

Quantitation of Mineralization

SaOS-2 cells were incubated in tissue culture flasks (24 cm^2). The formation of mineralized nodules was quantified by the method of Stanford et al. [50]. Briefly, after the indicated incubation period, medium was removed and the cells were washed with phosphate-buffered saline (PBS) and subsequently fixed with ice-cold 70% ethanol. Then, the cells were stained with 40 mM alizarin red-S (pH 4.2, 10 minutes, 20°C). Stained cell layers were rinsed with distilled water and then with PBS to remove nonspecific stain. After documentation, the bound stain was eluted with 10% (w/v) cetylpyridinium chloride and absorbance of the

solution was measured at 562 nm. The values were normalized to the DNA content as follows. Total DNA in SaOS-2 cells was determined in separate cultures using PicoGreen dsDNA Quantitation Reagent. The samples were diluted tenfold in TE buffer (pH 7.4; 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid [EDTA]) and mixed with PicoGreen (100 µL, diluted 1:200 in TE buffer). After incubation in the dark (5 minutes), the samples were measured in a fluorescence enzyme-linked immunosorbent assay plate reader (Fluoroscan version 4.0; Labsystems, Helsinki, Finland) at 485 nm excitation and 538 nm emission. Calf thymus DNA was used as a standard.

Electron Microscopy

Scanning electron microscopy (SEM) analysis was used to study the morphological characteristics of cells in culture [25]. Medium was removed and the cells were fixed in 2.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.8), dehydrated in graded ethanol series, and air-dried. Then, the plates were mounted on aluminum stubs (SEM-Stubs G031Z; Plano, Wetzlar, Germany) that had been covered with adhesive carbon (carbon adhesive Leit-Tabs G3347, Plano, Wetzlar, Germany) and inspected.

Gene Expression Studies: Northern Blot Analysis

RNA from stimulated and nonstimulated SaOS-2 cells was isolated with TRIzol reagent (GIBCO BRL, Grand Island, NY) as described [51] and then repurified using the SNAP Total RNA Isolation Kit (Invitrogen). Total RNA (5 µg) was electrophoresed and blotted onto a Hybond-N^+ nylon membrane (Amersham, Little Chalfont, UK). Hybridization was performed with the following probes: (1) human amelogenin ([52] accession number BC069118; nt_{245} to nt_{628} of the cDNA was amplified by polymerase chain reaction [PCR]), (2) human ameloblastin ([53] AF219994, nt_{331} to nt_{906}), (3) human enamelin ([54] AF125373, nt_{3180} to nt_{3711}). The probes were labeled with the PCR-DIG-Probe-Synthesis Kit according to the manufacturer's instruction manual (Roche, Mannheim, Germany). After washing, digoxigenin (DIG)-labeled nucleic acid was detected with anti-DIG Fab fragments (conjugated to alkaline phosphatase, dilution of 1:10,000) and visualized by a chemiluminescence technique using CDP according to the instructions of the manufacturer (Roche). In one series of experiments, RNA was loaded onto the gels to demonstrate the equalization of the RNA samples used for analysis; agarose gel was stained with ethidium bromide (1 µg/mL). Where indicated, the screens (blots) were scanned with the GS-525 Molecular Imager (Bio-Rad, Hercules, CA).

Statistical Analysis

An unpaired two-tailed Student's *t*-test was applied to determine significance [55]; results at $P < 0.05$ were considered to be statistically significant.

Results

Viability Testing

The effect of the inducers of mineralization used in this study on viability of SaOS-2 cells was determined by the MTT assay. Experiments were performed with 5 mM ß-glycerophosphate, 20 µM Na-silicate, 60 µM TEOS, or 50 µg/mL spicule nanoparticles. These nanoparticles were obtained from *S. domuncula* spicules by mechanical disintegration. They had sizes between 10 and 100 nm, with an average of 30 nm (Fig. 1A, B). The components (ß-glycerophosphate, Na-silicate, TEOS, or spicule nanoparticles) were added to the SaOS-2 cells for 1–14 days. In the MTT assays the absorbance values (570 nm) did not differ significantly between the controls and the test assays during the 3-day incubation period (data not shown), indicating that these components do not cause adverse effects to SaOS-2 cells under the conditions used.

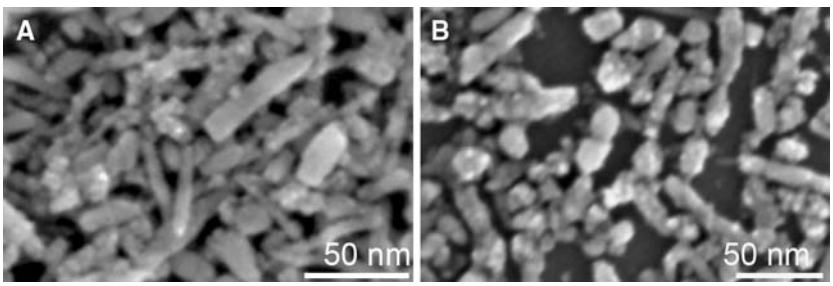

Fig. 1 Siliceous nanoparticles were obtained from spicules of the sponge *S. domuncula* by mechanical disintegration, as described under "Materials and Methods." The spicule nanoparticles (**A, B**) were inspected by SEM

Effect of Silica-Based Components on ß-Glycerophosphate-Induced Phosphate Mineralization

ß-Glycerophosphate is a well-established inducer of organophosphate donor/bone mineralization in mammalian cells [50]. For quantification, the alizarin red-S assay was applied, and the values were normalized to the DNA content in the samples. If ß-glycerophosphate was added to SaOS-2 cells, calcium mineralization started to increase strongly after 7 days (Fig. 2). The extent of mineralization increased from 0.054 nmole alizarin red-S/µg DNA (x10) at day 2 to 0.26 nmole/µg (x10) at day 7. Interestingly, if ß-glycerophosphate was applied together with a silica-based component(s), a stronger increase in calcium phosphate mineralization was measured. The increase was significant ($P < 0.05$) if 20 µM Na-silicate was added simultaneously with 5 mM ß-glycerophosphate (0.37 nmole/µg [x10]). If correlated with the mineral formation after exposure to ß-glycerophosphate alone (set to 100%), the stimulation caused by Na-silicate together with ß-glycerophosphate was 142% after 7 days or 288% after 14 days (0.75 nmole/µg [x10]) (Fig. 2). In controls it had been assured that in the absence of any inducer – or presence of Na-silicate alone – no considerable mineralization could be measured with alizarin red-S. Besides Na-silicate, also TEOS, which is an organo-silica compound hydrolyzing into silicic acid, was studied. This compound, administered together with ß-glycerophosphate, caused after an incubation period of 14 days a significant stimulation of mineralization compared to the amount measured with ß-glycerophosphate alone (Fig. 2). The increase in mineralization measured after exposure of the cells to nanoparticles together with ß-glycerophosphate (to 148% at day 14) was statistically not significant compared to ß-glycerophosphate alone (set to 100%).

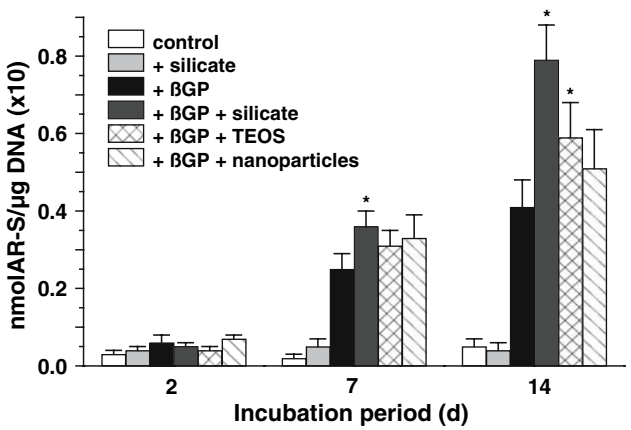

Fig. 2 Effect of silica-based components on ß-glycerophosphate-mediated phosphate mineralization. Mineralization was quantified by staining with alizarin red; values were normalized on the basis of DNA content in the culture. Cultures were incubated in the absence (*open bars*) or presence (*light gray bars*) of 20 µM Na-silicate. In a separate series of experiments, ß-glycerophosphate (5 mM) was incubated alone (*filled bars*) or was coincubated together with either 20 µM Na-silicate (*dark gray bars*), 60 µM TEOS (*cross-hatched bars*), or 50 µg/mL spicule nanoparticles (*hatched bars*) for a period of 2–14 days. Data are means ± standard deviation, $n = 4$ per value

To demonstrate that indeed mineralization occurred during the incubation period, SaOS-2 cells were incubated for up to 10 days in the presence of 5 mM ß-glycerophosphate. In the absence of ß-glycerophosphate, the surface of the flattened, lobulated cells remained smooth (Fig. 3A). If ß-glycerophosphate was added, the shape and the smooth surfaces of the cells remained like in the controls during the first 2 days (Fig. 3B). Subsequently, after incubation with ß-glycerophosphate for >5 days, 100-nm small lobules formed at the surfaces of the cells and 20–50 nm long small rods, which are present in clusters, appeared as nodules (Fig. 3C, D). During 10 days of incubation in the presence of ß-glycerophosphate, the number of rods increased under formation of 250-nm large clusters, or nodules (Fig. 3F); in control cells which were not treated with ß-glycerophosphate these rod clusters were absent (Fig. 3E).

Differential Expression of Amelogenin, Ameloblastin, and Enamelin in Response to Silica-Based Components

In Northern blot studies the differential expression of those genes which are involved in enamel formation was determined. SaOS-2 cells were incubated for 2 days in McCoy's medium (plus ascorbic acid) in the absence of any inducer or in the presence of 5 mM ß-glycerophosphate, 20 µM Na-silicate, 60 µM TEOS, or 50 µg/mL spicule nanoparticles. Subsequently, RNA was extracted and subjected to Northern blot analysis using the labeled human cDNAs for ameloblastin, amelogenin, or enamelin as probes. In a control series an aliquot of the extracted RNA was size-separated in an agarose gel and stained with ethidium bromide; almost the same intensities of the signals for 18S and 28S rRNA were seen in all RNA extracts (Fig. 4). In the absence of any inducer, only very low levels of ameloblastin and enamelin transcripts could be detected, while (almost) no expression was seen for the amelogenin gene (Fig. 4).

However, if the cells were incubated with either 5 mM ß-glycerophosphate, 20 µM Na-silicate, 60 µM TEOS, or 50 µg/mL spicule nanoparticles, strong signals were seen for amelogenin transcripts (0.6-kb band); the signals of transcripts in the RNA extract from cells incubated with ß-glycerophosphate and Na-silicate were almost similarly strong (Fig. 4). The amelogenin levels, obtained from RNA of cells incubated with TEOS or with nanoparticles,

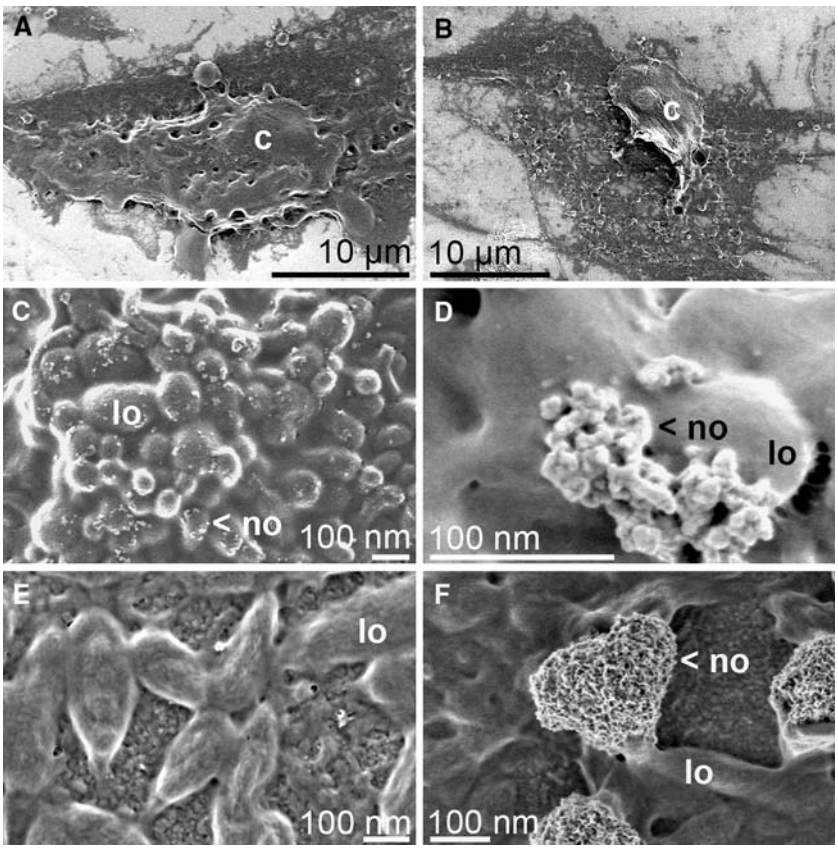

Fig. 3 Formation of rods on the surface of the lobules present on SaOS-2 cells. **A** SaOS-2 cells (*c*) grown in McCoy's medium, supplemented with 10% FCS, antibiotics, and 50 µM ascorbic acid for 2 days. **B** The cells incubated in the same medium, together with 5 mM ß-glycerophosphate for 2 days. **C, D** After 5 days in culture with 5 mM ß-glycerophosphate, small rods appear on the lobulated SaOS-2 cells, which represent mineralizing nodules (< *no*). **E** SaOS-2 cells with their lobulated surface (*lo*) grown for 10 days in cultures without ß-glycerophosphate; no mineralizing nodules are seen. **F** Large rod clusters, or nodules (< *no*), are formed during an extensive incubation period (10 days) in the presence of ß-glycerophosphate

reached 35% (TEOS) and 30% (nanoparticles) of the level measured in RNA from cells incubated with ß-glycerophosphate (set to 100%). Checking in parallel the expression level of the ameloblastin gene in RNA (1.6-kb band) from cells incubated with the silica-based components, lower transcript levels compared to RNA from ß-glycerophosphate-incubated cells (100%) were seen. The steady-state expression in SaOS-2 cells incubated with Na-silicate (15%) was low, while in cells exposed to TEOS or nanoparticles almost no transcripts were detectable. These experiments were performed in triplicate, and all confirmed that the effect of silicic acid, TEOS, or nanoparticles on the expression of the ameloblastin gene is low and varies between 5% and 15% compared to the transcript level seen with ß-glycerophosphate (100%). Finally, the steady-state level of transcripts was determined with the enamelin probe (5.3-kb band in Northern blots); here the level was highest in RNA from cells treated with ß-glycerophosphate (100%), still extensive in Na-silicate-treated cells (85%), and lower in cells treated with TEOS (45%) or nanoparticles (32%) (Fig. 4).

These results show that the silica-based components caused differential gene expression: upregulation of amelogenin and enamelin genes and only low (or no) expression of the ameloblastin gene.

Kinetics of Steady-State Expression of Amelogenin, Ameloblastin, and Enamelin in Response to Na-Silicate

In order to support the data summarized in the previous section, Na-silicate to increase the steady-state expression of amelogenin and enamelin, time kinetic experiments were performed (Fig. 5) and the intensities of the signals on the blots were densitometrically analyzed. The Northern blot experiments show that the increase of the steady-state expression of both the amelogenin and enamelin genes was measurable already after 1 day; the level of both transcripts increased over tenfold compared with the level at time 0. In contrast, the level of ameloblastin remained almost unchanged. A prolonged incubation for an additional 1 day (total 2 days) or 4 days (5 days) further increased expression of the amelogenin and enamelin genes. In contrast, the level of ameloblastin transcripts did not change during the 5-day incubation with Na-silicate.

Expression of Amelogenin, Ameloblastin, and Enamelin during Coincubation of Na-Silicate with ß-Glycerophosphate

A further result of the expression studies was that the level of steady-state transcripts of amelogenin and enamelin

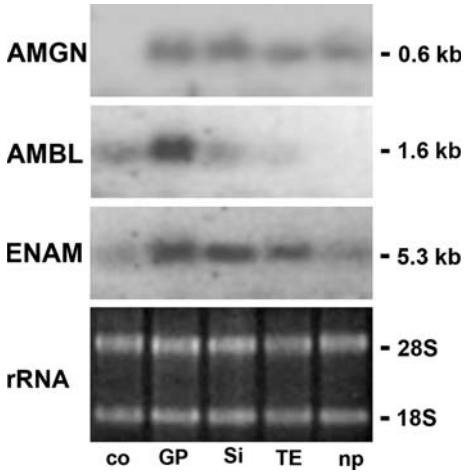

Fig. 4 Expression of the genes encoding the polypeptides amelogenin (AMGN), ameloblastin (AMBL), and enamelin (ENAM). RNA was isolated from SaOS-2 cells after an incubation period of 2 days in the absence of any inducer of mineralization (*co*) or in the presence of 5 mM ß-glycerophosphate (*GP*), 20 µM Na-silicate (*Si*), 60 µM TEOS (*TE*), or 50 µg/mL spicular nanoparticles (*np*). The same amounts (5 µg) of RNA were loaded onto the gels and size-separated. After blot transfer, the filters were hybridized with the labeled probes for human amelogenin (AMGN), human ameloblastin (AMBL), and human enamelin (ENAM). After washing, DIG-labeled nucleic acid was detected with anti-DIG Fab fragments (conjugated to alkaline phosphatase, dilution of 1:10,000) and visualized by chemiluminescence. To assure that the same amount of RNA was loaded onto the gels, a parallel gel was stained with ethidium bromide; the 18S and 28S rRNA bands are marked

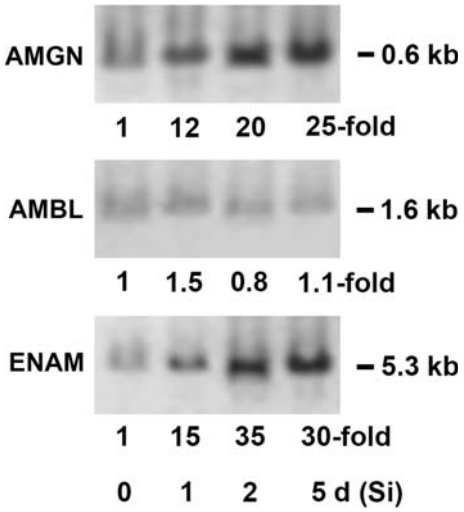

Fig. 5 Kinetics of expression of amelogenin, ameloblastin, and enamelin in SaOS-2 cells in response to Na-silicate. Cells were incubated with 20 µM Na-orthosilicate for 1–5 days. Total RNA was prepared, and 5-µg aliquots were electrophoresed, blotted onto a membrane, and hybridized with the labeled amelogenin (AMGN), ameloblastin (AMBL), and enamelin (ENAM) probes. The expression level of the genes was also determined at time point 0 days. The relative transcript levels of the three genes were determined by densitometric analysis and correlated to the respective expression level at 0 days

increased in SaOS-2 cells almost to levels seen for ß-glycerophosphate (Fig. 4). In order to clarify if this stimulatory effect on transcripts is additive or even potentiative, coincubation experiments were performed. For the (relative) quantification of the transcript levels, seen in the single- or double-incubation experiments (2-day incubation period), the expression level in SaOS-2 cells after treatment with ß-glycerophosphate was set to 100%. The Northern blot data showed that the expression level of amelogenin after exposure to silicic acid alone (95%) increased only to 105% if silicic acid was added with ß-glycerophosphate (Fig. 6). Similar was the effect of silicic acid on the ß-glycerophosphate-caused expression on enamelin; for this transcript species the level was 105% if ß-glycerophosphate was added together with silicic acid (Fig. 6). If Na-silicate was added alone, the expression level was 55% compared with that of ß-glycerophosphate alone. No considerable contribution of silicic acid on the ß-glycerophosphate-induced upregulation of ameloblastin expression was seen if the cells were incubated with this compound together with silicic acid. Addition of Na-silicate alone to the cells caused a slight increase in the transcript level (5%); when giving this component together with ß-glycerophosphate, the level amounted to 95%. These experiments were performed at least three times; after densitometric evaluation, signal strengths were measured and the results varied by 20% in maximum.

Discussion

In the present study we investigated if silica-based components, Na-silicate, TEOS, or nanoparticles obtained from sponge spicules, cause an induction/increase of the steady-state level of matrix proteins composing the enamel. Based on earlier studies [17, 25], we postulated that these silica molecules could exert, via increased gene expression, osteoinductive/osteoconductive activity. We selected as major genes/proteins the matrix proteins amelogenin, ameloblastin, and enamelin of the enamel for our investigations. During tooth formation, the enamel compartment is framed by outer and inner epithelia, which surround the stellate reticulum and the stratum intermedium. After a series of epithelial-mesenchymal interactions, the epithelial cells differentiate to form preameloblasts and finally the fully differentiated ameloblasts [56]. These highly prismatic cells secrete enamel proteins, e.g., amelogenin, ameloblastin, and enamelin. In addition, they are involved in the transport of mineral ions such as calcium and phosphate into the enamel matrix [3]. Amelogenin is secreted from the ameloblasts and undergoes posttranslational modifications under formation of aggregates [57]. The resulting 15-nm large nanospheres are composed of

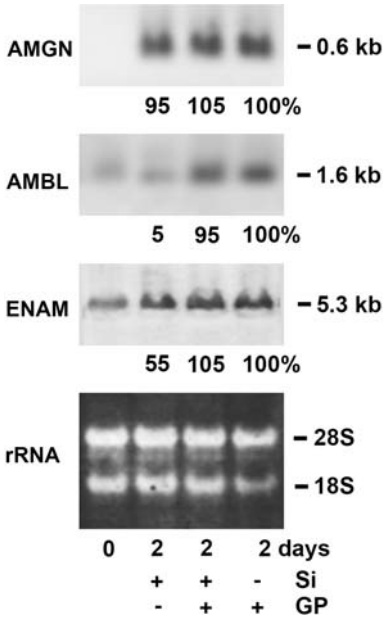

Fig. 6 Effect of silicic acid on amelogenin, ameloblastin, and enamelin expression levels in SaOS-2 cells exposed to ß-glycerophosphate. Cultures were incubated for 2 days in the absence (-) or presence (+) of either 20 µM Na-orthosilicate (*Si*) or 5 mM ß-glycerophosphate (*GP*) alone or with these two components together. RNA was isolated and 5-µg aliquots were subjected to Northern blotting to quantify the level of amelogenin (AMGN), ameloblastin (AMBL), and enamelin (ENAM) expression. In addition, RNA from cells at time 0 days was analyzed. In parallel gels, RNA was loaded onto the gels and, after size separation, stained with ethidium bromide to identify the 18S and 28S rRNA bands

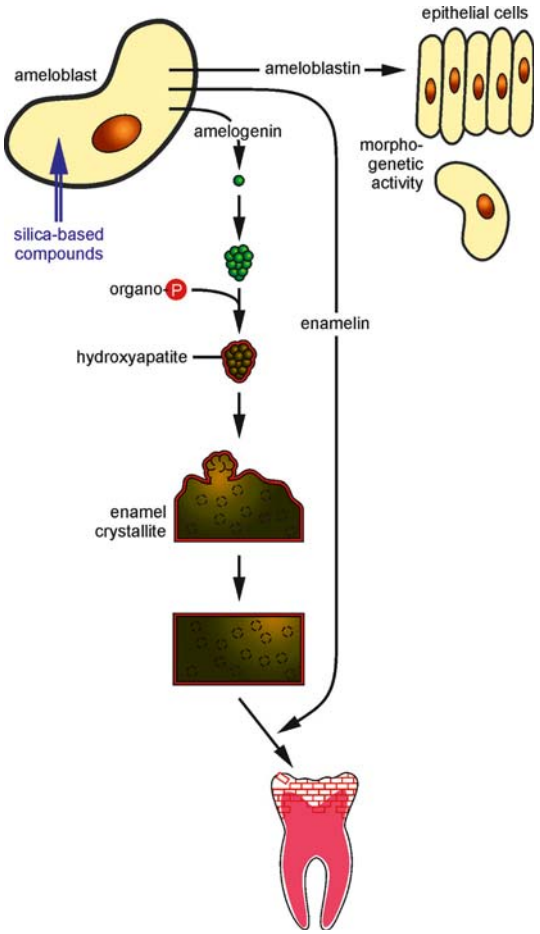

Fig. 7 Schematic outline of the effect of silica-based components (Na-silicate, TEOS, nanoparticles) on the expression of three genes (amelogenin, ameloblastin, and enamelin) in ameloblasts. After exposure to the silica-based components, expression of the amelogenin gene was strongly upregulated. After a series of modifications, the amelogenin molecules form nanospheres around which hydroxyapatite is deposited (for details, see text). Amelogenin becomes resorbed into the hydroxyapatite crystallites, which aggregate to larger crystallite entities in the presence of enamelin. The gene expression of this second protein is likewise upregulated by the silica-based components. The gene expression of the third protein formed in ameloblasts and studied here, ameloblastin, is not altered by the silica-based components. Ameloblastin displays morphogenetic activity and controls the differentiation state of the ameloblasts

100–200 amelogenin molecules [58]; the scaffold of the amelogenin nanospheres controls the orientation of the hydroxyapatite crystals [58–60]. After completion of enamel crystallite formation, the processed amelogenins are resorbed from the enamel matrix [61]. A schematic outline of the role of amelogenin during mineralization is given in Figure 7. It has been postulated that enamel matrix proteins, like amelogenin and amelogenin-like proteins, also induce expression of additional genes involved in mineralization, including tissue-specific marker proteins (bone sialoprotein). Focusing on amelogenin, it could be demonstrated that this is a critical signaling molecule required for appropriate development of the periodontium [62]. Hence, amelogenin exhibits osteoinductive activity [63]. Interesting is the finding summarized in the present study that silica-based components (Na-silicate, TEOS, nanoparticles) cause strong upregulation of the expression of the amelogenin gene.

The second protein involved in the assembly of the crystallites in the enamel is enamelin [reviewed in 4]. Enamelin is an essential component of the enamel matrix, required for correct biomineralization of the enamel matrix to mature enamel, and remains at the front of the mature enamel [64]. Hence, this structural, highly glycosylated protein controls as an extracellular matrix component the extent of enamel formation [65]. Like amelogenin, enamelin gene expression is also strongly upregulated by the silica-based components.

The third gene studied here which is expressed in the ameloblasts is ameloblastin. Ameloblastin is a cell adhesion molecule essential for amelogenesis [7] that regulates the differentiation/secretory state of the ameloblasts [66] and serves as a cell adhesion molecule on cells of the dental epithelium. It functions as a "growth factor" during

periodontium development and regeneration through modulation of BMP expression [67]. The expression state of ameloblastin remains unchanged after exposure of SaOS-2 cells to silica-based components.

In a parallel series of experiments, the effect of the organic phosphate ß-glycerophosphate on the expression of the three selected enamel proteins, amelogenin, ameloblastin, and enamelin, was studied. ß-Glycerophosphate has been described to induce mineralization in cell culture systems by serving as a source of inorganic phosphate [68]. In addition, ß-glycerophosphate promotes mineralization in vitro by modulating bone cell metabolic activity. This metabolite undergoes hydrolysis by bone cells, resulting in an increase of inorganic phosphate, which stimulates rapid mineral deposition [69]. Now we show that exposure of SaOS-2 cells to ß-glycerophosphate results in a strong increase in the steady-state expression of the three genes encoding the enamel proteins. This effect was seen – in contrast to the effect of the silica-based components – for all three genes. Therefore, we propose that ß-glycerophosphate causes a more general effect on gene expression in ameloblasts, like that seen for bone acidic glycoprotein-75 [70]. In an earlier study, using the sponge *S. domuncula* cell system as a model, it could be demonstrated that silica-based components (Na-silicate, TEOS, or nanoparticles) cause differential gene expression; besides the gene coding for the enzyme involved in the synthesis of bio-silica (silicatein) also the gene coding for the structural protein collagen was strongly upregulated. This effect of silica was not seen by exposure of the cells to the "growth/differentiation factor" myotrophin [30]. In mammalian cells, bioactive glasses (composed of 53–58% SiO_2) stimulate the expression of BMP-2 and other bone mRNAs [71]. However, very little is known about the molecular mechanisms which result in the differential gene expression in response to biogenic silica, in spite of the fact that this material displays its morphogenetic potential in plants and in animals [72]. To date, only one silica-dependent transcription factor has been identified in the sponge *S. domuncula*, the homeodomain protein Iroquois [73]. The present study broadens this view by the result that, besides morphogens, also the structural proteins of the enamel, amelogenin and enamelin, are highly expressed after exposure of SaOS-2 cells to silica-based components. We have extended the gene expression studies by *in vitro* investigations, which revealed that addition of Na-silicate together with ß-glycerophosphate causes an extensive biomineralization process in SaOS-2 cells. However, coincubation experiments using ß-glycerophosphate together with silicic acid components revealed that silicic acid does not contribute to the level of amelogenin, ameloblastin, and enamelin expression observed after incubation with ß-glycerophosphate alone.

In order to clarify if the silica-based components applied in the present study cause some toxic effects, viability studies were performed. Applying the MTT test, no indication could be obtained to suggest any adverse effect on SaOS-2 cells. It should be mentioned here that the enzyme silicase, which had been described to mediate in sponges dissolution of siliceous skeletal elements [2, 37], has high sequence similarity to the mammalian carbonic anhydrase, which can be taken as an indication that the silica nanoparticles, obtained from sponge spicules, might be dissolved in mammalian tissue as well. Further studies must show if the silica nanoparticles, used for the studies here, are metabolized to monomeric silica.

In conclusion, dental enamel formation is a highly specialized process which involves a series of novel proteins, such as amelogenin, ameloblastin, and enamelin, all synthesized in the ameloblasts. Among them, amelogenin and enamelin are genuine structural molecules, which contribute to hydroxyapatite crystallite formation. The genes encoding these two proteins are strongly upregulated in vitro (SaOS-2 cells) after exposure to silica-based components. In contrast, expression of the gene controlling the differentiation state of the ameloblasts, ameloblastin, is not altered in the presence of the components (Fig. 7). Hence, the data of this study contribute to the recent interest in silica-based bioactive glasses as promising bone substitutes. Parallel experiments might be performed with ameloblasts to underscore the (potential) differential expression of amelogenin, ameloblastin, and enamelin by the silica-based components. However, until now only little has been known about the molecular mechanisms of ameloblast differentiation [74]. Previously, it was demonstrated that bioactive glasses or glass ceramics can be used to form silica gel layers on surface bone-replacement materials, allowing more tight bone bonding [75–77]. In turn it was proposed that the unique properties of bioactive glasses and glass ceramics might be beneficial for clinical use in orthopedic, oral, maxillofacial, and otological surgery for bone and cartilage reconstruction [71]. The progress seen with the use of silica-based components from sponges is the fact that this material is produced enzymatically, by silicatein [29–31], allowing formation of bioactive glasses at ambient temperature and pressure. These properties are especially advantageous for the deposition/formation of bio-silica and in turn hydroxyapatite in situ at those bone defects which do not allow replacement surgery.

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