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REVIEW ARTICLE

The mineralization of bone tissue: a forgotten dimension in osteoporosis research

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Abstract Osteoporosis treatment should not only prevent the loss of bone tissue, not interfere with apatite and avoid bone mineral changes at the crystal level, but should also increase the mechanical resistance of bone and thus protect the skeleton against new fractures. Mineral substance is crystallized as nonstoichiometric carbonated apatite ionic crystals of small size and extended specific surface. Consequently, they have a very large interface with extracellular fluids, and numerous interactions between ions from the extracellular fluid and ions constituting apatite crystals are thus possible. It is generally agreed that bone strength depends on the bone matrix volume and the microarchitectural distribution of this volume, while the degree of mineralization of bone tissue is almost never mentioned as a determinant of bone strength. We now have evidence that the degree of mineralization of bone tissue strongly influences not only the mechanical resistance of bones but also the bone mineral density. In adult bone, our model is based on the impact of changes in the bone remodeling rate on the degree of mineralization of bone tissue. The purpose of this paper is to report the main results concerning the interactions of strontium (Sr) with bone mineral in animals and in osteoporotic women treated with strontium ranelate (SR). These studies aimed to evaluate using X-ray microanalysis, X-ray diffraction and computerized quantitative contact microradiography: (1) the relative calcium and Sr bone content, (2) the distribution of Sr in compact and cancellous bone, (3) the dose dependence of the deposition of Sr in bone, (4) the interactions between Sr and mineral at the crystal level (in monkeys), (5) the influence of Sr on the mean degree of mineralization of bone tissue and on the distribution of the degree of mineralization of bone tissue, and (6) the bone clearance of Sr over short periods of time (6 and 10 weeks) after cessation of SR administration (monkeys treated for 13 and 52 weeks, respectively). In monkeys killed at the end of exposure (13 or 52 weeks), Sr was taken up in a dose-dependent manner into compact and cancellous bone, with a higher content in new bone than in old bone. The Sr content greatly decreased (about 2-fold) in animals killed 6 or 10 weeks after the end of treatment but this affected new bone almost exclusively. After SR treatment, there were no significant changes in crystal characteristics. Easily exchangeable in bone mineral, Sr was slightly linked to crystals by ionic substitution (generally 1 calcium ion substituted by 1 Sr ion in each unit cell). The degree of bone mineralization was not significantly different in the various groups of monkeys. Thus, at the end of long-term SR treatment and after a period of withdrawal, Sr was taken up in a dose-dependent manner into new bone without alteration of the degree of bone mineralization and with no major modification of bone mineral at the crystal level. In postmenopausal osteoporotic women treated with SR (0.5, 1 and 2 g/day) for 2 years, Sr was dose7-dependently deposited into new bone without changes in the degree of mineralization of bone tissue. These findings could reflect dose-dependent stimulation of bone formation and are of potential value for the use of SR in the treatment of osteoporosis. In conclusion, the different studies performed on bone samples from monkeys and humans treated with various doses of SR showed that Sr was heterogeneously distributed between new and old bone but in a dose-dependent manner without alteration of the crystal characteristics and the degree of mineralization of bone tissue, even after long-term administration of often high doses of SR (the highest therapeutic dose used in humans is 4-fold lower than the lowest experimental dose administered to monkeys). This emphasizes the value, as antiosteoporotic treatment, of SR, which is safe at the bone mineral level.

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Introduction

Strontium ranelate (SR) has a dual effect on bone remodeling by increasing bone formation and decreasing bone resorption leading to prevention of bone loss and increase in bone mass and strength in rats [1, 2]. Strontium (Sr) induces no major change of mineral at the crystal level and is heterogeneously distributed in bone with higher concentrations in new bone than in old bone in monkeys treated for 13 weeks [3] and for 52 weeks [4]. Preliminary observations in postmenopausal osteoporotic women treated for 2 years with SR (2 g/day) confirmed the heterogeneous distribution of Sr in bone [5, 6].

In osteoporosis, the negative imbalance between bone resorption and bone formation is accelerated by the increase in the activation frequency of new remodeling units induced by the menopause. This imbalance persists until the end of life and shortens the duration of the secondary mineralization of basic structural units (BSUs) [7]. Osteoporosis treatment should not only prevent the loss of bone tissue, not interfere with apatite and avoid bone mineral changes at the crystal level, but it should also increase the mechanical resistance of bone and thus protect the skeleton against new fractures. The mechanisms by which Sr stimulates bone formation are not well known. Furthermore, the interactions between Sr and the bone mineral substance have not been fully elucidated.

The purpose of the present paper is to report the main results concerning the interactions of Sr with bone mineral in animals and in osteoporotic women treated with SR.

Bone mineral substance

The bone mineral substance has two main functions: a biomechanical one (stability of the skeleton) and a metabolic one (reservoir for many ions, control of mineral homeostasis).

Mature bone tissue is composed of 60–70% mineral substance and 30–40% organic matrix, mainly (85–90%) type I collagen fibrils. Mineral substance is crystallized as nonstoichiometric carbonated apatite ionic crystals of small size and extended specific surface (100–200 m^2/g); consequently, they have a very large interface with extracellular fluids. Bone mineral is metabolically active, thus various and numerous interactions between ions from the extracellular fluid and ions constituting apatite crystals are possible. The skeleton contains 99% of the calcium of the body, 35% of the body sodium, 80% of the body carbonates and 60% of the magnesium. From its initial formation to growth, maturation and dissolution, apatite crystals interact with the water from the bone matrix. Since crystals do not grow if ions do not diffuse from the milieu, the degree of mineralization does not progress when the water content is too low. Consequently, mineralization is rarely complete and stops at about 90–95% of the expected maximum level. In young bone tissue, the water content is high and ions are constantly exchanged with apatite. Conversely, in old bone tissue, these exchanges decrease considerably. A model with four compartments has been developed to summarize the crystal and its environment: from outside to inside the crystal there are the bulk solution easily separable from mineral, the hydrated shell (polarizable ions bound to crystal), the crystal surface, and the crystal interior. Some ions that constitute the crystal can be replaced by other ions, either during formation of the crystal (ionic substitutions) or at the surface of the crystal (surface ionic exchanges). According to the differences in size (ionic radius) between these different ions, these replacements may be followed by changes in shape, sizes at crystal and unit cell levels, and structure of the crystal (Table 1). Ionic exchanges are called isoionic when ions normally constituting apatite (calcium, phosphate) replace similar ones inside crystal. Conversely, in hetero-ionic exchanges, constituting ions are replaced by other ions normally absent from bone mineral (various cations instead of calcium, and anions instead of phosphate, carbonate and hydroxyl ions). Hetero-ionic exchanges are responsible for a great variety of exchanges. Often found at the surface of the crystal and in the hydrated shell, they are less frequent inside the crystal due to problems of charge and size. It is very important to take into account the different exchanges at the crystal level to better understand the mechanisms involved in the control of phosphate–calcium homeostasis by the skeleton.

Previous observations have shown that Sr is taken up at the bone tissue level by reaction with the mineral substance, i.e., apatite ionic crystals [8, 9, 10, 11, 12]. This uptake may be achieved by either ionic exchange or adsorption of Sr ions onto the surface of mature crystals, or alternatively by ionic substitutions with calcium ions into the crystal lattice of apatite. However, calcium ions are preferred to Sr ions and the amount of substitutions will always be relatively low in biological apatites. The ionic radius of the Sr ion is higher than that of the calcium ion (1.13 Å vs 0.99 Å). Consequently, since the Sr-hydroxyl distance is higher than that of

Table 1 Effects of some ionic substitutions on the lattice parameters of synthetic apatites (adapted from [8])

Substituent	Ionic radius (Å)	a-axis (Å)	c-axis (Å)
Calcium (apatite)	0.99	9.436	6.882
Strontium	1.12	9.768	7.214
Lead	1.20	9.880	7.410
OH (apatite)	1.53	9.436	6.882
Fluoride	1.36	9.383	6.882

calcium-hydroxyl, decreases in lattice energy (cohesion is inversely proportional to the distance) and thus of crystallinity are possible [11]. Thus, ionic substitutions between calcium and Sr in bone mineral may lead to modifications of lattice parameters, crystal sizes and crystallinity of mineral substance. However, at high concentrations it is impossible to ascertain the amount of Sr present in crystal lattice interstices and the amount of Sr taken up at lattice point positions by displacing calcium [13].

Degree of mineralization of bone tissue

It is generally agreed that strength of bones depends on the volume of bone matrix and the microarchitectural distribution of this volume, while the degree of mineralization of bone tissue (DMB) is almost never mentioned as a determinant of bone strength. We now have evidence that the DMB strongly influences not only the mechanical resistance of bones but also the bone mineral density (BMD) measured by dual-energy X-ray absorptiometry or other methods. From microradiographic observations made in the 1970s, it was clear that the DMB varies over BSUs, namely, the osteons in cortical bone and the trabecular packets in cancellous bone, the recently deposited ones being much less mineralized than the older ones. The ''young'' ones appear in dark gray on microradiographs, while the ''old'' ones are whiter (Fig. 1). This heterogeneity in the DMB is explained by the fact that bone formation which follows bone resorption in the remodeling sequence is a multistep process: following its deposition, the new matrix begins to mineralize after about 5–10 days from the time of deposition and the linear rate of this primary mineralization can be measured directly in vivo using double tetracycline labeling. After full completion of the BSUs, a phase of secondary mineralization begins. This process consists of a slow and gradual maturation of the mineral component, including an increase in the amount of crystals and/or an augmentation of crystal size toward their maximum dimensions. This secondary mineralization progressively augments the mineral content in bone matrix. At the end of the primary mineralization, mineral content represents only about 50% of the maximum degree of mineralization obtained at the end of the secondary mineralization phase.

In adult bone, the DMB depends on the rate of remodeling. In other words, the biological determinant of mineralization is the rate of turnover. Our model (Fig. 2) is based on the impact of changes in bone remodeling rate on the DMB, i.e., on the BMD at the tissue level. Thus, we hypothesized that agents (parathyroid hormone) or events (menopause, ovariectomy) which bring about an augmentation in the ''birthrate'' or activation frequency of basic multicellular units (BMUs) induce a decrease in the ''lifespan'' of BSUs, in other words in the time available for the secondary mineralization. This leads to new BSUs being resorbed before they have fully completed their secondary mineralization, as proven by the presence of a large amount of incompletely mineralized BSUs and a low mean DMB. Conversely, antiresorptive agents (bisphosphonates, calcitonin, estrogen, selective estrogen receptor modulators or SERMs) that cause a marked reduction in the birthrate of BMUs prolong the ''lifespan'' of the BSUs, allowing more complete secondary mineralization. This should finally bring about an increase in the DMB. Thus, it is important to place the effects of Sr salts on bone remodeling and mineralization within this model. This new approach of the determinants of bone strength and BMD and the results of our recent studies as well as others now in progress using antiresorptive (SERMs) and forming (SR) agents used in the treatment of osteoporosis emphasize that bone mineral substance is an important factor to take into account in the pathophysiology of osteoporosis and other bone conditions as well as in the mechanisms of action of compounds influencing the remodeling rate. These data also demonstrate that ''bone mass'' and ''bone mineral density,'' often used synonymously, are two different entities. For

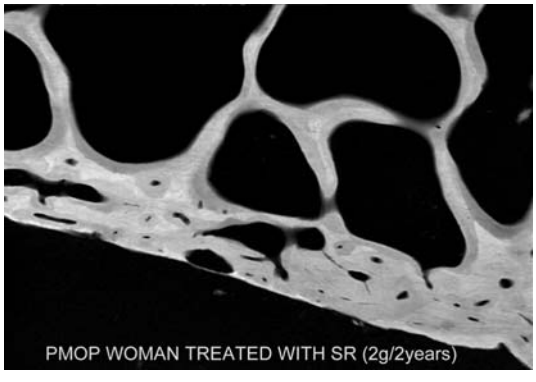

Fig. 1 Microradiograph of iliac compact bone tissue from a woman. The degree of mineralization of bone is heterogeneously distributed with the recently deposited basic structural units (BSUs) being much less mineralized than the older ones. The ''young'' BSUs appear in dark gray on the microradiograph and the ''old'' ones are almost white

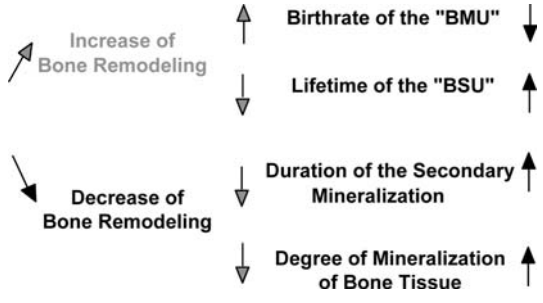

Fig. 2 Working model of the potential mechanisms by which events or compounds act on bone remodeling activity and, consequently, modify the degree of mineralization of bone. The *black arrows* follow the suggested sequence in a case of decreased bone remodeling activity, and the *gray arrows* illustrate the inverse sequence when bone remodeling activity is increased. *BMU*, basic multicellular unit; *BSU*, basic structural unit

these reasons, it is important to rediscover the mineral dimension of bone which has been forgotten for many years.

SR has a dual effect on bone remodeling by increasing bone formation and decreasing bone resorption leading to the prevention of bone loss and an increase in bone mass and strength in rats [14]. Previous observations [1, 2, 3] have shown that Sr was present at the mineral substance level (ionic exchange and/or surface adsorption of Sr ions onto the crystals, ionic substitutions with calcium ions within the crystal lattice of apatite of newly formed mineral deposits; Table 1). Sr induces no major change of mineral at the crystal level and is heterogeneously distributed in bone with higher concentrations in new bone than in old bone in monkeys treated for 13 weeks [3] and for 52 weeks [4]. Preliminary observations in postmenopausal osteoporotic women treated for 2 years with SR (2 g/day) confirmed the heterogeneous distribution of Sr in bone [4, 5] (G. Boivin, D. Farlay, E. Foos et al., unpublished data). In order to better understand the main interactions of Sr with bone mineral, the main results of these studies performed using X-ray microanalysis, X-ray diffraction and computed quantitative contact microradiography will be summarized. The purposes of these studies were to evaluate: (1) the relative calcium and Sr bone contents, (2) the distribution of Sr in compact and cancellous bone, (3) the dose dependence of the deposition of Sr in bone, (4) the interactions between Sr and mineral at the crystal level (monkeys), (5) the influence of Sr on the mean DMB and on the distribution of the DMB, and (6) the bone clearance of Sr over short periods of time (6 and 10 weeks) after cessation of SR administration (monkeys treated for 13 and 52 weeks, respectively).

Materials and methods

Iliac bone samples were first obtained from 20 male monkeys: (a) 4 untreated animals, (b) 12 animals at the end of a 13-week exposure period to SR (750, 275 or 100 mg/kg per day orally), (c) 4 animals 6 weeks following the end of a 13-week exposure period (750 or 100 mg/kg per day orally). In a second study, iliac bones were obtained from 30 monkeys: 7 untreated controls, 12 killed at the end of a 52-week period of SR treatment (200, 500 and 1250 mg/kg per day orally), and 11 killed 10 weeks after the end of a 52-week period of SR treatment (same three doses of SR).

Sr interactions with bone mineral were also investigated on the occasion of clinical studies. At the end of a 2-year placebo (PLA)-controlled study in postmenopausal osteoporotic women treated with three different SR doses, 27 transiliac bone biopsies (7 PLA controls, 6 SR-treated at 0.5 g/day, 6 SR-treated at 1 g/day, 8 SR-treated at 2 g/day) were analyzed.

Sr uptake and distribution in these samples were quantified by X-ray microanalysis, and the DMB was measured by quantitative microradiography. Changes at the crystal level were evaluated only in monkeys, by X-ray diffraction or Raman spectrometry.

Preparation of bone samples

Iliac bones were taken off and each divided into three parts. Some of the bone samples were fixed in 80% alcohol, dehydrated in absolute alcohol, then embedded in methyl methacrylate without prior decalcification [15]. Bone samples were oriented so as to cut the osteons perpendicular to the Haversian canals in at least one of the cortices (compact bone).

For the X-ray microanalysis [3, (G. Boivin, D. Farlay, E. Foos, et al. unpublished data)] embedded bone samples were surfaced, carbon-coated, then fixed in the specimen holder. The areas analyzed included old and new lamellar bone tissue from compact and cancellous bone. Fifteen different measurements were performed in each category of tissue (15 in new compact bone, 15 in old compact bone, and 15 in new and old cancellous bone), i.e., a total of 60 measurements in each bone sample were analyzed.

For quantitative microradiography [16, 17], thick sections were cut from embedded bone samples used for X-ray microanalysis, progressively ground to a thickness of 100 ± 1 μm, and polished. The bone sections were cleaned with ultrasound then microradiographed.

Finally, some of the non-decalcified iliac bone samples from monkeys were taken before embedding and treated for 3–4 days with a NaOH saturated solution in order to clean the bone from the organic material. These samples were either powdered for X-ray diffraction or kept dry for Raman microspectrometry [3]. This study was not performed on human material due to the small size of bone biopsies.

X-ray microanalysis

X-ray microanalysis was performed using a Camebax (Cameca, Courbevoie, France) equipped with an energy-dispersive spectrometer and with a detector of low elements (Voyager III, Tracor, USA). The Sr (Sr Lα), phosphorus (P Kα) and calcium (Ca Kα) elements were mainly analyzed at the same place at a voltage of 15 kV. Each measurement represented a count of 100 s at the same place. For each element, contents were expressed as a percentage of weight (100% corresponding to the total elements analyzed). Finally, the weight ratios Ca/P, Ca + Sr/P, Sr/Ca, Sr/P and Sr/Ca + Sr were also calculated. Secondary electron images (morphology of the area measured) and X-ray images (topographic distribution of the main elements analyzed) were performed to illustrate the Sr distribution in bone tissue.

Quantitative microradiography

Contact microradiography of 100 ± 1 μm-thick iliac bone sections was performed using an X-ray diffraction unit PW 1830/40 equipped with a diffraction tube PW 2273/20 (Philips, Limeuil Brévannes, France). Nickel-filtered copper Kα radiation was used at 25 kV and 25 mA. A Geola high-resolution film (VRP-M green sensitive) was exposed for 20 min (Slavich International Wholesale Office, Vilnius, Lithuania). For quantitative evaluation of the X-ray absorption by the bone section, a reference system composed of aluminum was exposed on each microradiograph [16, 17]. The DMB was quantified using a new combined contact microradiography-microdensitometry computerized method [7, 17, 18] described briefly as follows. A custom-developed software was used for the automatic analysis of gray levels of microradiographs with Visiolab 1000 (Biocom, France), a true color image processing workstation operating with Microsoft Windows. The image of the microradiograph was captured through a microscope using a 3-CCD color camera. Each field analyzed was divided into 4350 square measurement units (about 100 pixels each). After calibration using the aluminum reference step-wedge, the measured area was automatically selected and the gray levels were measured from the computer-generated map indicating the spatial distribution of the "measurement units". Data were converted from gray-level values into the degree of mineralization with the construction of a calibration curve based on the measurements obtained on the aluminum step-wedge. The DMB was finally expressed in grams mineral per cubic centimeter bone and results were measured separately in compact, cancellous, and total bone (compact + cancellous) tissues.

X-ray diffraction

X-ray diffraction study [3, 19] allows information to be obtained on the crystallographic species of the bone mineral substance, on crystal size and crystalline perfection, and on the amount of hetero-ionic substitutions in the mineral substance. Powdered samples were analyzed using a Siemens D500 diffractometer (Diffraction Center Henri Longchambon, Université Claude Bernard Lyon 1, Villeurbanne, France). Profiles were obtained between 0 and 70°2θ and counting of 2 s was performed at room temperature every 0.2°. Parameters a and c of the crystal unit cell were measured and compared with standard values of the JCPDS (Joint Committee for Powder Diffraction Standards; from apatite with Ca = 10 and Sr = 0 to compound totally substituted with Ca = 0 and Sr = 10) in order to evaluate the substitutions of Ca by Sr and the modifications at the crystal level.

Raman microspectrometry

Raman microspectrometry allows some information on the vibration of groups of atoms in the crystal lattice to be obtained. These vibrations are identified by different frequencies of inelastic diffused light, and they depend on the crystal arrangement. The profiles obtained are composed of peaks, characteristic for a given mineral structure. Changes in the shape and width at half-maximum of peaks allowed the evaluation of modifications of crystallinity and hetero-ionic substitutions. The technique is nondestructive and is performed on raw solid nonpowdered samples. The Raman microspectrometer used was a Dilor XY coupled with an Olympus microscope (objective ×50). This spectrometer uses an argon laser as exciting light (514 nm). The laser is focused on the sample through the microscope, then the diffused light is directed toward a charge coupled device detector. This diffused light is used to identify and localize the different polyatomic compounds.

Results

Monkeys treated or not with SR for 13 weeks

In controls, homogeneously distributed traces of Sr were detected in bone. In all treated monkeys, Sr was only detected in calcified matrix. In monkeys killed at the end of exposure, Sr was dose-dependently incorporated into the mineral substance of compact and cancellous bone. Sr was heterogeneously distributed with 3 to 4 times more Sr in new than in old compact bone, and about 2.5 times more Sr in new than in old cancellous bone. Sr was strongly decreased in animals killed 6 weeks after the end of exposure. Diffraction showed no significant changes in crystal lattice characteristics. Sr appeared easily exchangeable from bone mineral and was slightly linked to mature crystals through ionic substitution. Indeed, even with the highest dose, there was a mean substitution of only one of the 10 calcium ions by one Sr ion in each crystal. In conclusion, Sr when taken up by bone was heterogeneously distributed with a higher concentration in new than in old bone but without major modification of bone mineral at the crystal level.

Monkeys treated or not treated with SR for 52 weeks

After the study on short-term administration of SR, the interactions of Sr with bone mineral were investigated in monkeys after long-term SR treatment and at the end of a longer recovery period (10 weeks). In monkeys killed at the end of a 52-week period of SR treatment, Sr was taken up in a dose-dependent manner into compact and cancellous bone, with a higher content (1.6 times) in new bone than in old bone. Sr content greatly decreased (1.7 to 2 times) in animals killed 10 weeks after the end of treatment but this affected almost exclusively new bone. After SR treatment, there were no significant changes in crystal characteristics. Easily exchangeable in bone mineral, Sr was slightly linked to crystals by ionic substitution (generally 1 calcium ion substituted by 1 Sr ion in each unit cell). DMB was not significantly different in the various groups of monkeys. In conclusion, at the end of long-term SR treatment and after a period of withdrawal, Sr was taken up in a dose-dependent manner into new bone without alteration of the DMB and with no major modification of bone mineral at the crystal level. These findings confirm the safety of SR use at the mineral level and its potential value as an antiosteoporotic treatment which simultaneously increases bone formation and decreases bone resorption.

Postmenopausal osteoporotic women treated with SR for 2 years

Only traces of Sr were found in placebo (PLA)-treated bone. In SR-treated women, Sr was dose-dependently deposited into compact ($p = 0.0001$) and cancellous bone ($p = 0.0002$), with significantly higher contents in new bone than in old bone ($p = 0.0001$). The uptake of Sr in new bone was also dose-dependent ($p = 0.0001$ in compact, $p = 0.0002$ in cancellous). Old bone contained small amounts of Sr and only after treatment with 2 g/day. Mean DMB was not significantly different in SR and PLA groups in either compact or cancellous bone. However, the mean DMB tended to increase in the SR group treated with 0.5 g/day and to progressively decrease to the PLA value in the 1 and 2 g/day groups. In summary, after a 2-year period of treatment with 0.5, 1 and 2 g/day of SR, Sr was dose-dependently deposited into new bone without changes in the mean DMB. These findings could reflect a dose-dependent stimulation of bone formation and are of potential interest for the use of SR in the treatment of osteoporosis.

Conclusion

The different studies performed on bone samples from monkeys and humans treated with various doses of SR showed that Sr was heterogeneously distributed between new and old bone but in a dose-dependent manner without alteration of the crystal characteristics and DMB, even after long-term administration of often high doses of SR (the highest therapeutic dose used in humans is 4-fold lower than the lowest experimental

dose administered to monkeys). This emphasizes the value of SR as an antiosteoporotic treatment which is safe at the bone mineral level.

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