Normal Matrix Mineralization Induced by Strontium Ranelate in MC3T3-E1 Osteogenic Cells

A. Barbara, P. Delannoy, B.G. Denis, and P.J. Marie

There is growing evidence that strontium ranelate (SR; S12911-2, PROTELOS; Institut de Recherches Internationales Servier, Courbevoie, France), a compound containing 2 atoms of stable strontium (Sr), influences bone cells and bone metabolism in vitro and in vivo. We previously reported that SR increases bone mass in rats and mice by stimulating bone formation and inhibiting bone resorption. We also showed that short-term treatment with SR enhances osteoblastic cell recruitment and function in short-term rat calvaria cultures. Because Sr incorporates into the bone matrix, it was of interest to determine whether SR may affect matrix mineralization in long-term culture. To this goal, osteogenic mouse calvaria-derived MC3T3-E1 osteoblastic cells were cultured for up to 14 days in the presence of ascorbic acid and phosphate to induce matrix formation and mineralization. Matrix formation was determined by incorporation of tritiated proline during collagen synthesis. Matrix mineralization was quantified by measuring the number and surface of mineralized nodules using a digital image analyzer. In this model, 1,25(OH)2 vitamin D (1 nmol/L) used as internal control, increased alkaline phosphatase (ALP) activity, an early osteoblast marker, on days 4, 10, and 14 of culture. Treatment with SR (1 mmol/L Sr^{2+}) increased ALP activity at days 4 and 14 of culture. SR also increased collagen synthesis at days 4 and 10 of culture. In contrast, 1,25(OH)2 vitamin D (1 nmol/L) inhibited collagen synthesis at 4 to 14 days of culture. Long-term treatment with SR (0.1 to 1 mmol/L Sr^{2+}) dose dependently increased Sr concentration into the calcified nodules, but did not alter matrix mineralization in long-term culture, as shown by the ratio of the surface of mineralized nodules to the number of mineralized nodules on day 14 of culture. These results show that long-term treatment with SR increases collagenous matrix formation by MC3T3-E1 osteoblasts without inducing deleterious effect on matrix mineralization.

THERE IS growing evidence that strontium (Sr) influences bone cells and bone metabolism in vitro and in vivo.[1,2] Our previous experimental studies in animals and humans showed that low doses of Sr inhibit bone resorption and/or stimulate bone formation.[3-6] Other studies also showed that Sr can stimulate bone formation and inhibit bone resorption in vitro.[7,8] Consistently, it was found that strontium ranelate (SR), a compound containing 2 atoms of stable Sr, increases osteoblastic cell replication and function in rat calvaria cell cultures[9] and inhibits the differentiation and resorbing activity of osteoclasts in vitro.[10,11] This discovery led to the hypothesis that this compound may have therapeutic implications in osteopenic disorders. Indeed, the administration of SR was found to reduce bone resorption in estrogen-deficient rats,[12] immobilized osteopenic rats,[13] and intact monkeys[14] and to increase bone mass in ovariectomized rats,[12] intact rats,[15] and mice[16] and to prevent the vertebral and nonvertebral fracture risk in postmenopausal osteoporosis.[17-19]

Sr is known to be incorporated into the exchangeable part of hydroxyapatite. A small fraction (about 10%) can exchange with calcium (Ca) within the hydroxyapatite crystals.[20] In normal animals, the incorporation of Sr in bone following SR administration at low or high doses does not lead to abnormal crystal structure or defective mineralization measured either by biophysical or histomorphometric analyses.[21] In contrast, high Sr levels in dialyzed bathes may be associated with defective bone mineralization in dialyzed patients with severe renal insufficiency.[22] Strontium administration at high doses in rats with renal failure was also found to result in abnormal mineralization of the bone matrix.[23,24] It is therefore important to ensure that long-term treatment with SR at doses equivalent to those that are efficient for fracture prevention in clinical studies[17-19] maintains normal mineralization of the matrix.

The aim of the present study was therefore to investigate whether long-term treatment (14 days) with SR may affect the formation of mineralized nodules induced by MC3T3-E1 osteogenic cells in vitro. The data show that SR, which increases Sr content in the mineralized matrix, increases collagenous matrix formation by MC3T3-E1 osteoblasts without inducing deleterious effect on the mineralized matrix in the long term.

MATERIALS AND METHODS

Cell Cultures and Treatment

MC3T3-E1 are clonal osteogenic cells derived from newborn mouse calvaria[25] that have a high mineralization potential.[25-29] MC3T3-E1 cultured in the presence of ascorbic acid display a time-dependent and sequential expression of osteoblast characteristics analogous to the bone formation process in vivo.[27] The cells actively replicate, then express alkaline phosphatase (ALP) activity and synthesize a collagenous extracellular matrix, which progressively undergoes mineralization.[27] In this study, MC3T3-E1 cells (kindly provided by Drs D. Wang and R. Transceschi, University of Michigan, Ann Arbor, MI) by courtesy of Dr F. Dunn (Aventis Pharma, Romainville, France) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 72.5 mg/L Ca and 28.2 mg/L phosphorus, supplemented with 10% fetal calf serum (Gibco BRL, Cergy Pontoise, France) and 1% penicillin/streptomycin (Gibco BRL) in 5% CO_2 at 37°C. Cultures were conducted for 14 days in the continuous presence of ascorbic acid (25 μg/mL) and phosphate (3 mmol/L) for the last 7 days of culture to induce matrix formation and mineralization.[29] The cells were cultured

From INSERM U349 affiliated to CNRS, Lariboisière Hospital, Paris, France; and Elemental Research, North Vancouver, British Columbia, Canada.

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Address reprint requests to P.J. Marie, PhD, INSERM U349, 2 rue Ambroise Paré 75475 Paris cedex 10, France.

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in the presence or absence of SR at doses up to 1 mmol/L Sr^{2+}, a dose that we found to be efficient to promote osteoblast replication and collagen synthesis in short-term studies.[9]

ALP Activity

At the end of treatment, the cells were rinsed with phosphate-buffered saline (PBS) at 4°C, scraped in distilled water, sonicated, and ALP activity in the cell lysates was determined by a colorimetric method using phenyl phosphate (PNP) as substrate (Alkaline Phosphatase kit, bioMerieux, Marcy l'Etoile, France). Protein content was determined colorimetrically (BioRad, Ivry, France). 1,25(OH)2D is known to stimulate ALP activity in MC3T3-E1 cultures,[26] and this compound was used as an internal control in these studies.

Collagen Synthesis

To determine collagen synthesis, tritiated proline (2 μCi/mL; NEN, Boston, MA) was added 24 hours before the end of the culture. The samples were then precipitated in 10% trichloracetic acid with 1 mmol/L cold proline, then dissolved in 0.1 N NaOH for 1 hour at 65°C, and tritiated proline incorporation into collagen was determined by liquid scintigraphy counting.[9] The data were corrected for protein content.

Ca, Magnesium, and Sr Measurements

At the end of the cultures, the wells were washed with cold calcium-free PBS, scraped, centrifuged, and the pellets were dissolved in 10% nitric acid for 24 hours at room temperature. Ca, magnesium (Mg), and Sr contents were determined by inductively coupled plasma mass spectrometry (ICP/MS), and protein content was determined using the DC Protein Assay (Bio-Rad, München, Germany). Results are expressed as the ratio of the element (Sr, Ca, Mg) on the total Sr + Ca + Mg/mg protein.

Analysis of Matrix Mineralization

Matrix mineralization was quantified by measuring the number and surface of mineralized nodules using a digital image analyzer (Biocom, Lyon, France). The surface and the number of all mineralized nodules were quantified in 3 wells per condition at day 14 of culture.

Statistics

All parameters are expressed as the mean ± SEM. Differences between untreated and treated groups were analyzed using the statistical package super-analysis of variance (ANOVA) with a subsequent Scheffe F Test (Macintosh, Abacus Concepts, Berkeley, CA). Differences between the mean values were evaluated with a minimal significance of $P < .05$.

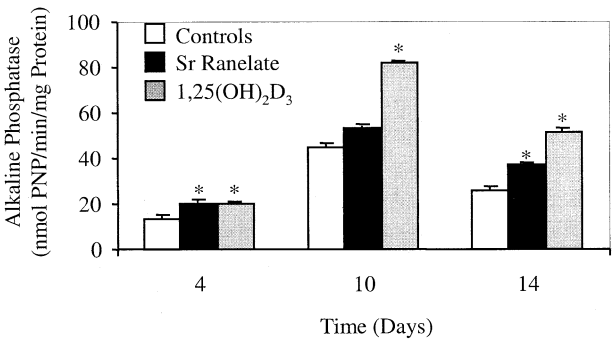

Fig 1. Effects of SR (1 mmol/L Sr^{2+}) and 1,25(OH)2D (1 nmol/L) on ALP activity in MC3T3-E1 osteoblastic cells. Data are expressed as the mean ± SEM (n = 4 to 5 replicates per group). *$P < .05$ *v* control group.

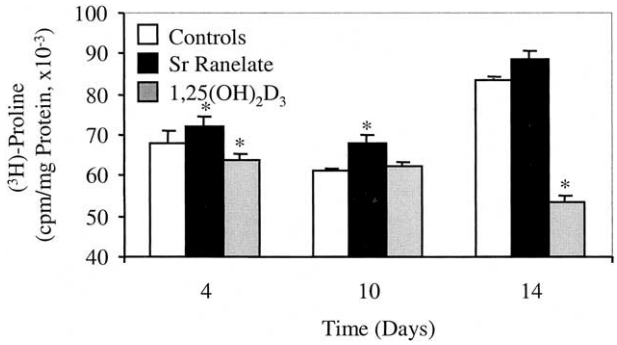

Fig 2. Effects of SR (1 mmol/L Sr^{2+}) and 1,25(OH)2D (1 nmol/L) on collagen synthesis in MC3T3-E1 osteoblastic cells. Data are expressed as the mean ± SEM (n = 4 to 6 replicates per group). *$P < .05$ *v* control group.

RESULTS

SR Increases ALP Activity

The effects of SR (0.1, 0.5, and 1 mmol/L Sr^{2+}) were first analyzed in MC3T3-E1 cells cultured from 4 to 14 days. In control cells, ALP activity increased transiently at 10 days. Treatment with 1,25(OH)2D (1 nmol/L) significantly increased ALP activity on day 4 (+45%), day 10 (+81%), and day 14 (+100 %) of culture compared with controls. Treatment with SR (1 mmol/L Sr^{2+}) significantly increased ALP activity at day 4 (+45 %) and day 14 (+45 %) of culture compared with control conditions (Fig 1). Thus, SR increased ALP activity in long-term cultures of MC3T3-E1 cells. The magnitude and time-course of the effect differed, however, from that of 1,25(OH)2D.

SR Increases Collagen Synthesis

We used 1,25(OH)2D as a control because this compound is known to inhibit collagenous synthesis by a transcriptional mechanism in mouse osteoblastic cells.[30] We found that treatment with 1,25(OH)2D (1 nmol/L) inhibited collagen synthesis at 4 days (−24%) and 14 days of culture (−36%) (Fig 2), which validates the use of the model. We previously reported that SR stimulates type I collagen synthesis in rat calvaria and rat calvaria cells in short-term culture.[9] However, the effects of SR on collagen synthesis in mouse calvaria cells in long-term culture was not known. Here we found that SR (1 mmol/L Sr^{2+}) significantly increased collagen synthesis at days 4 (+6%) and day 10 (+11.5%) of culture (Fig 2).

SR Maintains Bone Mineralization

Besides its effect on ALP activity and collagen synthesis, we determined whether SR might affect the mineralization of the matrix formed by MC3T3-E1 cells. Consistent with previous data,[27] MC3T3-E1 cells cultured in the presence of ascorbic acid and phosphate, form 3-dimensional nodular structures at

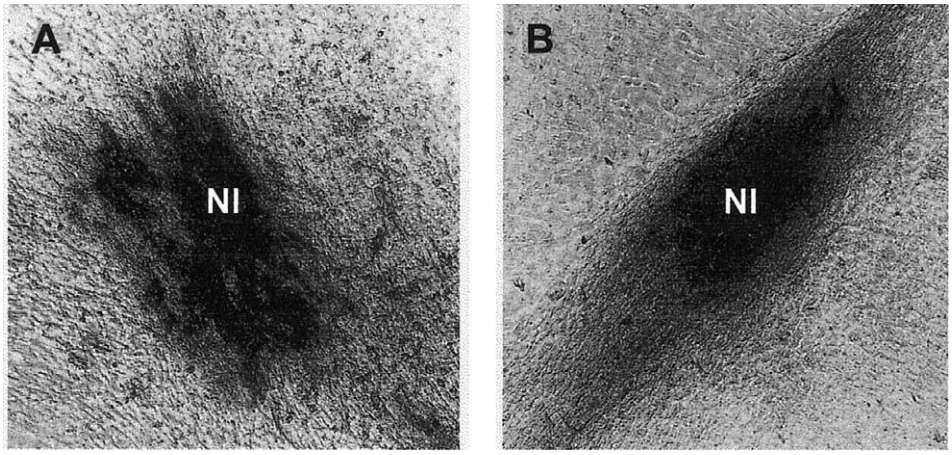

Fig 3. Formation of nodules (NI) by MC3T3-E1 osteoblastic cells after 14 days of culture in (A) untreated cells or (B) in the presence of SR (0.5 mmol/L Sr^{2+}).

day 10 to 14 of culture that became mineralized with time (Fig 3). Nodules formed under treatment with SR (Fig 3B) did not differ from those formed with untreated cells (Fig 3A) at any dosage level. As shown in Fig 4, continuous treatment with SR at Sr^{2+} concentrations of 0.1, 0.5, and 1 mmol/L, did not significantly affect the surface of mineralized nodules. In addition, the ratio of the surface to the number of mineralized nodules was unaffected by SR treatment for up to 14 days (Table 1). This indicates that SR maintains a normal mineralization induced by MC3T3-E1 cells in long-term cultures.

Sr Incorporates Into Mineralized Nodules

We then verified that Sr incorporated into calcified nodules. As shown in Fig 5A, Sr content measured at 14 days of culture in nodules was very low in control nodules and increased dose-dependently under treatment with SR. To further determine whether SR incorporation may affect mineral uptake in the nodules, we determined the mineral content in relation to the Sr content. As shown in Fig 5B, Ca content was slightly reduced after treatment with SR. In contrast, Mg uptake increased after treatment (Fig 5C).

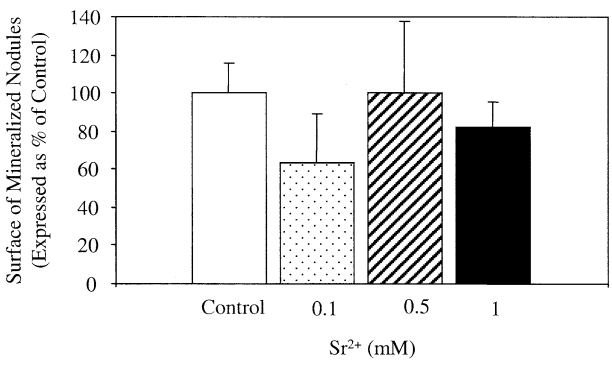

Fig 4. Surface of mineralized nodules formed by MC3T3-E1 osteoblastic cells cultured for 14 days in the absence (control) or presence of SR. The results are expressed as % of control. Data are expressed as the mean ± SEM (n = 4 to 5 replicates per group). There was no significant difference between SR and control groups.

DISCUSSION

We previously showed that SR has beneficial effects on bone mass in experimental models of osteopenia (reviewed in

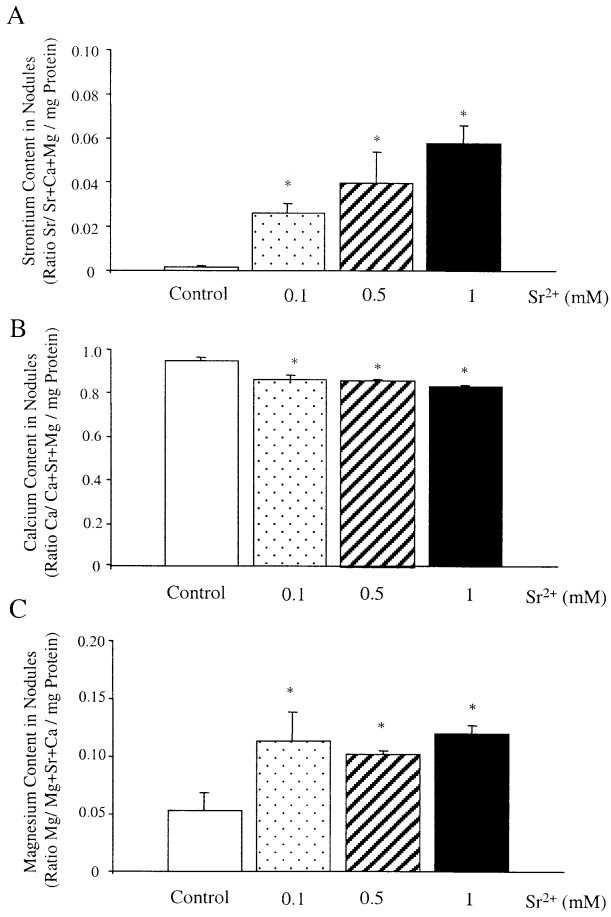

Fig 5. (A) Sr, (B) Ca, and (C) Mg content in the mineralized nodules formed by MC3T3-E1 osteoblastic cells cultured for 14 days in the absence (control) or presence of SR. Data are expressed as the mean ± SEM (n = 5 to 6 replicates per group). *$P < .05$ *v* the control group.

Table 1. Effects of SR on the Ratio of the Surface to the Number of Nodules in MC3T3-E1 Osteoblastic Cells at 14 Days of Culture

Control	SR 0.1 mmol/L Sr^{2+}	Control	SR 0.5 mmol/L Sr^{2+}	Control	SR 1.0 mmol/L Sr^{2+}
1.24 ± 0.55	2.04 ± 0.91	1.32 ± 0.59	4.58 ± 3.24	1.86 ± 0.93	1.63 ± 0.82

NOTE. (n = 3 to 5 replicates per group). There was no significant difference between SR and control groups.

Marie[31]). Because Sr incorporates into bone (reviewed in Dahl et al[20]), it was important to determine whether SR might alter mineralization of the matrix in long-term culture. The present study shows that long-term in vitro treatment with SR does not hamper matrix mineralization induced by murine osteogenic cells.

MC3T3-E1 are osteogenic cells that are a widely used model to study in vitro matrix mineralization. These cells are able to differentiate into osteoblasts that express strong ALP activity and to form a collagenous matrix organized in 3-dimensional nodules, which progressively become mineralized[25,27,29,32,33] in the presence of ascorbic acid and phosphate. In this system, MC3T3-E1 cells display a time-dependent and sequential expression of osteoblast characteristics analogous to in vivo bone formation.[27] Therefore, this model has previously been used to study the expression of osteoblast specific genes[33] and the effects of calciotropic agents, such as prostaglandins,[34,35] retinoic acid,[36] 1,25(OH)2D,[26] parathyroid hormone,[37] bone morphogenetic protein-2 (BMP-2), and transforming growth factor-β (TGF-β)[38,39] on osteoblast differentiation and mineralization. Moreover, the model was used to analyze the effects of pharmacologic agents, such as statins[40] and bisphosphonates,[41] as well as metals, such as zinc,[42] aluminum,[43] and cadmium.[44] Therefore, this culture system offers a useful model for making a quantitative estimation of osteoblast-mediated mineralization in vitro. In this culture system, the expression of ALP is time dependent with a peak occurring at 10 days of culture.[27] We found that SR increased ALP activity in MC3T3-E1 osteoblasts. However, the effect of SR was weaker than the effect of 1,25(OH)2D. This is consistent with the previous finding that 1,25(OH)2D promotes markedly ALP activity in MC3T3-E1 osteoblasts at the early phase of culture.[26] Although the mechanism of action of SR on ALP activity is unknown at the moment, the increased ALP activity suggests that this compound may act directly on osteoblastic cells in this model. This is consistent with the increased plasma bone specific ALP activity found in animals treated orally with SR (reviewed in Marie[31]).

The addition of ascorbic acid in MC3T3-E1 cells is known to induce the deposition of collagen in the extracellular matrix.[27] We found that matrix deposition accumulated maximally at 10 days, which is consistent with previous findings in this model.[29] We also found that SR increased collagen synthesis in MC3T3-E1 cultures, which confirms our previous finding that short-term treatment with SR increased collagen synthesis in both rat calvaria organ cultures and rat calvaria cells.[9] In the present study, the increase in collagen synthesis appeared to be biphasic. This may be due to a different response to Sr of the cultured cell population, which progressively differentiates into mature osteoblasts.[29] This effect clearly differs from the inhibitory effect of 1,25(OH)2D that is known to transcriptionally inhibit type I collagen gene expression in rodent osteoblasts.[30] Our finding that SR increased collagen synthesis in MC3T3-E1 cells is consistent with previous studies showing that Sr increases trabecular bone formation in vivo in rats, mice, and humans as demonstrated by bone histomorphometric analysis (reviewed in Marie[31]).

The most important issue of this study was to determine whether SR might affect matrix mineralization once present in the bone matrix. MC3T3-E1 are known to deposit minerals in the collagenous matrix in the presence of β-glycerol phosphate.[25] Minerals formed in vitro were found to consist of Ca and phosphorus deposited on well-banded collagen fibrils, and some of the crystals matured into hydroxyapatite crystals.[25,28] In the system culture used here, the addition of phosphate, a natural substrate, allowed mineralization of the matrix at 10 to 14 days of culture. In the presence of SR, we found that Sr incorporated dose-dependently in nodules. These in vitro data are similar to the dose-dependent uptake of Sr in bone obtained in vivo in rats, mice, or monkeys treated with SR (reviewed in Marie[31]). We found that nodule calcification occurred normally despite Sr supplementation and incorporation in the mineralized nodules, indicating that supplementation with SR at the dose used did not affect matrix mineralization in vitro. This is consistent with the lack of defective bone mineralization observed by histomorphometry in several species (rat, mouse, monkey) treated with SR.[14-16] Our finding that long-term treatment with SR did not inhibit mineralization of the nodules formed by MC3T3-E1 cells is in contrast with the effects of zinc,[42] aluminum,[43] and cadmium,[44] which were all found to inhibit osteoblast-mediated mineralization in MC3T3-E1 cultures. Interestingly, we found that Sr uptake led to increased Mg uptake. Because Mg is known to be located exclusively in the external hydrophilic layer of the crystal,[45] this did not affect the mineralization of the matrix in long-term culture, but resulted in lower Ca levels in this layer.

The present in vitro data are in accordance with previous in vivo studies in normal or ovariectomized rodents showing that SR had no deleterious effect on bone matrix mineralization at effective doses on bone formation, resorption, or bone mass.[31] Consistently, it was previously found that long-term treatment with SR inducing a maximal bone Sr content of 2% (Sr/Sr + Ca, molar ratio) does not affect bone mineralization in animals with normal renal function.[2,31] The present study shows that SR at a dose that promotes ALP activity and collagen synthesis in osteoblasts induces normal matrix mineralization in long-term cultures without deleterious effect of SR on matrix mineralization.

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