Dose-dependent effects of strontium on osteoblast function and mineralization

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Background. Strontium-ranelate is now being used in the treatment of osteoporosis in elderly patients. As the majority of these patients already have a decreased renal function they are at an increased risk for accumulation of the element. Recent findings from epidemiologic studies in dialysis patients and experimental data obtained in a chronic renal failure (CRF) rat model established a dose-related multiphasic effect of strontium (Sr) on bone formation. To confirm these in vivo findings an in vitro set-up, consisting of primary rat osteoblast cultures, was applied. Sr was added to the culture medium at concentrations of 0, 0.5, 1.0, 2.0, 5.0, 20, and 100 μg/mL, respectively.

Methods. Calcium incorporation (index of mineralization) and alkaline phosphatase activity were determined in the medium during the culture period, while at the end of the experiment, nodule formation (mineralized + unmineralized area) was quantified using a digital imaging system. mRNA synthesis of various osteoblast specific genes was assessed by means of reverse transcription polymerase chain reaction (RT-PCR).

Results. Compared to the control group (0 μg/mL Sr), a significantly reduced nodule formation in the presence of an intact mineralization was found for the lowest 0.5 and 1 μg/mL Sr doses, suggesting an impaired in vitro osteoblast differentiation. Both nodule formation and mineralization were normal for the 2 and 5 μg/mL doses. For the highest Sr doses (20 and 100 μg/mL) a reduced mineralization was observed in the presence of an intact nodule formation indicating an inhibitory effect on the hydroxyapatite formation. The alkaline phosphatase activity reflected the multiphasic pattern of the nodule formation while the calcium incorporation corresponded with the pattern of nodular mineralization. No variations in cell proliferation were found. RT-PCR revealed that Sr interfered with the osteoblast at the level of the mRNA synthesis of several relevant genes.

Conclusion. Using the proposed in vitro model we confirmed the multiphasic effect of Sr on bone formation previously demonstrated in a CRF rat model. The data presented allow us to suggest that at low concentrations Sr interferes with the bone formation at the level of cell differentiation, whereas at high concentrations the disturbed mineralization is

Due to its chemical analogy to calcium (Ca), strontium (Sr, molecular weight 87.62) is a bone-seeking element that readily exchanges for Ca in diverse tissues, including the hydroxyapatite lattice in bone [1, 2]. Evidence has been presented that in subjects with normal renal function, a high Sr diet goes along with an increased amount of the element in the bone mineral [3]. Because the kidney is the main excretion route of Sr (clearance 4.0 to 5.4 mL/min) [4] and dialysis fluids may be contaminated with the element, dialysis patients are at an increased risk for accumulation/toxic effects of the element [5–7].

Data from previous studies of our group showed that: (1) bone Sr concentrations are increased in dialysis patients with osteomalacia—a type of renal osteodystrophy characterized by a defective mineralization in the presence of large osteoid seams—as compared to those presenting the other types of renal osteodystrophy [5, 6]; (2) osteomalacia can be induced in a chronic renal failure (CRF) rat model after oral administration of the element (0.3% in the drinking water during 12 weeks) [8], indicating that Sr may play a causative role in the development of this particular type of renal osteodystrophy; and (3) Sr exhibits a multiphasic and dose-dependent effect on bone formation/mineralization in CRF rats. Indeed, whereas a low Sr dose (0.03% in the drinking water during an 18-week loading period) leads to the development of an adynamic bone, normal bone histology was noted with the intermediate dose (0.075%), while administration of a high Sr dose (0.150%) again resulted in the development of severe osteomalacic lesions [9]. A dose-dependent effect of Sr on bone has previously been reported by Marie et al [10] in rats with normal renal function also. Here, at low doses (0.19%) a stimulation of bone formation was observed, whereas at higher doses (0.40%) a defective mineralization was noticed. Similar ambiguous effects have also been demonstrated for aluminum,
which, dependent on the degree of exposure, are expressed by either the development of adynamic bone or osteomalacia [11–13]. The clinical relevance of the present investigations needs to be emphasized since Sr is now being used as the Sr-ranelate compound for the treatment of osteoporosis in elderly patients in doses up to 2 to 3 g/day [14]. As the majority of these patients already have a decreased renal function (<60 mL/min), they are at an increased risk for accumulation of the element. Although the effects of Sr on bone have substantially been investigated in vivo [2, 6–10], until now, no long-term in vitro cell culture experiments have been performed, which contributes to the fact that [10, 15] the mechanisms underlying the element’s ambiguous effects on bone are poorly understood. To investigate whether the multiphasic effects of Sr observed in vivo can be reproduced and confirmed under standardized in vitro conditions, the present study, using a cell culture model consisting of primary osteoblasts isolated from 20-day-old fetal rat calvaria, was set up. The influence of various concentrations of stable Sr on a series of relevant physicochemical and biochemical parameters involved in the bone formation/mineralization process was examined under well-controlled conditions, aiming to investigate whether the effects of Sr on bone formation either are osteoblast-mediated and/or result from a direct physicochemical effect of the element on the hydroxyapatite formation.

**METHODS**

**Experimental design**

Primary osteoblasts were isolated from fetal Wistar rat calvaria. Calvaria were dissected aseptically from 20-day-old fetuses of timed-pregnant Wistar rats. After dissection, loosely adherent soft connective tissue was removed and calvaria were minced into 1 mm³ fragments which were placed on an 80 µm nylon mesh, pressed between two rings in α-minimal essential medium (MEM) medium (Life Technologies, Merelbeke, Belgium) supplemented with 10% fetal bovine serum (FBS) and 50 µg/mL ascorbic acid. After 2 or 3 days a significant cellular outgrowth from the calvaria fragments was observed. Medium was then refreshed every 2 days during 7 to 9 days until the cultures reached confluence. Nylon meshes containing the confluent cultures were subsequently cut from the rings and trypsinized to harvest the outgrown cells. These cells were seeded in 6-well plates at a density of 6000 cells/cm² in α-MEM medium enriched with ascorbic acid (100 µg/mL) until confluence was reached. After confluence both ascorbic acid (100 µg/mL) and β-glycerol phosphate (2 mmol) were added to obtain culture conditions ensuring a reproducible mineralizing culture. To minimize dedifferentiation of the primary osteoblasts, no further passages were used in these experiments. Sr was added to the medium of the confluent cultures at various amounts as the chloride compound SrCl₂.6H₂O (Merck, Leuven, Belgium), yielding final Sr concentrations of 0.5, 1.0, 2.0, 5.0, 20, and 100 µg/mL. Cells were kept in culture for three weeks. The choice of the concentrations was based on the results of an in vitro pilot study and on the serum Sr levels at which a dose-dependent effect of the element on bone was demonstrated in a CRF rat model [8, 9]. As control, we used medium to which: (1) no Sr; (2) 10⁻⁴ mol levamisole; and (3) 60 µmol pyrophosphate (Na₄P₂O₇H₂O) (Acros, Geel, Belgium) were added. Here, levamisole acts as an inhibitor of alkaline phosphatase, while pyrophosphate is a well-known crystal poison of apatite crystal formation/growth [16–19]. Hence, these compounds served as internal controls for the in vitro osteoid (i.e., nodule) deposition and bone mineralization. Three independent parallel experiments were set up to check the reproducibility of the effects of Sr on the in vitro bone formation. During the course of the experiment the culture medium was refreshed 3 times per week during the three-week period. Before each refreshment medium samples were taken and frozen at −80°C until measurement of the various parameters under study (see below).

**Hydroxyapatite analysis**

Mineralized cultures (to which no Sr was added) were fixed in 90% ethanol, air-dried, and analyzed with a JEOL JSM 6300 scanning electron microscope (JEOL Europe, Zawentem, Belgium) for morphologic examinations. Using the same apparatus, the elemental composition of the deposited mineral was determined by means of Electron Probe X-ray Micro Analysis (EPXMA) (JEOL Europe).

In addition to this technique, unfixed mineralized cultures were air dried for Fourier Transform Infra Red (FTIR) analysis to further identify and characterize the deposited mineral. After mixing the samples with potassium bromide, FTIR spectra were recorded on a Nicolet 20 DXB FTIR-spectrometer (Nicolet Instrument Corporation, Madison, WI, USA). Three hundred scans were taken in the mid infrared (IR)-range (400 to 4000 cm⁻¹).

**Sr determination**

Measurement of Sr in the culture medium was done using a Zeeman 3030 atomic absorption spectrometer equipped with an HGA-600 graphite furnace (Perkin Elmer Corporation, Ueberlingen, Germany), as previously described in detail [20]. With this method atomization was performed from the wall of pyrolytically coated graphite tubes. Calibration was performed against matrix-matched calibration curves. The medium samples were diluted in a 0.5 mL/L Triton X-100 – 1 mL/L HNO₃ solution.
Ca incorporation

Medium samples were taken before and after each incubation period to measure the Ca concentration. The decline in the Ca concentration in the medium after incubation was used as a measure of Ca incorporation into the mineralized nodules (i.e., hydroxyapatite formation). After integration over the different time points the Ca incorporation was expressed as the absolute amount of incorporated Ca (μg) over time [area under the time-concentration curve (AUC) of 11 time points]. This method was used as an alternative for the previously described radioactive ⁴⁵Ca-incorporation method [18, 21]. Ca was determined by means of flame atomic absorption spectrometry using a Perkin Elmer 372 (Perkin Elmer Corporation) instrument by a modification of the technique of Hansen [22]. To overcome the problem of chemical interference due to the presence of phosphate and high salt content of the samples, 1/50 dilutions of the samples were made in 0.1% La(NO₃)₃.

At the end of the experiment, for each of the cultures the total amount of incorporated Ca was compared to the area of the mineralized sections of the nodules measured by quantitative microscopic digital image analysis (KS-400; Kontron Embedded Computer AG, Munchen, Germany).
Germany). Within the nodules, differentiation between mineralized and unmineralized nodule area was done based on the difference in gray value following a modified protocol from Nefussi et al [23] (Fig. 1A, upper panel).

**Phosphorous measurement**

Total phosphorous content was measured in the cell culture medium before and after incubation on a Vitros 950 AT Autoanalyzer System (Ortho Clinical Diagnostics, Johnson & Johnson, Raritan, NJ, USA). 950 AT Autoanalyzer System (Ortho Clinical Diagnostics, Johnson & Johnson, Raritan, NJ, USA).

**Alkaline phosphatase assay**

Alkaline phosphatase activity in the conditioned culture medium was determined spectrophotometrically by measurement of the release of p-nitro phenol from p-nitro phenolphosphate. The enzyme activity was determined as the change in absorbance at 405 nm over a 10 minutes period. This procedure is based on the kinetic method for measuring total alkaline phosphatase activity acknowledged by the International Federation of Clinical Chemistry (IFCC) [24].

**Electrophoresis of alkaline phosphatase**

To check whether Sr has a direct effect on the enzymatic activity or electrophoretic mobility, a serum sample with a known bone alkaline phosphatase activity was incubated with various Sr concentrations, after which the isoenzymes were separated by agarose gel electrophoresis according to the method described by Van Hoof et al [25]. Neuraminidase was used to separate liver and bone alkaline phosphatase during electrophoresis. Afterwards, the enzymatic activity was quantified by densitometry at 640 nm after incubation with 2-bromo-4-chloro-3-indoxyl phosphate at pH 10.6.

**DNA assay**

Using the Fluoreporter® Blue Fluorometric dsDNA quantitation kit (Molecular Probes, Leiden, The Netherlands), the concentration of double-stranded DNA (dsDNA) was determined in cultures of the various study groups to check for possible Sr-mediated differences in cell number. DNA measurements were performed at one time point before confluence and at three different time points after confluence.

**5-bromo-2’-deoxyuridine (BrdU) incorporation**

The effects of Sr on the proliferation of primary osteoblasts were determined using a cell proliferation enzyme-linked immunosorbent assay (ELISA) BrdU kit (Roche, Basel, Switzerland). At two different time points preconfluence, as well as at five time points postconfluence, cells cultured in 48-well plates and exposed to different Sr concentrations were incubated with BrdU labeling solution for 24 hours at 37°C. Following the manufacturer’s instructions, cells were fixed for 30 minutes and incubated with the anti-BrdU antibody for 90 minutes. After several washing steps and addition of the substrate, the color intensity was measured spectrophotometrically at 450 nm.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from primary osteoblasts exposed to the different Sr concentrations 18 days’ post-confluence using the standard protocol from the High Pure RNA Isolation Kit (Roche), including a DNase I treatment to eliminate possible DNA contamination. The total RNA concentration in the isolates was quantified by spectrophotometry at 260 nm. One µg of the RNA extract was used to precheck the RNA quality by electrophoresis on a 2% agarose gel, after which aliquots of the RNA isolate were frozen at ~80°C until analysis.

Reverse transcription-polymerase chain reaction (RT-PCR) was performed for the following genes: alkaline phosphatase (ALP); bone sialoprotein (BSP); osteopontin (OPN); bone morphogenic protein-2 (BMP-2); core binding factor alpha 1 (CBFa-1); and glyceraldehyde-phosphate dehydrogenase (GAPDH), which was applied as housekeeping gene. PCR primer sequences and fragment length for the different genes under study are, respectively: ALP F: 5’-GCTACACCCAACACAACCG GCCA-3’, R: 5’-TCCAAATGCTGATGAGGTTCA-3’, 550 bp; BSP F: 5’-AAGAAGGCTGGAGATGCAGA GGG-3’, R: 5’-GAGGTTGTCATCATTCCACTCG-3’, 350 bp; OPN F: 5’-TGAGACCTTGACATTGTGCT TTGC-3’, R: 5’-CTCATCTTGTGGCATGGGGAATAC TG-3’, 519 bp; BMP-2 F: 5’-GCTGTCTTCTAGTGTTG TG-3’, 485 bp; GAPDH F: 5’-ACCAGACGTGCTGATTCTC ACC-3’, R: 5’-AGAAGGTTTGTGTCACGGT-3’, 286 bp; ACC-3’, R: 5’-CCAACACCTGTTGGTTGTA-3’, 450 bp. Using the Superscript One Step RT-PCR kit (Invitrogen, Merelbeke, Belgium), reverse transcription was performed at 50°C for 30 minutes for all samples following the standard procedures supplied by the manufacturer. After amplification in a Gene Amp 9600 PCR System (Perkin Elmer), a 10 µL quantity of each PCR product was then electrophoresed on a 2% agarose gel (E-gel system; Invitrogen), visualized, and quantified on a Lumi Imager (Boehringer Mannheim).

**Statistics**

Data are expressed as mean ± SD unless otherwise stated. For each of the various study parameters, comparison of the various study groups (i.e., different Sr concentrations) versus the control group (0 µg/mL Sr) was done by ANOVA. In case more than two groups were compared, a post-hoc Bonferroni test was applied to correct for the number of comparisons. Statistical significance was obtained when \( P < 0.05 \) at a two-tailed level.
RESULTS
Mineralization
Cells seeded at 6000 cells/cm² in 6-well culture plates reached confluence within 7 days. Nodule formation was observed from day 7 post-confluence. As assessed in control cultures these nodules were mineralized within 15 days after confluence (Fig. 1A, upper panel). EPXMA spectra of the mineralized nodules revealed a Ca/P ratio of 10/6, which reflects the stoichiometric composition of the bone mineral \[ \text{Ca}_{10} \left( \text{PO}_4 \right)_6 \left( \text{OH} \right)_2 \] in the in vivo situation (Fig. 1A, middle panel). Moreover, comparison of the FTIR spectra of the in vitro deposited mineral with those of synthetic Ca hydroxyapatite and pulverized animal bone revealed the typical absorbance peaks of hydroxyapatite at 1030, 600, and 560 cm⁻¹ (Fig. 1B, upper panel, and lower panel), confirming the EPXMA data. These results thus present evidence that the in vitro formed mineral is hydroxyapatite, the main compound of bone.

Effect of Sr on expression of the phenotypic characteristics
Determination of the Sr content in the culture medium revealed values accurately reflecting the values noted for the Sr additions of 0, 0.5, 1, 2, 5, 20, and 100 μg/mL Sr²⁺.
Nodule formation. Quantitative microscopic analysis of the nodule formation by measuring the nodule area in the culture showed a Sr-related multiphasic pattern (Fig. 2A). In the control group, a total nodule area (mineralized + unmineralized sections) of \(31.5 \pm 10.3\) mm\(^2\)/cm\(^2\) was found, 76.8 \pm 10.3\% of which was mineralized \((N = 10)\). Compared to the control group, total nodule area was significantly \((P < 0.05)\) decreased in the groups treated with 0.5 and 1 \(\mu g/mL\) Sr \((11.2 \pm 7.8, 13.9 \pm 12.2\) vs. 31.5 \pm 10.3 mm\(^2\)/cm\(^2\); \(N = 10\) in all groups), whereas no difference was found for the 2, 5, 20, and 100 \(\mu g/mL\) treatment groups \((25.0 \pm 7.0, 29.8 \pm 7.0, 28.9 \pm 8.8, 24.8 \pm 7.9\) vs. 31.5 \pm 10.3 mm\(^2\)/cm\(^2\); \(N = 10\) in all groups). The mean size of the nodules was not different between the different groups (data not shown).

Differentiation between the mineralized and unmineralized nodule areas allowed us to calculate the degree of mineralization as the percentage of the total nodule area covered by hydroxyapatite deposits (mineralized area/total area \(\times 100\)) (Fig. 2B). By doing so, in the 0.5 and 1 \(\mu g/mL\) Sr groups, the mineralization appeared to be intact despite the decreased nodule formation \((64.4 \pm 34.4, 46.4 \pm 41.4\%\) vs. 76.8 \pm 10.3\% in the control group; NS; \(N = 10\) in all groups). The intermediate groups \((2.0\) and 5.0 \(\mu g/mL\) Sr) did not differ from the control group, as the normal nodule formation was accompanied by an intact mineralization \((79.9 \pm 8.7\) and 75.0 \pm 10.1 vs. 76.8 \pm 10.3\%; NS; \(N = 10\) in all groups) However, in the 20 and 100 \(\mu g/mL\) treatment groups, the mineralization was significantly impaired in the presence of an intact nodule formation \((14.7 \pm 20.4, 9.1 \pm 15.0\) vs. 76.8 \pm 10.3\%; \(P < 0.05\); \(N = 10\) in all groups).

For the levamisole group (alkaline phosphatase inhibitor) both the total nodule area \((9.6 \pm 3.5\) mm\(^2\)/cm\(^2\); \(N = 10\)) and the ratio of the mineralized area/total nodule area \((4.2 \pm 13.2\%)\) were significantly \((P < 0.05\) lower than the control group \((31.5 \pm 10.3\) mm\(^2\)/cm\(^2\) and 76.8 \pm 10.3\%, respectively; \(N = 10\)). The pyrophosphate group revealed a pattern comparable to that noted for the 20 and 100 \(\mu g/mL\) Sr groups (i.e., a decreased mineralized area versus the control group in the presence of an unchanged total nodule area \((47.5 \pm 15.3\%\) vs. 76.8 \pm 10.3\%; \(P < 0.05\); \(N = 10\)).

Ca incorporation. The Ca incorporation showed an Sr-dependent multiphasic pattern similar to the calculated mineralized nodule areas determined by quantitative microscopic imaging (Fig. 2C). Indeed, in the 0.5, 1, 20, and 100 \(\mu g/mL\) Sr groups, the amounts of incorporated Ca were significantly lower than the values measured for the control group \((221.8 \pm 28.3, 189.4 \pm 23.6, 222.4 \pm 49.1, 124.2 \pm 10.3\) vs. 323.6 \pm 13.1 \(\mu g\) Ca incorporated; \(P < 0.05\); \(N = 18\) in all groups), while the 2 and 5 \(\mu g/mL\) Sr groups did not differ significantly \((252.3 \pm 62.4\) and 285.1 \pm 25.6 vs. 323.6 \pm 13.1 \(\mu g\) Ca incorporated; \(N = 18\) in all groups). In both the pyrophosphate, and even to a higher extent in the levamisole group, the Ca incorporation was significantly decreased versus the 0 \(\mu g/mL\) control group \((211.8 \pm 53.1, 147.4 \pm 38.3\) vs. \(323.6 \pm 13.1 \(\mu g\) Ca incorporated; \(P < 0.05\); \(N = 18\)), which again was in agreement with the data obtained by digital image analysis.

Measurement of the total phosphorous concentration before and after incubation revealed no significant differences between the different treatment groups, which was due to the high background phosphorous level resulting from the large excess present in the culture medium.

Alkaline phosphatase. The addition of Sr to the culture medium affected the alkaline phosphatase activity in a multiphasic way, similar to that seen for the quantification of the total nodule formation. Enzymatic activity was significantly \((P < 0.05)\) decreased in the 0.5, 1, and 2 \(\mu g/mL\) groups versus the control group \((49.7 \pm 6.3, 79.7 \pm 12.8, 88.7 \pm 10.0\) vs. 121.1 \pm 15.5 U/L, respectively; \(N = 18\) in all groups). The inhibitory effect in these groups was comparable to that noted for the levamisole group (enzymatic activity, 50.8 \pm 0.7 U/L; \(N = 18\)). No significant difference was found in the 5, 20, and 100 \(\mu g/mL\) groups versus control. Alkaline phosphatase activity was significantly \((P < 0.05)\) increased in the pyrophosphate group \((168.2 \pm 15.1 U/L; N = 18)\) versus all other groups.

To examine whether the observed multiphasic effect of Sr on alkaline phosphatase was due to an interference of the element with the cellular secretion/synthesis of the enzyme rather than being the result of a direct interaction with the enzyme activity, the electrophoretic mobility and enzymatic activity of a serum sample incubated with the different Sr-concentrations was also assessed. No difference in the electrophoretic pattern or in signal
intensity could be observed between the various groups. The absence of any difference in enzymatic activity was further confirmed using the standard procedure with p-nitro phenolphosphate as substrate (data not shown).

Multiphasic effects of Sr on both Ca incorporation and alkaline phosphatase were consistent in function of time for all the groups under study and no time-dependent difference or time shift was observed (Fig. 2D).

Total DNA content and cellular proliferation. To examine whether the observed Sr-induced effects on the different parameters were due to an interference of the element with the cellular proliferation, total DNA content and BrdU-incorporation of the different groups under study were determined at different time points pre- and post-confluence. No difference in the DNA content or in the BrdU-incorporation (Fig. 3) between the groups could be observed.

RT-PCR analysis. Reverse transcription-polymerase chain reaction analysis on cell lysates of the different groups at sacrifice of the culture (i.e., 18 days’ post-confluence) also revealed a multiphasic effect of Sr on the mRNA expression of alkaline phosphatase and bone sialoprotein, the effect being pronounced most intensively in the 0.5 µg/mL group, as shown in Figure 4. A multiphasic effect was also seen for the osteoblast-specific transcription factor CBFa1 and BMP-2, which were particularly decreased in the 1 µg/mL group. No such effect was found on the mRNA expression of osteopontin. RT-PCR analysis was performed in duplo on samples taken from 3 independent cultures (i.e., 6 measurements for each gene).

DISCUSSION

In previous studies, increased bone Sr levels were noted in dialysis patients with osteomalacia [5, 6]. A causative role for Sr in the development of osteomalacia was demonstrated by our group in a rat model with chronic renal insufficiency [8]. In addition, in these experimental studies the effect of Sr on the bone formation/mineralization was found to be dose dependent [9]. Here, at low doses an adynamic, and at high doses an osteomalacic bone lesion could be induced. In rats with normal renal function Marie et al [10] found also a dose-dependent effect of Sr on the bone metabolism. The mechanisms underlying these effects are poorly understood. The present in vitro model consisting of mineralizing osteoblasts was used to investigate whether (1) it was possible to reproduce the causal, dose-dependent effects of Sr as previously observed in vivo; (2) the effects of Sr on bone mineralization are osteoblast-mediated; and (3) and/or result from a direct physicochemical effect.

Primary rat osteoblast cultures isolated from 20-day-old fetal rat calvaria were used. This model has previously been applied by others studying the in vitro osteogenesis [26–30], and to investigate the effects of trace elements other than Sr on the in vitro mineralization [31–35]. With the in vitro set-up of the present study, the temporal evolution of the major phenotypic characteristics (alkaline phosphatase and Ca incorporation) involved in the mineralization process (data not shown) agrees with previous literature data [28] and is assumed to represent well the in vivo situation. Moreover, EPXMA analysis
of the mineralized nodules revealed a Ca/P ratio of 10/6, which corresponds with the ratio found in the hydroxyapatite lattice \([\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]\) of animals and humans. These results were further confirmed by FTIR yielding spectra of the deposited mineral similar to those of synthetic apatite and pulverized animal bone.

Quantification of nodule formation was primarily done by measuring the nodule area. Following a modified protocol from Nefussi et al [23], by using a microscopic digital imaging system it was possible to distinguish between the mineralized and unmineralized portions of the nodules without the need for staining since the deposited mineral in the nodules can be observed as highly contrasting dark areas, while the unmineralized portions can be easily differentiated from the background (Fig. 1). Aside from nodule counting, this method has the advantage of providing quantitative information about the degree of mineralization of the nodules. Since the mean size of the nodules was not different between the different groups, the quantification of the total nodule area also reflects the number of the nodules. Additional to this microscopic method, quantification of the degree of mineralization was assessed by measuring the daily decrease of the Ca concentration in the conditioned medium. This decrease was calculated by comparing the total Ca content in the medium before and after cellular incubation with each refreshment of the medium. By doing so, measurement of the Ca incorporation offers the possibility to easily quantify the in vitro mineralization in one and the same culture at various time points without the need to sacrifice the cultures or to use radioactive isotopes [18]. Results obtained by this method also indicated a multiphasic effect of Sr on the mineralization process. It should be mentioned that at the highest concentrations the variation in the Ca incorporation does not seem to match very well with the variation in the mineralized nodule areas seen by image analysis. This might be explained by our preliminary results of the x-ray diffraction analysis of the in vitro deposited hydroxyapatite, performed at the European Synchrotron Radiation Facility (ESRF, Grenoble, France, beamline ID18F), revealing that high Sr concentrations give rise to diffusely deposited amorphous mineral reflecting a defective mineralization [abstract; Verbeckmoes SC et al. J Am Soc Nephrol 12:577A, 2002]. This type of defective mineralization cannot be quantified by visual examination in unstained cultures (image analysis), but goes along with a sustained Ca consumption that will be detected by measuring the Ca incorporation. Moreover, whereas the microscopic quantification of the mineralized area only gives two-dimensional information (nodule area), the Ca incorporation provides three-dimensional information (nodule volume) of the Ca consumption during the total mineralization process.

Using this validated model of in vitro bone formation we could confirm the multiphasic effects previously observed in vivo and present evidence for Sr to exert a cell biological effect at low doses, and a physicochemical effect on bone formation/mineralization at high doses.

The reduced nodule formation at the lowest Sr concentrations (0.5 to 1 \(\mu\)g/mL) was not accompanied by an impaired mineralization, since in these groups the ratio of the mineralized area/total nodule area did not differ from those of the control group. It is generally assumed that nodule formation goes along with the differentiation of osteoprogenitor cells toward mature osteoblasts [26, 28, 32, 36]. Since in the present study Sr was added to the culture from the moment of confluence onward, and because there was not any difference in DNA content and BrdU incorporation between the different groups, the observed differences in total nodule formation (mineralized + unmineralized) between the different doses could not be due to a change in proliferation. Hence, as previously proposed by others in the context of different experimental set-ups [26–28, 31, 32, 37], the altered nodule formation most reasonably must be due to alterations in osteoblastic differentiation.

The electrophoretic data of alkaline phosphatase indicate that the observed effect of Sr on the activity of the enzyme in the culture medium was not due to a direct interaction of Sr with the intact metalloenzyme, but rather occurs secondary to a cellular response. This suggestion is further supported by the results obtained by RT-PCR analysis. Here, the mRNA expression of alkaline phosphatase was also affected in a multiphasic way similar to the pattern for its enzymatic activity. This multiphasic pattern, and more particularly, the inhibition of the expression at low Sr concentrations, was also observed on other osteoblast-specific factors, such as bone sialoprotein, BMP-2, and CBFa-1 [26, 28]. Data from the literature clearly show that BMP-2 and CBFa-1 are closely involved in the osteoblast differentiation process [38–42]. Hence, the RT-PCR data, together with the effects on the nodule formation, support the suggestion that the observed effects of Sr at low doses are mediated by an inhibitory effect on osteoblast differentiation. No clear significant effect could be demonstrated for osteopontin, which most probably was due to its very low expression and, hence, relatively low signal/noise ratio.

At the intermediate concentrations (2 to 5 \(\mu\)g/mL), no significant difference with the control group could be observed in contrast to the highest concentrations (20 to 100 \(\mu\)g/mL). In the latter, the normal osteoblast function is reflected by an intact nodule formation and a normal alkaline phosphatase activity. However, the nodules are only partly mineralized, indicating a defective mineralization, most likely occurring secondary to a physicochemical interference of Sr with hydroxyapatite formation/dissolution. These observations are also in line with in vitro data reported by Christoffersen et al [43], evidencing that synthetically prepared hydroxyapatite, in which up to 10 mol % Ca was replaced by Sr, has a higher solubility than the Sr-free hydroxyapatite.
Complex dose-dependent effects of Sr on Ca metabolism have recently been published by other investigators [44, 45]. An explanation for the multiphasic interference with the osteoblastic activity may be found in the calcimetric properties of the element. Brown [46] has reported that Sr has the potential to activate the extracellular Ca-sensing receptor in both the kidney and in the parathyroid gland. Moreover, Somlyo and Somlyo [47] found that Sr can be stored in the sarcoplasmic reticulum and mitochondria of vascular smooth muscle cells, cellular compartments known as intracellular Ca stores that play an important role in intracellular signaling. In view of this, together with the fact that several cellular responses are strongly dependent on the intra- or extracellular Ca concentrations, one can reasonably expect that Sr may exhibit a multiphasic effect by either stimulating or inhibiting the osteoblastic activity, depending on the dose.

In agreement with our previous observations in the CRF rat [8, 9], and previous reports in literature [1, 10, 15, 48–50], a complex dose-dependent effect of Sr on nodule formation, as well as on mineralization, could be reproduced in our cell culture model. With regard to the phenotypic characteristics monitored in the present in vitro study (alkaline phosphatase, Ca incorporation) a striking similarity with the bone histomorphometric data of Sr-loaded CRF rats seen in a previous experimental study [9] was found. Indeed, in these animals at serum Sr concentrations around 1 μg/mL, a reduced mineral apposition and bone formation rate were noticed in the presence of a reduced osteoblastic activity (cell biological effect), characteristic for the presence of an adynamic bone disease. At the intermediate concentration, corresponding with the 5 μg/mL Sr group of the present study, a “normal” bone histology (i.e., a normal amount of osteoid in combination with an intact mineral apposition and bone formation rate) was found. Finally, in the presence of a serum Sr level of 10 μg/mL, the animals presented an impaired mineralization in the presence of an increased amount of osteoid featuring the hallmarks of osteomalacia. It is worth mentioning that the range of the Sr concentrations applied in the present study covers the serum concentrations found in both hemodialysis patients with osteomalacia and adynamic bone disease (serum Sr level 0.1 to 1 μg/mL) [5] and postmenopausal women with osteoporosis (serum Sr level 10 to 20 μg/mL) treated with ranelate using doses up to 2 to 4 g/day [44, 51]. Recently, osteomalacic lesions have also been associated with increased bone Sr levels in European dialysis patients [52]. Interestingly, a comparable complex dose-dependent interference with bone metabolism has previously been shown for aluminum (Al) also in both dialysis patients and CRF rats. In these studies the development of adynamic bone and osteomalacia was also seen at low and high doses, respectively [12, 13].

Our primary cell culture data to a certain extent do not fully agree with the in vitro findings reported by Canalis et al [48] using calvaria organ cultures. They found a dose-dependent stimulating effect on osteoblast number, labeled surface, and bone formation rate. Several important differences in experimental setup should be taken into account when comparing our data with the results obtained by these investigators. In contrast to our long-term osteoblast cultures, in which consistent multiphasic effects of Sr exposure were seen over the whole culture period (21 days), the latter investigators incubated their organ cultures with Sr for only 24, 48, and 96 hours.

CONCLUSION

Using the proposed model we have been able to demonstrate a multiphasic effect of Sr on the in vitro bone formation. Moreover, these data allow us to suggest that, at lower concentrations, the interference of Sr with the bone formation is cell-mediated, most probably at the level of osteoprogenitor cell differentiation. At high Sr concentrations no effect on the osteoblast differentiation could be observed; therefore, the disturbed mineralization is suggested to result from a physicochemical interaction.

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