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Strontium Ranelate Increases Cartilage Matrix Formation

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ABSTRACT

Based on previous studies showing that strontium ranelate (S12911) modulates bone loss in osteoporosis, it could be hypothesized that this drug also is effective on cartilage degradation in osteoarthritis (OA). This was investigated in vitro on normal and OA human chondrocytes treated or not treated with interleukin-1β (IL-1β). This model mimics, in vitro, the imbalance between chondroformation and chondroresorption processes observed in vivo in OA cartilage. Chondrocytes were isolated from cartilage by enzymatic digestion and cultured for 24–72 h with 10^{-4}–10^{-3} M strontium ranelate, 10^{-3} M calcium ranelate, or $2 \cdot 10^{-3}$ M $SrCl_2$ with or without IL-1β or insulin-like growth factor I (IGF-I). Stromelysin activity and stromelysin quantitation were assayed by spectrofluorometry and enzyme amplified sensitivity immunoassay (EASIA), respectively. Proteoglycans (PG) were quantified using a radioimmunoassay. Newly synthesized glycosaminoglycans (GAGs) were quantified by labeled sulfate ($Na_2{}^{35}SO_4$) incorporation. This method allowed the PG size after exclusion chromatography to be determined. Strontium ranelate, calcium ranelate, and $SrCl_2$ did not modify stromelysin synthesis even in the presence of IL-1β. Calcium ranelate induced stromelysin activation whereas strontium compounds were ineffective. Strontium ranelate and $SrCl_2$ both strongly stimulated PG production suggesting an ionic effect of strontium independent of the organic moiety. Moreover, 10^{-3} M strontium ranelate increased the stimulatory effect of IGF-I (10^{-9} M) on PG synthesis but did not reverse the inhibitory effect of IL-1β. Strontium ranelate strongly stimulates human cartilage matrix formation in vitro by a direct ionic effect without stimulating the chondroresorption processes. This finding provides a preclinical basis for in vivo testing of strontium ranelate in OA. (J Bone Miner Res 2001;16:299–308)

Key words: strontium ranelate, chondrocytes, proteoglycans, stromelysin, osteoarthritis

INTRODUCTION

CARTILAGE IS constantly being turned over in the balance between extracellular matrix synthesis and degradation. Chondroformation is stimulated by growth factors such as insulin-like growth factor I (IGF-I).[1] Chondroresorption, which involves metalloproteinases like stromelysin,[2,3] is stimulated by cytokines such as interleukin-1β (IL-1β).[4] In osteoarthritis (OA), this delicate equilibrium is progressively disrupted, leading to an excess of tissue degradation and, finally, tissue disappearance. The imbalance between stromelysin and proteoglycans (PGs) constitutes an important pathophysiological process. The levels and activity of stromelysin were found to be high in OA cartilage, synovia, and synovial fluid,[5–7] whereas PG synthesis and content decreased with lesion severity.[8] The metabolic disruption is characterized by PG release, fixed charge density, and cation concentration decrease.[9] As the rate of PG synthesis decreases when the ionic strength is below the physiological strength, cation administration could have beneficial effects on OA chondrocyte metabolism.[10,11] Strontium, a divalent cation, has shown evidence of beneficial effects on bone metabolism[12] and interference in cartilage metabolism.[13] Strontium ranelate (S12911), an uncoupling agent devel-

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oped as a preventive and curative treatment of postmenopausal osteoporosis,[12,14–16] prevents bone loss induced by estrogen deficiency by inhibiting bone resorption and stimulating bone formation.[17]

The present study investigated the effects of strontium ranelate on stromelysin and PG synthesis in human chondrocytes cultured with or without IL-1β or IGF-I. In chondrocyte culture, IL-1β reproduces the decoupling effect leading to a decrease in PG and an increase in stromelysin synthesis,[4,18] whereas IGF-I stimulates PG production.[1] The effects of strontium ranelate were compared with the effects of calcium ranelate and strontium chloride ($SrCl_2$), which were used as controls.

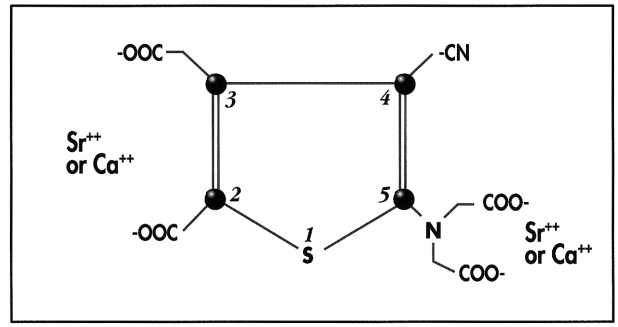

FIG. 1. Structure of strontium ranelate and calcium ranelate.

MATERIALS AND METHODS

Human chondrocyte culture

Freshly isolated human cartilage chondrocytes were cultured at a high density for a short-term period (24–72 h) to retain their phenotype. Cartilage samples were obtained from the knees of 13 cadavers (23–83 years old) immediately after death. They were excised from the superficial and medium layer of cartilage, avoiding the calcified layer. Pathological cartilage modifications were evaluated on femoral and patellar articular surfaces using the Moskowitz scale.[8] Each culture was run with chondrocytes from a single patient. The cartilage was cut into small fragments. Chondrocytes were isolated from their extracellular matrix by enzymatic digestion. Cartilage fragments (3 g) were first treated (30 minutes, 37°C, 200 rpm) with 10 ml hyaluronidase solution (Sigma Chemie, Bornem, Belgium) previously dissolved (0.5 mg/ml) in Dulbecco's modified Eagle's medium (DMEM; Biowhittaker, Brussels, Belgium). Cartilage fragments were then treated (1 h at 37°C) with 10 ml pronase solution (1 mg/ml in DMEM; Merck-Belgolabo, Overijse, Belgium). Finally, they were incubated (20 h, 37°C, 200 rpm) with 10 ml collagenase (Sigma Chemie) solution (1 mg/ml in DMEM containing 1% Ultroser G, a serum substitute supplied by Gibco, Gent, Belgium). The cells were filtered through a nylon mesh (70 μm), washed three times, counted (range, $1–2 \cdot 10^6$ cells/ml), and resuspended in 1 ml of adequate culture medium (CM; DMEM supplemented with 1% insulin, transferin, and seleium (ITS+) or TS+, 2 mM glutamine, and 50 μg/ml ascorbate). ITS+ is a culture supplement containing 0.625 mg/ml insulin, 0.625 mg/ml transferrin, 0.625 μg/ml selenious acid, 125 mg/ml bovine serum albumin (BSA), and 0.530 mg/ml linoleic acid. When chondrocytes were incubated with IGF-I, ITS+ was replaced by TS+ (TS+ is ITS+ without insulin). DMEM contained 1.8 mM calcium. Cells were kept in this CM for 48 h to eliminate in vivo contamination with drugs that the donors may have taken before death. After this washout period, cells were seeded in 10 ml polypropylene Falcon tubes ($1–2 \cdot 10^6$ cells/ml) and maintained under constant agitation (100 rpm) in a 95% air/5% CO_2 atmosphere. At the end of each culture period (1, 2, or 3 days), cells (chondrocyte pellet [CP]) and supernatant (CM) were separated by centrifugation (1000 rpm for 5 minutes). The chondrocytes recovered after collagenase digestion of the tissue were 95% viable (trypan blue test). CPs were extracted before PG and DNA analysis (CP extract). They were washed twice with Hanks' buffered saline solution (HBSS; Gibco) and homogenized by ultrasonic dissociation (10 s, 50 W/cm^2) in phosphate-buffered saline, pH 7.5, containing proteinase inhibitors. All the chemicals for which the source was not mentioned were of the purest grade commercially available.

Culture treatment

Human chondrocytes ($1–2 \cdot 10^6$ cells/ml) were cultured for 24–72 h in the absence or presence of strontium ranelate ($15 \cdot 10^{-4}$–10^{-3} M), $SrCl_2$ ($2 \cdot 10^{-3}$ M), or calcium ranelate (10^{-3} M) with or without IL-1β (10^{-11} M and 10^{-10} M) or IGF-I (10^{-3} M and 10^{-8} M). The tested compounds were directly dissolved in the CM, which was then sterilized by filtration before cellular culture. Strontium ranelate (S12911; Servier, Courbevoie, France) is made up of two stable strontium atoms and ranelic acid as carrier (5-{bis(carboxymethyl)amino}-2-carboxy-4-cyano-3-thiophenacetic acid). In calcium ranelate (S12911–0), strontium atoms are replaced by two calcium atoms (Fig. 1). Three flasks were used for each concentration of the drug and for the corresponding controls.

DNA assay

Chondrocyte DNA content is correlated directly to the cell number of each culture. DNA content was measured in the CP extract using a fluorometric method.[19] This measurement ensures elimination of result variations caused by the different number of chondrocytes in each culture.

Stromelysin activity measurement

Stromelysin activity released in the CM was measured on resorufin-labeled casein from cow's milk (Boehringer, Mannheim, Germany) using a spectrofluorometric method. Latent stromelysin (prostromelysin) was activated or not activated by *p*-aminophenylmercuric acetate[20] (APMA; Sigma Chemie) treatment of the CM (0.5 mM for 4 h at 37°C). APMA-activated CM (100 μl) was then incubated with 20 μg resorufin-labeled casein for 18 h at 37°C in a standard buffer (Tris-HCl 0.2 M, pH 7.5) containing 0.02 M

$CaCl_2$. The enzymatic reaction was stopped by adding trichloroacetic acid at a final concentration of 1.8% and 20 mM EDTA. The samples were centrifuged at 7000*g* for 15 minutes. Cleaved casein was then measured fluorometrically in 600 μl of supernatant previously buffered with 900 μl of Tris-HCl (0.5 M, pH 8.8). Excitation and emission wavelengths were 574 nm and 584 nm, respectively. Parallel samples containing 20 mM EDTA added before incubation were measured similarly, providing blank values. A standard curve obtained by incubation of increased quantities of purified stromelysin (Biosource Europe, Nivelle, Belgium) allowed the conversion of the measured fluorescence values into the stromelysin concentration equivalent (μg Eq strom/ml). The molecular weight of the detected proteinase was assessed by electrophoresis on a casein-containing gel (Zymogram) as previously described. [21]

Stromelysin quantitation

Total stromelysin released in the CM was assayed using an enzyme amplified sensitivity immunoassay (EASIA; Biosource Europe). In this immunoassay, monoclonal antibodies are directed against prostromelysin, activated stromelysin, and stromelysin bound to tissue inhibitor of metalloproteases (TIMP)-1 and TIMP-2. Total stromelysin corresponds to the level of stromelysin synthesized in the CM including prostromelysin, activated stromelysin, and stromelysin bound to TIMP-1 and TIMP-2. The method was linear between 5 and 20 ng/ml with a limit of detection of 2.5 ng/ml.

PG quantitation

PGs were quantified in CM and CP extracts by radioimmunoassay (RIA) as previously described.[22,23] Rabbit polyclonal antibodies used are specific to the antigenic determinant of the PG core protein. No cross-reaction was observed with glycosaminoglycans (GAGs) such as chondroitin sulfate or keratan sulfate or with PG treated with various enzymes. Only treatment with proteolytic enzymes (papain, trypsin, and chymotrypsin) abolished or diminished immunoreactivity as opposed to treatment with chondroitinase ABC and neuraminidase. Antiserum did not cross-react with other cartilage matrix constituents such as type II, type IX, and type XI collagens. The lack of cross-reaction with bone PG or skin, cornea, liver, heart, and lung tissue extracts, showed that the antiserum used in the study contains only specific chondrocyte-aggrecan antibodies.

Sulfated GAG quantitation

PG monomers are made up of sulfated GAG bound on a linear protein core. The quantitation of the newly synthesized PG was carried out by determination of radioactivity incorporated in sulfated GAG (^{35}S-GAG). $Na_2{}^{35}SO_4$ was added to the CM (5 μCi/ml) during the last 24 h of the culture. To eliminate unlabeled $Na_2{}^{35}SO_4$, CM was dialyzed three times against Tris-HCl 0.05 M (pH 9) containing protease inhibitors. The protease inhibitors used throughout the study were $5 \cdot 10^{-8}$ M 6-aminohexanoic acid, $5 \cdot 10^{-8}$ M trypsin inhibitor, 0.01 M EDTA, and $6.7 \cdot 10^{-3}$ M sodium azide. CP previously washed three times with HBSS was extracted (24 h, at 4°C, 100 rpm) by 500 μl of guanidine hydrochloride (GuHCl, 4 M, in 0.5 M sodium acetate solution containing protease inhibitors, pH 5.8). Three CP extracts treated in the same conditions were pooled and dialyzed against distilled water and Tris-HCl, 0.05 M (pH 9).

Radioactivity was measured in the samples using an LKB 1214 RackBeta liquid scintillation counter (Amersham International, Brussels, Belgium).

Determination of the PG size

The size of the PG produced by the chondrocytes was determined by chromatography. CM and CP extract were eluted on gel filtration chromatography under associative conditions. These conditions protect PG complexes from hydrolysis and allow PG separation according to their molecular weight. For analytical purposes, a column (0.8 cm $\times$ 60 cm) was packed with Sepharose CL2B (Pharmacia, Uppsala, Sweden). The column was equilibrated and eluted with 0.05 M Tris-HCl (pH 9) containing enzymatic inhibitors. The flow rate was 6 ml/h and 1-ml fractions were collected. The column was calibrated under the same conditions with a 1-ml sample adjusted to 20,000 cpm ($Na_2{}^{35}SO_4$). The chromatogram was established by determining the radioactivity in 0.5 ml of each fraction. The chromatographic profile of the ^{35}S-GAG was characterized by a distribution coefficient [$K_d = (V_e\text{-}V_0)/(V_s\text{-}V_0)$], where V_0 is the void volume of the column, V_s the total volume of the column, and V_e is the elution volume of the molecule under consideration. The peak fraction containing ^{35}S-PG aggregates and $Na_2{}^{35}SO_4$ were used to determine V_0 and V_S, respectively. Radioactivity allowed repartition of PG into three groups: PG-hyaluronic acid complexes ($K_d \leq 0.13$), PG monomers and small complexes ($0.13 < K_d < 0.7$), and small PG and PG fragments ($K_d \geq 0.7$).

Expression of the results and statistical analysis

The results were expressed as the rate of stromelysin or PG released into the CM or present inside the CP per microgram of DNA. The values presented are the cumulative amounts of stromelysin or PG found in the CM at the end of the specified culture period. Total PG production (TPG) was calculated by adding the results measured for the CM and the corresponding CP. Cumulative release rates of ^{35}S-GAG were obtained by adding the amounts found in the CM at the end of each incubation period. The mean $\pm$ SD of each variable was calculated. Comparison of mean values was performed for each culture individually using the unpaired Student's *t*-test. Production curves were compared for each experimental condition by means of Zerbe's randomization test.[24] Values were treated with a generalized linear mixed model to compare the effects of strontium ranelate, calcium ranelate, and strontium chloride on young normal and old OA cartilage groups; treated groups were compared between themselves and with the controls. Correlations between the variables were investigated by a linear

TABLE 1. DNA CONTENTS

	Controls		*S12911*			*S12911-0*	*$SrCl_2$*
	T_{0h}	T_{72h}	10^{-4} M	$5 \cdot 10^{-4}$ M	10^{-3} M	10^{-3} M	$2 \cdot 10^{-3}$ M
Without IL-1β	12.5 ± 1.3	13.0 ± 0.8	12.9 ± 1.1	12.3 ± 0.3	12.1 ± 0.7	12.3 ± 0.2	11.1 ± 0.7
With IL-1β	12.9 ± 2.0	13.1 ± 1.0	12.7 ± 1.3	13.9 ± 0.3	13.4 ± 0.4	11.5 ± 0.7	13.0 ± 1.2

Effects of strontium ranelate (S 12911), calcium ranelate (S 12911-0) or $SrCl_2$ on DNA (μg) contained in CP after 72h of normal human chondrocyte culture. The DNA content at the begining (T_{0h}) and the end of the culture (T_{72h}) were also compared for the controls. Results are mean values and SD of triplicate primary chondrocyte cultures in a representative experiment.

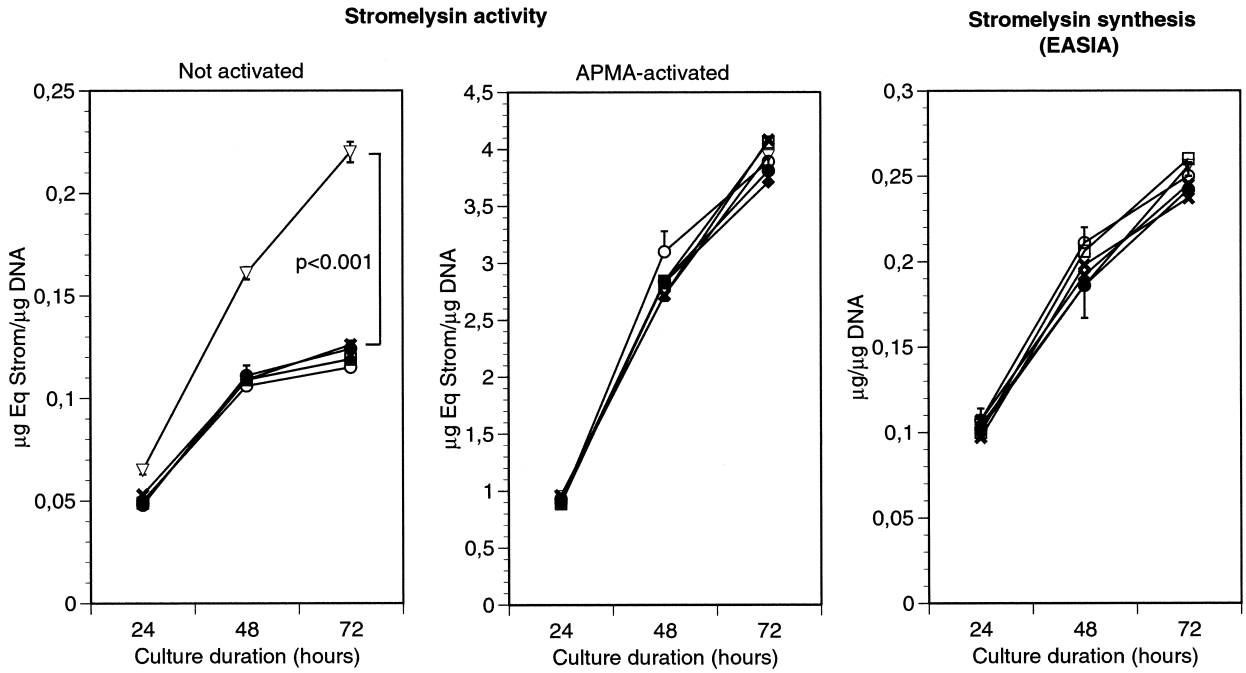

FIG. 2. Effect of increased amounts of strontium ranelate, $SrCl_2$, or calcium ranelate on stromelysin activity and synthesis by normal human chondrocytes. Primary chondrocytes were cultured for 72 h in the absence (-○-) or presence of 10^{-4} M (-×-), $5 \cdot 10^{-4}$ M (-◆-) or 10^{-3} M (-□-) strontium ranelate, $2 \cdot 10^{-3}$ M $SrCl_2$ (-●-), or 10^{-3} M calcium ranelate (-▽-). Results are the mean values of triplicate primary chondrocyte cultures in a representative experiment and error bars represent SD. The values represent the cumulative amounts of stromelysin over the specified culture periods. Differences between curves are tested by the Zerbe test.

regression analysis and a one-way analysis of variance (ANOVA) was performed.

RESULTS

Chondrocyte viability

Preliminary tests (trypan blue, ^{51}Cr release) showed the absence of cytotoxicity of the tested compounds. Cell viability was always superior to 90% whatever the culture treatment. The DNA content remained stable during the culture period (no significant difference between 0 and 72 h) and was not significantly affected by the presence of strontium ranelate (10^{-4}–10^{-3} M), calcium ranelate (10^{-3} M), or $SrCl_2$ ($2 \cdot 10^{-3}$ M; Table 1).

Stromelysin synthesis and activity

In basal conditions (no IL-1β stimulation), stromelysin activity measured in the CM of unactivated chondrocytes was very low (Fig. 2) or undetectable (Fig. 3), depending on the donor. However, stromelysin synthesis increased linearly with culture duration. Strontium ranelate and $SrCl_2$ did not activate stromelysin, whereas calcium ranelate induced enzyme activation in the culture where stromelysin activity was detectable ($p < 0.001$; Fig. 2).

APMA-activated stromelysin activity measured in the CM increased as a function of culture duration but was not affected by the presence of calcium or strontium compounds (Fig. 2). Furthermore, strontium ranelate, calcium ranelate, and $SrCl_2$ did not increase stromelysin protein synthesis (Fig. 2).

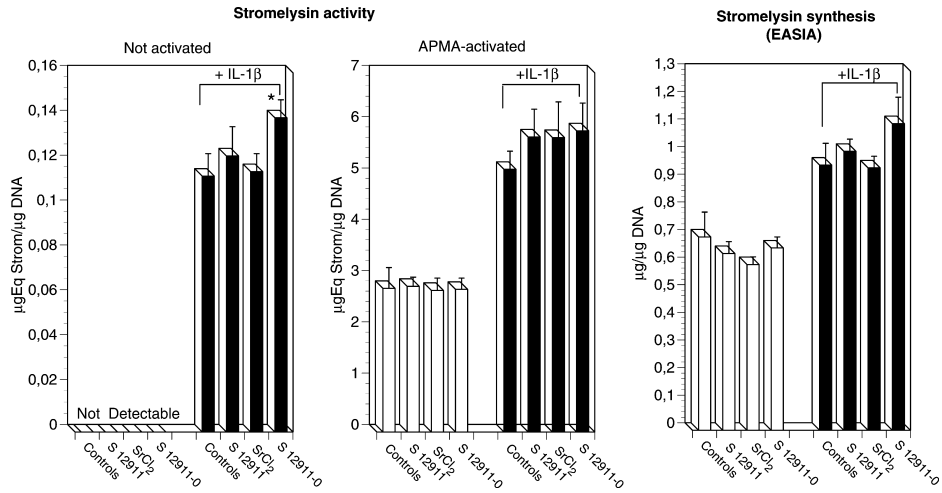

FIG. 3. Stromelysin activity and synthesis in the CM by unstimulated or IL-1β–stimulated (10^{-11} M) normal human chondrocytes were measured after 72 h of treatment with 10^{-3} M strontium ranelate (S12911), $2 \cdot 10^{-3}$ M $SrCl_2$, and 10^{-3} M calcium ranelate (S12911–0). A significant increase in the stromelysin activity was observed in the presence of 10^{-3} M calcium ranelate in comparison with unactivated stromelysin. Response is significant with $p < 0.05$.

IL-1β stimulated stromelysin synthesis and activity with a maximal effect after 72 h of incubation ($p < 0.01$; Fig. 3). The 72-h incubation period was retained to test the compounds with IL-1β stimulation. Strontium compounds did not significantly modify IL-1β–stimulated stromelysin synthesis and activity whereas calcium ranelate increased IL-1β–induced stromelysin activity ($p < 0.05$; Fig. 3).

PG production

In control conditions and in strontium ranelate–treated cultures, the PGs that were released in the CM (Fig. 4A) and contained in the CP (Fig. 4B) of normal chondrocyte cultures increased as a function of the culture duration. After 24 h of culture, strontium ranelate did not significantly modify PG production by normal chondrocytes whatever the concentration used. After 48 h of culture, strontium ranelate significantly increased the amount of PG released in the CM only at the concentration of 10^{-3} M and the PG contained in the CP at the concentrations of $5 \cdot 10^{-4}$ M and 10^{-3} M. After 72 h of culture, strontium ranelate dose-dependently increased the PG released in the CM ($r = 0.92$; $p < 0.01$). This increase was significant at concentrations of $5 \cdot 10^{-4}$ M and 10^{-3} M in the CP ($p < 0.05$). TPG production was strongly and significantly increased (approximately 20–35%) by $5 \cdot 10^{-4}$ M or 10^{-3} M strontium ranelate after 48 h and 72 h of culture (data not shown).

The effects of strontium ranelate were then compared between normal and OA chondrocyte cultures. Six different cartilage samples were used. They were excised from 3 normal young donors (<40 years old) and 3 OA donors (>60 years old). OA samples showed one or more lesions that scored III on the Moskowitz scale.[8] As expected, chondrocytes isolated from the cartilage of normal donors always produced higher amounts of PG than OA chondrocytes (Table 2). The PG amount released into the CM and contained in the CP after 72 h of culture with or without strontium ranelate was significantly lower in the OA group than in the normal group ($p < 0.001$; generalized linear mixed model; Table 2). Strontium ranelate (10^{-4}–10^{-3} M) significantly increased TPG ($0.0005 < p < 0.0001$) in normal chondrocyte cultures. No significant increase was observed at the concentration of 10^{-4} M when strontium ranelate was tested on OA cartilage, whereas higher concentrations ($5 \cdot 10^{-4}$ M and 10^{-3} M) induced a significant increase in TPG ($0.002 < p < 0.02$). $SrCl_2$ ($2 \cdot 10^{-3}$ M) is as efficient as strontium ranelate in increasing TPG produced by normal and OA chondrocytes (Table 2). Nevertheless, strontium ranelate increased PG contained in the CP of normal chondrocyte cultures whereas $2 \cdot 10^{-3}$ M $SrCl_2$ did not significantly modify this parameter. Calcium ranelate (10^{-3} M) also increased TPG in normal chondrocyte cultures to the same extent as strontium ranelate (Table 2).

Chondrocyte responses to IGF-I and IL-1β

The effects of strontium ranelate on chondrocyte responses to IGF-I and IL-1β were studied in normal human chondrocyte cultures. As expected, IGF-I dose-dependently stimulated TPG ($p = 0.02$) whereas IL-1β drastically inhibited PG synthesis after 72 h of culture. IGF-I (10^{-9} M and 10^{-8} M) increased TPG by 15% and 40%, respectively. When IGF-I (10^{-9} M) and strontium ranelate ($5 \cdot 10^{-4}$ M or 10^{-3} M) were added simultaneously, the stimulatory effect of IGF-I was higher than the effect of IGF-I alone ($0.03 < p < 0.004$; unpaired Student's *t*-test; Fig. 5). No similar effects were observed in the presence of 10^{-8} M IGF-I.

In the presence of 10^{-11} M or 10^{-10} M IL-1β, TPG drastically decreased by 60% and 68%, respectively. As shown in Fig. 6, 10^{-3} M strontium ranelate did not significantly modify the IL-1β–inhibiting effect on TPG.

PG size

In the culture conditions used, the newly synthesized PGs were built up with ^{35}S-GAG. The amounts of ^{35}S-GAG incorporated into the newly synthesized PGs during the last 24 h of 1, 2, or 3 days of normal human chondrocyte cultures are presented in Fig. 7.

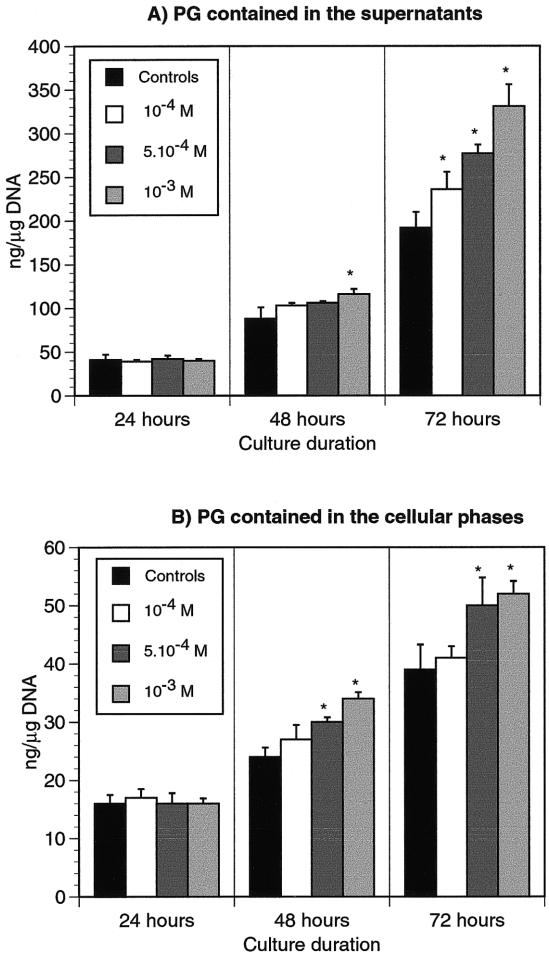

FIG. 4. Effect of strontium ranelate on human articular chondrocyte cultures. Normal human chondrocytes were incubated for the time specified in the absence (control condition) or presence of varying concentrations of strontium ranelate. At the end of the culture period, CM and CP were separated by centrifugation and were assayed for PG by a specific RIA. The data are expressed as amounts of PG found in the CM or in the CP per micrograms of DNA and presented as the mean and SD of triplicate cultures. Statistical significance in comparison to the controls, $*p < 0.05$.

PG released in the CM: In the absence of treatment, the daily production of ^{35}S-GAG released in the CM reached a maximum after 48 h of culture (Fig. 7A, same slope after 24 h and after 48 h). Strontium ranelate (10^{-3} M) and $SrCl_2$ ($2 \cdot 10^{-3}$ M) significantly increased the ^{35}S-GAG synthesis after 48 h and 72 h of incubation.

The newly synthesized ^{35}S-GAG released in the control CM were eluted through a CL2B chromatographic column in two major peaks (Fig. 8A). At each culture period, a large part of the newly synthesized ^{35}S-GAG was eluted with a $K_d \leq 0.13$ (Table 3). The percentage of ^{35}S-GAG eluted with a $K_d \leq 0.13$ (high-molecular weight PG complexes) increased with the culture duration (36–49%) whereas the ^{35}S-GAG eluted with a $K_d \geq 0.7$ (small PG and PG fragments) decreased (37–25%). In the presence of 10^{-3} M strontium ranelate or $2 \cdot 10^{-3}$ M $SrCl_2$, the percentage of the ^{35}S-GAG eluted with a $K_d \leq 0.13$ increased, with a K_d between 0.13 and 0.7 it was not modified, and with a $K_d \geq 0.7$ it decreased (Table 3, Fig. 8A).

PG contained in the cellular phase: The amount of ^{35}S-GAG contained in the CP of the culture increased as a function of the culture duration (Fig. 7B). Strontium ranelate (10^{-3} M) and $SrCl_2$ ($2 \cdot 10^{-3}$ M) significantly increased the amount of ^{35}S-GAG contained in the CP.

The chromatographic profile of ^{35}S-GAG contained in the CP of untreated chondrocyte cultures presented two major peaks (Fig. 8B). In the untreated cultures, 29–37% of the ^{35}S-GAG were eluted with a $K_d \leq 0.13$, the majority of the ^{35}S-GAG (43–52%) was eluted with a K_d between 0.13 and 0.7, whereas only 11–28% was eluted with a $K_d \geq 0.7$ (Table 3). As in the CM, the high-molecular weight PG complex rate contained in the CP increased with the culture duration whereas the rate of small PG and PG fragments decreased. Strontium ranelate (10^{-3} M) and $SrCl_2$ ($2 \cdot 10^{-3}$ M) treatments increased the PG contained in the CP without any effect on the PG size (Table 3; Fig. 8B).

DISCUSSION

In basal conditions and without any treatment, stromelysin activity was very low whereas stromelysin production increased linearly with the culture duration. This finding suggests that stromelysin is produced as prostromelysin (inactive form) and that only a small part of the proenzyme is activated. Interindividual variability in stromelysin synthesis and activity was observed (Figs. 2 and 3 after 72 h). Stimulation of the chondrocytes by IL-1β drastically increased stromelysin activity released in the CM. The mechanism involved may be the plasminogen activator/plasmin system, which can transform the proenzyme to the active form of stromelysin.[25] This hypothesis agrees with previous studies in which IL-1β was shown to increase plasminogen activator synthesis by the main cells of the joint, including human chondrocytes. [26,27]

Strontium ranelate and $SrCl_2$ did not activate stromelysin whereas calcium ranelate increased enzyme activity only in basal conditions. Calcium ranelate must act on the proenzyme activation process, because it did not increase stromelysin synthesis and did not modify the activity of APMA-activated stromelysin. This hypothesis agrees with a previous study that reported that Ca^{2+} is required for prostromelysin activation and stability.[20]

In the condition used, chondrocytes cultured without any treatment produced spontaneously aggregating PG and type II collagen. The TPG measured in the cultures from the different chondrocyte donors shows interindividual variation (Table 2; Figs. 4–6; after 72 h without any treatment). A large part of the newly synthesized PG (10–20%) was contained in the CP of the culture, suggesting the formation of a new matrix surrounding the chondrocytes. The qualitative analysis of the newly synthesized PG showed that the majority of the PG released in the CM (56%) and extracted from the CP (57%) is high-molecular weight PG complexes.

Strontium ranelate (10^{-3} M) strongly stimulated the synthesis of PG by human chondrocytes in vitro. This cartilage

TABLE 2. PG PRODUCTION MEASURED BY RIA

		S12911			*S12911-0*	*$SrCl_2$*
	Controls	*10^{-4}M*	*$5 \cdot 10^{-4}$ M*	*10^{-3} M*	*10^{-3} M*	*$2 \cdot 10^{-3}$ M*
		Proteoglycans contained in the culture supernatants				
Normal	622 ± 184	**707 ± 146**	**814 ± 191**	**800 ± 176**	**752 ± 145**	**844 ± 206**
		$p = 0.0012$	$p = 0.0001$	$p = 0.0001$	$p = 0.0001$	$p = 0.0001$
OA	212 ± 68	216 ± 62	**267 ± 91**	**292 ± 90**	—	302 ± 12
		$p = 0.86$	$p = 0.03$	$p = 0.0088$		$p = 0.0006$
		Proteoglycans contained in the cellular phases				
Normal	68 ± 25	73 ± 24	**82 ± 20**	**85 ± 18**	**82 ± 26**	71 ± 20
		$p = 0.13$	$p = 0.0003$	$p = 0.0001$	$p = 0.0001$	$p = 0.3$
OA	36 ± 15	36 ± 15	41 ± 17	45 ± 18	—	40 ± 12
		$p = 0.9$	$p = 0.13$	$p = 0.27$		$p = 0.24$
		Total proteoglycan production				
Normal	686 ± 210	**780 ± 167**	**894 ± 210**	**885 ± 194**	**833 ± 169**	**914 ± 223**
		$p = 0.0005$	$p = 0.0001$	$p = 0.0001$	$p = 0.0001$	$p = 0.0001$
OA	248 ± 81	253 ± 75	**309 ± 105**	**362 ± 156**	—	**340 ± 115**
		$p = 0.85$	$p = 0.02$	$p = 0.002$		$p = 0.0007$

Effects of strontium ranelate (S 12911), calcium ranelate (S 12911-0), or $SrCl_2$ on PG production (ng/μg DNA) by normal or OA human chondrocytes at 72h. Values are the means and SD of three separate chondrocyte cultures. Values with $p < 0.05$ are significantly different from the corresponding controls (in bold).

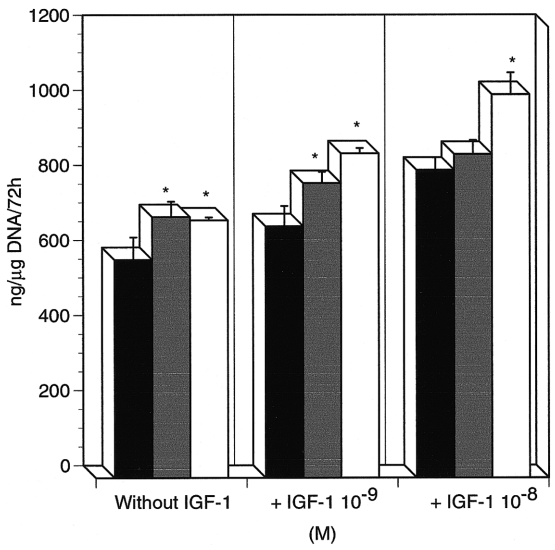

FIG. 5. Effect of strontium ranelate on normal human chondrocyte response to IGF-I. At the concentrations of 10^{-9} M and 10^{-8} M, IGF-I significantly stimulated TPG (sum of the PG amount released in the CM and contained in the corresponding CP) of normal human chondrocytes cultured for 72 h in the absence (black column) or in the presence of 10^{-3} M strontium ranelate (gray column) or $2 \cdot 10^{-3}$ M $SrCl_2$ (white column). Values are mean and SD ($n = 3$). Strontium increased the IGF-I–stimulating effect with $p < 0.05$.

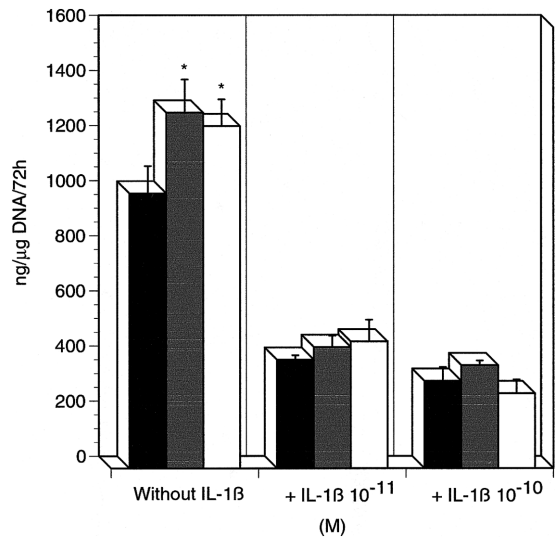

FIG. 6. Effects of strontium ranelate on normal human chondrocyte response to IL-1β: TPG produced by human chondrocytes cultured for 72 h in the absence (black column) or in the presence of 10^{-3} M strontium ranelate (gray column) or $2 \cdot 10^{-3}$ M $SrCl_2$ (white column).

matrix synthesis was not the result of cell proliferation, as shown in bone formation,[28] because the addition of strontium ranelate had no effect on the DNA level. Because $SrCl_2$ ($2 \cdot 10^{-3}$ M) showed similar effects in the CM, the stimulatory effect of strontium ranelate on PG production is independent of the ranelic acid and strontium must be responsible for this effect. These results differ from those of Svensson[29] who showed that $SrCl_2$ inhibited the synthesis of GAG and collagen in freshly isolated rat chondrocytes. This discrepancy could be explained by the higher strontium concentrations used ($3.2 \cdot 10^{-3}$ M and 10^{-2} M) in his experiment. Interestingly, strontium and calcium ranelate, but not $SrCl_2$, increased both the PG that were released in the CM and contained in the CP of the culture. This result indicates that strontium ranelate promotes the synthesis of PG capable of integrating the extracellular matrix. The qualitative analysis of the newly synthesized PG showed that strontium ranelate (10^{-3} M) and calcium ranelate (10^{-3}

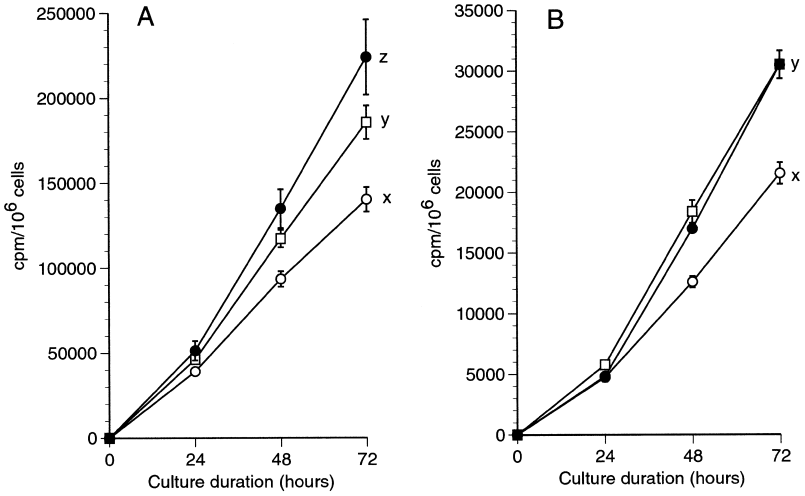

FIG. 7. Normal human chondrocyte effects of 10^{-3} M strontium ranelate (-□-) or $2 \cdot 10^{-3}$ M $SrCl_2$ (-●-) compared with the controls (-○-) on the cumulative amount of ^{35}S-GAG (A) released in the CM or (B) contained in the CP as a function of the culture duration (cumulative values; means ± SD). Statistical significance (A) in the 72-h CM in which *x* versus *y*, $p < 0.025$, and *x* versus *z*, $p < 0.01$, and (B) in the 72-h CP in which *x* versus *y*, $p < 0.01$.

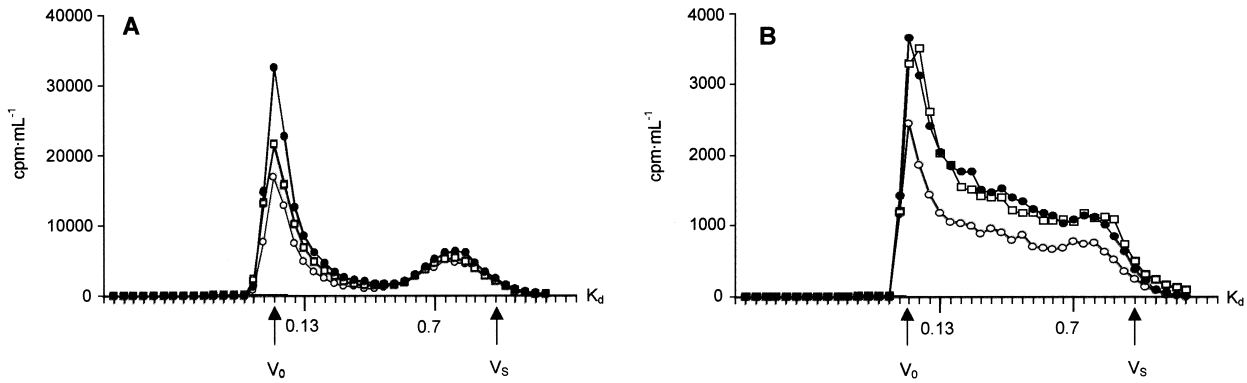

FIG. 8. Associative size exclusion chromatograms of ^{35}S-labeled PG produced by normal chondrocytes cultured for 72 h in the absence (-○-) or in the presence of 10^{-3} M strontium ranelate (-□-) or $2 \cdot 10^{-3}$ M $SrCl_2$ (-●-). (A) CM and (B) cellular phases.

M) promoted the synthesis of high-molecular weight PG complexes, which were shown to decrease in OA. [30]

The mechanism by which extracellular strontium ranelate influenced PG synthesis is not known. However, changes in the extracellular ionic environment would be expected to affect synthesis rates by altering the intracellular composition. It was previously shown that high extracellular Ca^{2+} increases intracellular free Ca^{2+} concentrations in parathyroid cells[31] and that this effect can be mimicked by several other divalent cations including strontium.[32] Intracellular Ca^{2+} concentration increases could be the result of inositol 1,4,5-triphophate receptor (InsP3R) stimulation,[33] which is regulated by multiple calcium binding sites and at least four different Ca^{2+} interaction sites.[31] The ionic strength of the aqueous environment also can profoundly affect gating calcium channels. Sr^{2+} and Ca^{2+} modulate the opening and closing of Ca^{2+} channels and in this way may affect cellular functions. [34]

Strontium could be hypothesized to be effective in reversing the IGF-I–stimulating effect, because factors that lead to increase PG synthesis were reported as causing an initial decrease in cytosolic free Ca^{2+} concentration.[35,36] The effect of strontium ranelate and IGF-I added simultaneously were tested on PG synthesis by normal human chondrocytes. Surprisingly, IGF-I stimulation of PG synthesis was increased by 10^{-3} M strontium ranelate treatment. This effect could be consequent to chondrocyte proliferation because IGF-I is required for cell proliferation.[37,38] However, in the culture conditions used, neither ^{3}H- thymidine incorporation nor DNA content was elevated in the presence of IGF-I added alone or in combination with strontium ranelate (data not shown). Autocrine regulation of chondrocyte metabolism by IGF-I produced in response to a high amount of strontium also might be responsible for the PG increase. Indeed, recently, it was reported that a high extracellular Ca^{2+} concentration significantly increased the secretion of IGF-I as well as the expression of IGF-I messenger RNA (mRNA) by osteoblastic MC3T3-E1 cells.[39] This finding could explain why strontium ranelate increased the IGF-I effect when it was added at a low concentration (10^{-9} M) but not at a high concentration (10^{-8} M).

Strontium ranelate, calcium ranelate, and $SrCl_2$ had similar stimulating effects on TPG. Nevertheless, $SrCl_2$ showed no significant effect on the PG contained in the CP, and calcium ranelate (10^{-3} M) increased the stromelysin activity measured in the CM. Because strontium ranelate also stimulated the production of type II collagen synthesis,[40] it can be concluded that 10^{-3} M strontium ranelate is more

TABLE 3. PHYSICOCHEMICAL FORM OF THE NEWLY SYNTHESIZED PG

	$K_d \leq 0.13$			$0.13 < K_d < 0.7$			$K_d \geq 0.7$		
	Controls	*S12911*	*$SrCl_2$*	*Controls*	*S12911*	*$SrCl_2$*	*Controls*	*S12911*	*$SrCl_2$*
				^{35}S-GAG released in the culture supernatants (%)					
0–24 h	36	42	46	27	30	26	37	28	28
24–48 h	45	45	49	25	26	26	30	29	25
48–72 h	49	55	61	26	24	20	25	21	19
				^{35}S-GAG contained in the cellular phases (%)					
0–24 h	29	29	28	43	40	41	28	31	31
24–48 h	29	36	29	48	44	50	23	20	21
48–72 h	37	34	37	52	52	51	11	14	12

Effects of strontium compounds on the physicochemical form of the newly synthesized PG by normal human chondrocytes. The chomatogram was established by determining the radioactivity in each fraction and dividing into 3 groups according to a distribution coefficient. The values are the percentage of radioactivity contained in 3 groups.

efficient in promoting cartilage formation than the other compounds tested. Moreover, 10^{-3} M strontium ranelate increased TPG in OA chondrocyte cultures and could have beneficial effects in the prevention and the treatment of OA lesions. This hypothesis agrees with a previous study that showed that in strontium-induced rachitic rats the size of the side chains of PG monomers was increased considerably in the lower part of the growth plate. [13]

In parallel, previous in vitro and in vivo works have shown that strontium ranelate reduced bone resorption and stimulate bone formation. In vitro, strontium ranelate inhibits bone resorption both in osteoclast and in organ cultures and stimulates preosteoblastic cell replication in rat calvaria cell and organ culture.[17,28,41] Furthermore, administration of 2 g/day of strontium ranelate to postmenopausal osteoporotic women for a 2-year period resulted in uncoupling between bone resorption (decrease of collagen cross-links excretion) and bone formation (increase in bone-specific alkaline phosphatase), which led to a significant increase in the lumbar spine bone density and a 44% reduction in the number of patients experiencing a new vertebral fracture during the second year of treatment.[15,42] In a prevention 2-year study, 1 g/day of strontium ranelate given to early postmenopausal women without prevalent osteoporosis, induced a significant increase in spine bone mineral density (BMD). Frequency and distribution of adverse reactions were similar in strontium ranelate and placebo-treated patients.[16] These data are consistent with the very good tolerance observed in toxicology studies carried out with strontium ranelate. Taken together, these data suggest that strontium ranelate has beneficial effects on both bone and cartilage remodeling.

In conclusion, 10^{-3} M strontium ranelate strongly increases cartilage matrix formation by ionic stimulation of chondrocyte anabolism without affecting cartilage resorption. In this way, it could restore the imbalance between chondroformation and chondroresorption observed during OA. This finding provides a preclinical basis for in vivo testing of strontium ranelate in prevention and treatment of OA.

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