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Effects of Zinc on Cell Proliferation and Proteoglycan Characteristics of Epiphyseal Chondrocytes

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Abstract Zinc has been postulated as an important nutritional factor involved in growth promotion; however, the cellular mechanisms involved in the effects of zinc on linear growth remain to be elucidated. This study was conducted to evaluate the effects of zinc on the proliferation rate of epiphyseal growth plate chondrocytes and on the structural characteristics of the proteoglycans synthesized by these cells. For these purposes, hypertrophic and proliferating chondrocytes were isolated from the tibiae of 1- and 5-week-old chickens, respectively. Chondrocytes were cultured under serum-free conditions and primary cultures were used. The results showed that zinc stimulated proliferation by 40–50% above the baseline in the case of proliferating chondrocytes, but it had no effect on hypertrophic chondrocytes. Zinc had neither any effects on mean charge density of proteoglycans synthesized by hypertrophic chondrocytes nor in their hydrodynamic size. In contrast, zinc induced an increase in mean charge density and a decrease of hydrodynamic size of proteoglycans synthesized by proliferating chondrocytes. In both cell types zinc had no effect on the composition and hydrodynamic size of the glycosaminoglycan chains. The increased ability of proliferating chondrocytes cultured in the presence of zinc to synthesize 3′-phosphoadenosine 5′-phosphosulfate (PAPS) could be explained by the induction of enzymes participating in the sulfation pathway of proteoglycans. Therefore, the increase in mean charge density of proteoglycans observed in this study may be explained by an increase of the degree of sulfation of proteoglycan molecules. We speculate that the effect of zinc on linear growth may be explained at a cellular level by: a) an increase in proliferation rates of proliferating chondrocytes, and b) increased synthesis of highly charged proteoglycan molecules which decreases mineralization. J. Cell. Biochem. 82: 501–511, 2001. © 2001 Wiley-Liss, Inc.

Key words: zinc; epiphyseal chondrocytes; proteoglycans; sulfation; growth plate; longitudinal growth

Longitudinal growth is a highly regulated physiological process, which occurs as a consequence of cell proliferation and synthesis of extracellular matrix components by the chondrocytes of the epiphyseal growth plate. The control of this process involves endogenous and systemic factors stimulating a sequence of events that involve both cell proliferation and differentiation, which eventually lead to chondrocyte hypertrophy and mineralization [Isaksson et al., 1987]. These cells respond to the stimuli represented by hormones, growth factors, and nutrients by changing proliferation rates, volume, and rate of collagen and proteoglycan (PG) synthesis; the net result is an increase in linear growth [Allen, 1994; Price et al., 1994]. Postnatal longitudinal growth ends with the closure of the epiphyseal growth plate [Ianotti, 1990], and gross abnormalities of the growth plate result in severe impairment of longitudinal growth [Leach and Nesheim, 1965; Maroteaux et al., 1983; Boden et al., 1987]. Over the past decades multiple studies have demonstrated that zinc (Zn) is a key factor for normal growth and development of skeletal tissue [Hambidge et al., 1986; O'Dell and Reeves, 1988; Dorup and Clausen, 1991; Dorup et al., 1991]. Zn-deficient animals show a severe reduction in growth rate, which can be reverted by supplementation of the element in the diet [Giugliano and Milward, 1984]. In humans, the positive effects of Zn supplementation on longitudinal growth have been demonstrated in numerous epidemiological and clinical interventional studies [Ronaghy et al., 1974; Castillo-Durán et al., 1987; Gibson et al., 1989; Allen, 1994]. Epidemiological and clinical evidences have confirmed the role of Zn as a

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determinant element in longitudinal growth of children. Thus, zinc has been suggested as a nutritional factor involved in stunted growth, and the need for supplementation at early stages of development has been proposed [Gibson et al., 1989; Favier, 1992; Walravens et al., 1992; Ruz et al., 1997; Rivera et al., 1998; Castillo-Duran and Cassorla, 1999]. However, little is known about the molecular and cellular mechanisms involved in the regulation of this process. Moderate zinc deficiencies have been shown to be associated with impaired growth in children. These deficiencies do not appear to alter Zn-dependent enzymes or gene-regulatory elements. The growth impairment, however, points to a pronounced effect of altered Zn metabolism on bone formation [Koyano et al., 1996].

Mineral deposition is essential for proper bone formation, which occurs in the extracellular matrix and is mediated by proteins and other components. Zinc has long been known to play an important role in the mineralization of skeletal tissues, but the mechanisms of Zn action are not well understood [Kirsch et al., 2000]. Experimental Zn deficiencies have been demonstrated to result in abnormal cartilage development and irregular and increased cartilage mineralization, resulting in bone deformities [Westmoreland and Hoekstra, 1969]. On the other hand, increased levels of Zn are associated with decreased mineralization [Kirsch et al., 2000]. PG are major components of the growth plate extracellular matrix that interact with collagen, cell membranes, and tissue-specific proteins [Farquharson et al., 1994]. Several studies have shown that the concentration of PG within the growth plate plays an important role in calcification, the sulfate content being low in calcified cartilage [Boyd and Shapiro, 1980]. Studies on the brachymorphic mouse, which lacks the enzyme responsible for glycosaminoglycan sulfation (GAG) suggest that the decreased sulfation facilitates the onset of mineralization [Sugahara and Schwartz, 1982a].

Different studies have also analyzed the effects of Zn on the metabolism of growth plate chondrocytes [Koyano et al., 1996; Litchfield et al., 1998]. These studies examined the effect of this trace metal on the cell protein, alkaline phosphatase activity, and the synthesis of some extracellular matrix components produced by growth plate and articular chondrocytes under different culture conditions. It has been hypothesized that Zn may serve as a regulator of chondrocyte matrix protein turnover acting through an as of yet undefined mechanism [Koyano et al., 1996].

It is know also that growth plate chondrocytes are responsible for the earliest stages of bone formation and are thus critical to the understanding of the way in which essential and toxic metal ions may influence overall skeletal development. For these reasons, the main objective of this study was to evaluate the effects of Zn on proliferation rates and metabolic activity of the cells responsible for longitudinal growth; i.e., epiphyseal growth plate chondrocytes. For this purpose, avian proliferating and hypertrophic chondrocytes were cultured under serum-free conditions, and primary cultures were used to determine cell proliferation and synthesis of PG in response to different concentrations of zinc.

MATERIALS AND METHODS

Avian chodrocytes were used in this study since they have been shown to be a suitable and practical experimental model for the study of the endocrine and metabolic regulatory mechanisms underlying longitudinal growth regulation [Rosselot et al., 1992, 1994].

Chondrocyte Isolation and Cell Cultures

Proliferating chondrocytes were isolated from the epiphyseal growth plate as previously described [Rosselot et al., 1992]. Briefly, 5-week-old chicken were killed by cervical dislocation, the epiphyseal growth plates from the tibia were aseptically removed, and treated with a 0.2% collagenase solution for 2 h at 37°C. Cells liberated by this treatment were washed three times with Dulbecco's Modified Eagle medium (DME) (Sigma, St Louis, MO). Cells were cultured in DME medium supplemented with 10% of fetal calf serum (FCS), 100 μg/ml streptomycin, and 100 U/ml penicillin, at 37°C in a humidified atmosphere of 5% CO_2. Cells were plated in culture dishes (multiwells, 96 wells, Nunc) at a density of 7×10^4 cells/well. The medium was changed every 4 days. Hypertrophic chondrocytes were isolated similarly from one-week-old chickens [Rosselot et al., 1992]. The tibias were removed, hypertrophic zones were dissected, and any remaining soft tissue was removed and discarded. The hypertrophic tissue was incubated with 3 ml of a 0.2%

collagenase solution (type I, Worthington, Freehold, NJ) for 10 min at 37°C. Cells liberated by this treatment, mainly red blood cells and damaged cells, were discarded. The remaining tissue was incubated again with the collagenase solution for 2 h at 37°C. Cells liberated by this second enzymatic treatment were washed three times with DME medium and cultured in DME medium supplemented with 10% FCS, 100 μg/ml streptomycin, and 100 U/ml penicillin, at 37°C in a humidified atmosphere of 5% CO_2. Cells were plated in culture dishes (multiwells, 96 wells, Nunc, Naperville, IL) at a density of 7×10^4 cells/well. The medium was changed every fourth day. After one week of culture, proliferating and hypertrophic chondrocytes were cultured for an additional 4 days in a serum-free defined medium (MWM) containing aminoacids, lactalbumin, transferrin, and growth factors (IGF-I, FGF) [Rosselot et al., 1992], and supplemented with $ZnCl_2$ at different concentrations (0, 0.05, 0.1, 0.2, 0.5, 1, 10, 50, and 100 μM).

Characterization of Proliferating and Hypertrophic Chondrocytes: Expression of Type II and Type X Collagens

Cells were incubated for 7 days in DME medium. The medium was removed, a fresh medium containing ascorbic acid (50 μg/ml), β-aminopropionitrile (100 μg/ml), and ^{14}C-proline (5 μCi/ml) was added, and incubation continued for 24 h. After incubation with ^{14}C-proline the medium was removed and a stock of protease inhibitors was added to the medium to yield a final concentration of 5 mM EDTA, 0.2 mM phenylmethyl-sulfonylfluoride, 5 mM N-ethylmaleimide, and 1 mM p-aminobenzamidine hydrocloride. Medium was exhaustively dialyzed at 4°C against 0.15 mM NaCl and 50 mM Tris–HCl buffer containing the above mixture of protease inhibitors. Each sample was digested with 100 mg/ml pepsin in 0.5 M acetic acid for 6 h at 15°C to convert the procollagens to collagens and remove their noncollagenous domains. The digested samples were dialyzed at 4°C against 0.15 mM NaCl and 50 mM Tris–HCl containing protease inhibitors and an aliquot was used for electrophoresis. The samples were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, the gels were processed for fluorography as described previously [Rosselot et al., 1992].

Cell Proliferation

^{3}H-thymidine incorporation has been shown to be a good indicator of chondrocyte proliferation by Rosselot et al. [1994]. For this, chondrocytes were plated into 96-well plates at 1×10^5 cell/cm^2 and cultured in MWM [Rosselot et al., 1994] containing the various $ZnCl_2$ concentrations. The medium was supplemented with 1 μCi/ml of ^{3}H-thymidine (2.0 Ci/mM, ICN, Irvine, CA), and cells were incubated for 8 h. After the labeling period, cells were liberated from the culture dishes by a mild treatment with pronase (0.2%, 10 min, 37°C), and collected by filtration [Rosselot et al., 1994]. Filters were exhaustively washed with distilled water, and the radioactivity retained was measured.

Proteoglycans Synthesis

Chondrocytes were cultured in MWM containing the different $ZnCl_2$ concentrations studied. The medium was supplemented with 20 μCi/ml sodium ^{35}S-sulfate (ICN, 43 Ci/mg S) as a glycosaminoglycan radioactive precursor, and cells were incubated for 8 h at 37°C. At the end of the labeling period, the culture medium was removed and the cell layer was washed twice with phosphate-saline buffer (PBS). The washes were combined with the culture medium (culture medium fraction) and 0.4 g/ml solid guanidine-HCl, 1.25 mg/ml N-ethylmaleimide, and 0.18 mg/ml phenylmethylsulfonylfluoride were added [Rodríguez et al., 1998].

To remove unincorporated $^{35}SO_4$, and guanidine–HCl, the culture medium fractions were applied into a Sephadex G-50 column (0.8 × 15 cm) equilibrated with a buffer containing 8 M urea, 0.05 M sodium acetate, 0.15 M sodium chloride, and 0.5% Triton X-100, at pH 7.0 (buffer A).

Ion-Exchange Chromatography

Fractions containing the excluded material (Vo) from Sephadex G-50 were pooled and loaded into a DEAE-Sephacel column (0.6 × 10 cm) equilibrated and washed with buffer A. The retained material was eluted with a linear gradient of sodium chloride (0.15–0.8 M) prepared in buffer A. Fractions (0.8 ml) were collected and aliquots measured for radioactivity and sodium chloride content [Rodríguez, 1995].

Molecular Sieve Chromatography

To analyze the hydrodynamic size of intact PG and their GAG chains, the species eluted from the DEAE-Sephacel column were pooled, dialyzed exhaustively against distilled water, lyophilized to dryness and reconstituted in a solution containing 4 M guanidine–HCl, 0.05 M sodium acetate, 0.5% Triton X-100, at pH 6.0 (buffer B); it was then applied to a Sepharose CL-6B column. Fractions (0.45 ml) were collected and aliquots taken for radioactivity measurements [Rodríguez et al., 1995].

Characterization of Glycosaminoglycan Chains

To further characterize the PG molecules synthesized by proliferating and hypertrophic chondrocytes, glycosaminoglycan (GAG) chains were released from the core protein and analyzed by ion-exchange and molecular sieve chromatography, and their sensitivity to enzymatic treatment was studied. For these purposes, PG were treated with 0.1M sodium hydroxide in the presence of sodium borohydride (75.5 mg/ml) for 48 h at 45°C, to release the GAG chains from the core protein [Rodríguez et al., 1998]. To analyze the hydrodynamic size of GAG chains, the released chains were applied to a Sepharose CL-4B column, equilibrated in buffer B. GAG chains were subjected to chondroitinase ABC treatment [Minguell and Tavassoli, 1989]. The extent of the enzymatic digestion was detected by the appearance of low-molecular weight-labeled species after Sephadex G-25 chromatography [Keller et al., 1989].

Synthesis of 3′-Phosphoadenosine 5′-Phosphosulfate (PAPS)

To analyze whether changes in the incorporation of $^{35}SO_4$ into PG molecules could be due to changes in enzymatic activities in the sulfation pathway of PG, we measured the ability of chondrocyte homogenates to synthesize an intermediary of the sulfation pathway, PAPS.

Proliferating chondrocytes cultured in the presence of different concentrations of $ZnCl_2$ during 4 days, were removed by trypsination, collected by centrifugation, washed three times with 50 mM Tris–HCl buffer and subjected to sonication for 5 min.

The incorporation of $^{35}SO_4$ into PAPS was measured in a reaction mixture containing 50 mM Tris–HCl buffer (pH 7.0), 5 mM ATP, 5 mM $MgCl_2$, 0.1 M NaF, 10 μCi $^{35}SO_4$ and an aliquot of the cell homogenate. The reaction mixture was incubated for 90 min at 37°C. After incubation, NaOH to a 0.5 N final concentration, to favor the stability of PAPS, and 0.1 mg of unlabeled PAPS, used as a carrier, were added to the reaction mixture. Synthesized PAPS was separated by paper chromatography using saturated ammonium sulfated solution:0.1 M ammonium acetate:isopropanol (79:19:2) as solvent. The presence of PAPS was evaluated in a U.V. transilluminator and quantitated by measuring the radioactivity associated with the carrier.

RESULTS

Cell Proliferation

Cell proliferation was evaluated by the incorporation de ^{3}H-thymidine into DNA of proliferating and hypertrophic chondrocytes.

The phenotypic expression of the cells, as proliferating and hypertrophic chondrocytes, was confirmed through analyses of collagen type released to the culture media [Rosselot et al., 1992]. As shown in Figure 1, proliferating

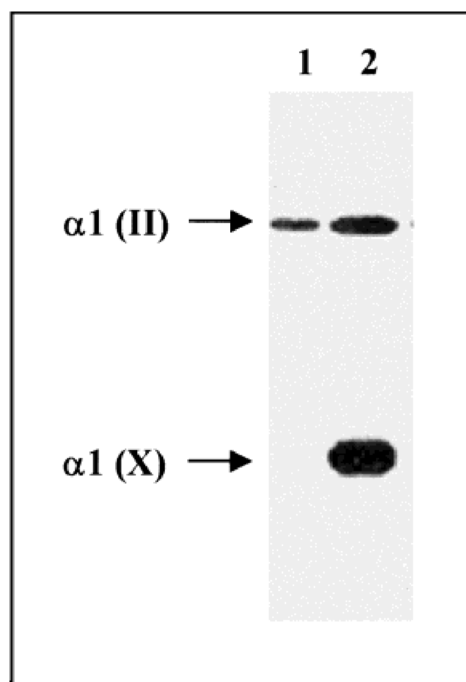

Fig. 1. Electrophoretic analysis of collagen synthesis by proliferating and hypertrophic chondrocytes. **Lane 1** corresponds to the collagen produced by proliferating chondrocytes, and **lane 2** to the collagen produced by hypertrophic chondrocytes.

chondrocytes produce type II collagen, and the hypertrophic chondrocytes synthesized both type II collagen and type X collagen. Type II collagen is the most abundant of the collagens in the growth plate, and since it is found almost exclusively in cartilage it is a specific phenotypic marker for chondrocytes. The synthesis of type X collagen has been associated with chondrocytes hypertrophy.

Figure 2 shows that $ZnCl_2$ present in the culture medium exhibits a differential effect on the proliferation rate of hypertrophic and proliferating chondrocytes. Low concentrations of $ZnCl_2$ (up to 0.5 μM) stimulated (40–50%) the incorporation of ^{3}H-thymidine into DNA from proliferating chondrocytes, with maximum stimulation at a concentration of 0.2 μM. This stimulatory effect was specific for proliferating chondrocytes, since at the same range of $ZnCl_2$ concentrations, no effect was observed in the proliferation rate of hypertrophic chondrocytes. At $ZnCl_2$ concentrations above 1 μM, there was increasing inhibition of the incorporation of ^{3}H-thymidine into DNA in both proliferating and hypertrophic chondrocytes. This suggests that higher concentrations of $ZnCl_2$ (up to 10 μM) appear to stop completely the proliferation of proliferative chondrocytes, which may be interpreted as a possible cytotoxic effect of zinc in the proliferating as well as hypertrophic chondrocytes. More specific studies are necessary to perform to evaluate a possible cytotoxic effect of zinc.

Proteoglycan Synthesis

Both, proliferating and hypertrophic chondrocytes in culture actively synthesize and release PG to the culture medium.

Proliferating chondrocytes cultured in the presence of low concentrations of $ZnCl_2$ (up to 1.0 μM) incorporate 2 to 6 times more ^{35}S-sulfate into PG than chondrocytes cultured in the absence of $ZnCl_2$ (Fig. 3). At the same time, $ZnCl_2$ (up to 1 μM) showed no effect on the incorporation of ^{35}S-sulfate into PG molecules produced by hypertrophic chondrocytes. At concentrations of $ZnCl_2$ higher than 10 μM, the incorporation of ^{35}S-sulfate was inhibited in both proliferating and hypertrophic chondrocytes.

It is interesting to note that proliferating chondrocytes, either in the absence or in the

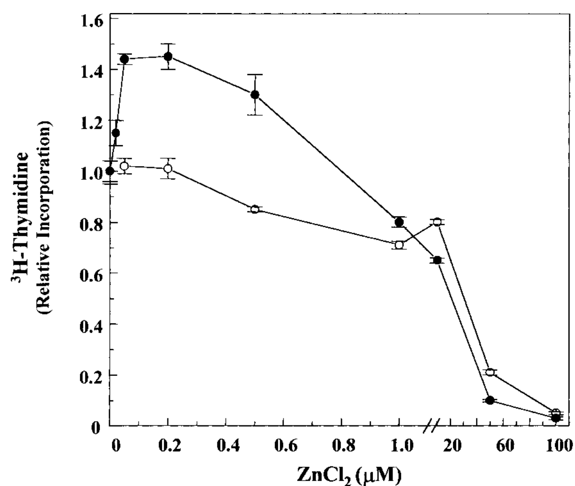

Fig. 2. Incorporation of ^{3}H-Thymidine. Proliferating (•-•) and hypertrophic (○-○) chondrocytes proliferation was evaluated by the incorporation of ^{3}H-thymidine into DNA molecules. Chondrocytes were cultured in MWM containing different $ZnCl_2$ concentrations, and supplemented with 1 μCi/ml of ^{3}H-thymidine. Cells were incubated for 8 h, liberated from the culture dishes by a mild treatment with pronase (0.2%, 10 min, 37°C), and collected by filtration. Filters were washed exhaustively with distilled water, and the radioactivity retained was measured. The results, at each concentration of $ZnCl_2$, were expressed as the relative incorporation of ^{3}H-thymidine respect to the incorporation at 0 μM $ZnCl_2$. Each experiment was performed in triplicate and results are expressed as mean + SD.

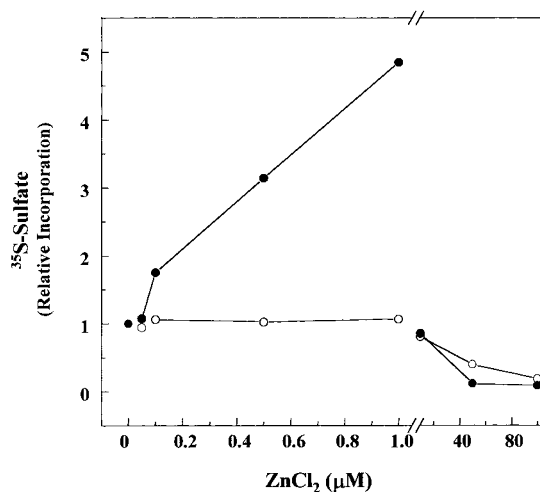

Fig. 3. Incorporation of ^{35}S-sulfate into proteoglycans. Hypertrophic (○-○) and proliferating (•-•) chondrocytes were cultured in MWM containing different $ZnCl_2$ concentrations (up to 100 μM), and supplemented with 20 μCi/ml sodium ^{35}S-sulfate. Cells were incubated for 8 h at 37°C. For each concentration of $ZnCl_2$, the culture media from four different wells were removed, pooled and applied into a Sephadex G-50 column (see Materials and Methods) to remove the unincorporated $^{35}SO_4$, guanidine-HCl, and other chemicals. The incorporation of ^{35}S-sulfate into proteoglycans, at each concentration of $ZnCl_2$, were expressed in a relative manner respect the incorporation at 0 μM $ZnCl_2$.

presence of $ZnCl_2$, incorporate more ^{35}S-sulfate into PG than hypertrophic chondrocytes (data not shown). Thus, in the absence of zinc, proliferating chondrocytes incorporate 1.5–2 times more ^{35}S-sulfate than hypertrophic chondrocytes. This difference is more evident when chondrocytes were cultured in the presence of increasing concentrations of $ZnCl_2$.

PG Characterization

Ion-Exchange Chromatography. After labeling with ^{35}S-sulfate, the different fractions of culture medium were prepared as described in Materials and Methods and applied into a DEAE-Sephacel column. Analysis by ion-exchange chromatography of PG released to the culture medium revealed the presence of a single component in proliferating as well as hypertrophic chondrocytes, but eluting from the DEAE-Sephacel columns at different salt concentrations: 0.36 and 0.47 M NaCl, respectively (Fig. 4A and D). The PG synthesized by hypertrophic chondrocytes cultured in the presence of 1 μM $ZnCl_2$ eluted from the DEAE-Sephacel column at the same concentration of sodium chloride than the control (0.47 M) (Fig. 4D). However, the PG synthesized by proliferating chondrocytes showed a shift of the elution peak of the DEAE-Sephacel from 0.36–0.45 M NaCl, depending on whether the cells were cultured in the presence or the absence of $ZnCl_2$; this effect depends of the concentration of zinc (Fig. 4A). Thus, PG produced by proliferating chondrocytes cultured in the absence of $ZnCl_2$ eluted from the DEAE-Sephacel column at 0.36 M NaCl. When chondrocytes were cultured in the presence of 0.1 or 1 μM $ZnCl_2$ the PG synthesized eluted from the DEAE-Sephacel column at 0.4 M and 0.45 M NaCl, respectively (Fig. 4A).

Molecular Sieve Chromatography. To further analyze the structural characteristics of PG synthesized by hypertrophic and proliferating chondrocytes, PG were examined by molecular sieve chromatography in Sepharose CL-4B columns. Hypertrophic chondrocytes cultured in the presence or absence of 1 μM $ZnCl_2$ synthesized a PG eluting from the column as a single and well defined peak with a Kav = 0.38 (Fig. 5). GAG chains released from the PG synthesized in the presence or in the absence of $ZnCl_2$, eluted from the column as a single peak with a Kav = 0.54.

Conversely, proliferating chondrocytes synthesized PG molecules with different hydrodynamic sizes when cells were cultured in the absence or in the presence of 1 μM $ZnCl_2$ (Fig. 6). In the absence of $ZnCl_2$, proliferating chondrocytes synthesized a PG specie eluting from the Sepharose CL-4B column with a Kav = 0.4. In

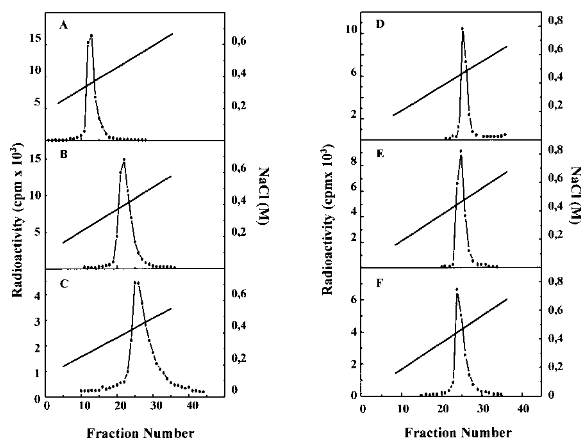

Fig. 4. DEAE-Sephacel chromatography of proteoglycans produced by proliferating chondrocytes (**A**, **B**, **C**) in the presence of different $ZnCl_2$ concentration (0, 0.1, and 1 μM, respectively) and hypertrophic chondrocytes (**D**, **E**, **F**) in the presence of different $ZnCl_2$ concentration (0, 0.1, and 1 μM, respectively). Radioactive material (see Materials and Methods) from chondrocytesin culture, was applied to the DEAE-Sephacel column. Retained material was eluted with a linear gradient of sodium chloride (0.15–0.8 M), and aliquots taken for measurements of radioactivity and salt concentration.

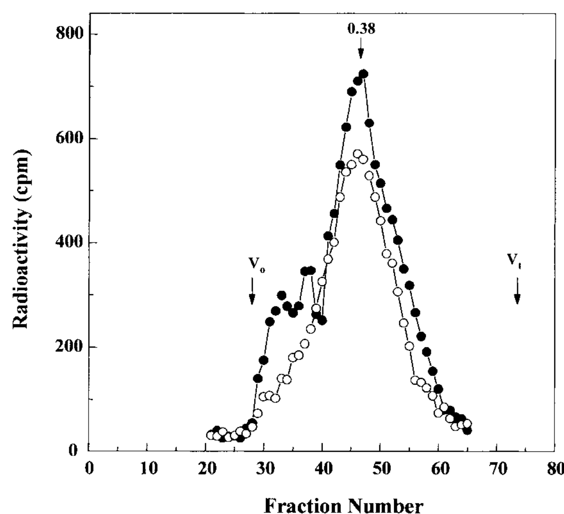

Fig. 5. Sepharose CL-4B chromatography of proteoglycans produced by hypertrophic chondrocytes in the absence (●-●) and in the presence of 1μM $ZnCl_2$ (○-○). Aliquots of the column fractions were taken for measurement of radioactivity. Arrows indicate Kav values.

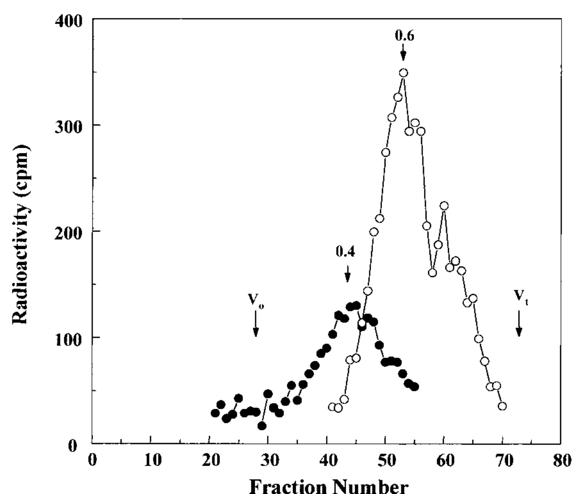

Fig. 6. Sepharose CL-4B chromatography of proteoglycans produced by proliferating chondrocytes in the absence (•-•) and in the presence of 1 μM $ZnCl_2$ (o-o). Aliquots of the column fractions were taken for measurement of radioactivity. Arrows indicate Kav values.

the presence of 1 μM $ZnCl_2$ these cells synthesized a PG specie with a smaller hydrodynamic size than the control, (Kav = 0.6). However, the analysis of GAG chains revealed the same hydrodynamic size for GAG chains released from PG synthesized in the presence or in the absence of $ZnCl_2$ (Kav = 0.64).

Characterization of GAG Chains. To investigate whether $ZnCl_2$ induces changes in the composition of the GAG chains associated with the PG synthesized by proliferating and hypertrophic chondrocytes, PG were subjected to chondroitinase ABC and ACII treatments. Between 85–90% of the radioactivity associated to the GAG chains of PG synthesized by proliferating chondrocytes, in the absence or in the presence of $ZnCl_2$, are sensitive to chondroitinase ABC treatment. At the same time, 80–85% of the GAG chains derived from PG synthesized by hypertrophic chondrocytes, cultured in the presence or in the absence of $ZnCl_2$, are sensitive to chondroitinase ABC treatment. These results suggest that addition of 1 μM of $ZnCl_2$ to the culture medium does not modify the proportion of chondroitin sulfate GAG chains associated with the core protein of PG synthesized by either proliferating or hypertrophic chondrocytes.

The remaining radioactivity (10–20%) that was not sensitive to the enzymatic treatment, presumably corresponds to keratan sulfate, the other GAG chains found in cartilage PG, as described previously [Carney and Muir, 1988].

Synthesis of PAPS

Analysis by ion-exchange chromatography of PG synthesized by proliferating chondrocytes cultured in the presence of $ZnCl_2$ revealed that these cells synthesized PG molecules with a higher mean charge density as compared with the PG synthesized by proliferating chondrocytes cultured in the absence of $ZnCl_2$. To determine whether the observed changes in mean charge density correlate with changes in the activity of some enzymes involved in the sulfation pathway of PG, we measured the capacity of these cells to synthesize PAPS.

The cell homogenate prepared from proliferating chondrocytes catalyzed the incorporation of ^{35}S-sulfate into PAPS, a metabolic intermediary of the sulfation pathway (Fig. 7). The ability of the homogenate derived from proliferating chondrocytes to catalyze the incorporation of labeled sulfate into PAPS was associated with the different concentrations of $ZnCl_2$ in the culture medium during the culturing period. Thus, the amount of PAPS produced by proliferating chondrocytes cultured in the presence of 1 μM $ZnCl_2$ was higher than that produced by chondrocytes cultured in a culture medium supplemented with 0.1 μM $ZnCl_2$; it was also higher than the amounts of PAPS synthesized

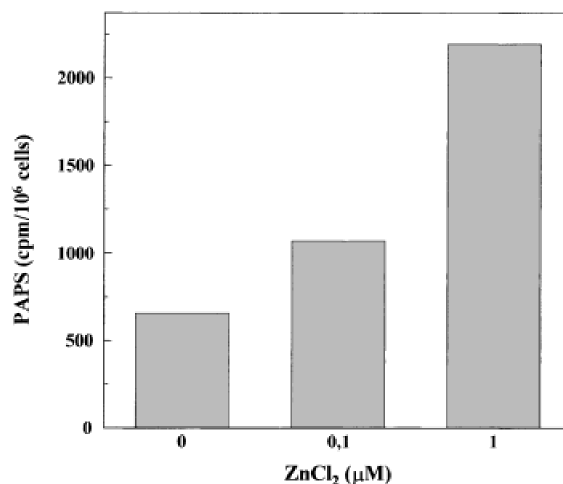

Fig. 7. Synthesis of 3′-phosphoadenosine 5′-phosphosulfate (PAPS). Proliferating chondrocytes were cultured in the presence of different concentrations of $ZnCl_2$ for 4 days. For each concentration of $ZnCl_2$, the cells from four different wells were removed by tripsinization, collected by centrifugation, pooled and subjected to sonication for 5 min. PAPS synthesized was separated by a paper chromatography using unlabeled PAPS (0.1 mg) as carrier. The presence of PAPS was evaluated in an U.V. transilluminator and quantified measuring the radioactivity associated with the carrier.

by proliferating chondrocytes cultured in the absence of $ZnCl_2$ (Fig. 7).

Incorporation of sulfate into PAPS by the cell homogenates from proliferating chondrocytes was dependent on the concentration of $ZnCl_2$ in the culture medium.

Addition of 1 μM $ZnCl_2$ directly to the reaction mixture did not increase the incorporation of ^{35}S-sulfate into PAPS (data not shown). This suggests that the effect of $ZnCl_2$ on sulfation is not mediated by an increase in the activity of enzymes involved in the sulfation pathway, but to an increased level of some of these enzymes, modifying the rate of synthesis and/or degradation of enzymes of the sulfation pathway.

DISCUSSION

The physiological functions of Zn are mostly explained by the activity of several enzymes in which zinc is an essential component in their structure, and which participate in different metabolic pathways [Williams, 1989]. Zn is required for the activity of DNA polymerase, and it is an important component of several nuclear proteins [Chesters, 1989], which bind to distinct sequences in the promoter region of specific genes regulating transcription rates [Chesters, 1992].

Several endocrine factors regulate chondrocyte activity in the epiphyseal growth plate [Rosselot et al., 1992; Nilsson et al., 1994; Rosselot et al., 1994]. Growth hormone (GH) and insulin-like growth factor I (IGF-I), together with fibroblast growth factor (FGF) are the main regulatory factors of cellular activity in the epiphyseal growth plate [Rosselot et al., 1992; Nilsson et al., 1994; Luan et al., 1996].

It is possible to speculate that Zn may modify longitudinal growth through local stimulation of gene expression in cells of the epiphyseal growth plate. This could be mediated by changes in the transcription rates of the genes encoding for IGF-I and its receptor, GH receptor, and IGF-II and/or FGF and their receptors. Increased expression of one or several of these genes in response to Zn, would explain the stimulating effect of this micronutrient on longitudinal growth. In addition, the effects of Zn on the synthesis and activity of hormones and growth factors should be considered [Favier, 1992; Droke et al., 1993].

The effect of Zn on reversing or preventing growth stunting could be explained by the direct stimulation of proliferative and metabolic activity of the cells responsible for longitudinal growth: proliferating chondrocytes, and by the production of extracellular matrix favoring the persistence of unmineralized tissues.

Our results show that zinc specifically increases the proliferation rate of proliferating chondrocytes in culture, since no effect was observed in hypertrophic chondrocytes. Thus, the number of proliferating chondrocytes increased by 40–50% when cells were cultured with low concentrations of zinc (up to 0.5 μM), but no effect was observed in hypertrophic chondrocytes when cultured using the same concentrations of zinc (Fig. 2). We also observed that zinc (0.1 and 1 μM) markedly increased the incorporation of $^{35}S{-}SO_4$ into PG molecules (5–6 times as compared to controls) in proliferating chondrocytes, but no effect was found in sulfate incorporation into PG molecules produced by hypertrophic chondrocytes. Elution profiles in ion-exchange chromatography showed an increase in mean charge density of PG synthesized by proliferating chondrocytes cultured in the presence of $ZnCl_2$ (Fig. 4A) as compared with chondrocytes cultured in the absence of zinc. No changes in mean charge density of the PG synthesized by hypertrophic chondrocytes were observed in the presence or absence of zinc (Fig. 4D). The addition of zinc changed the hydrodynamic size of PG produced by proliferating (Fig. 6) as opposed to hypertrophic chondrocytes in culture (Fig. 5).

Change in sulfate concentrations may reflect either changes in total proteoglycan content or alterations in their composition. Undoubtedly, the latter possibility is important, as the degree of sulfation has been shown to influence hydroxyapatite formation [Chen et al., 1984].

Our results strongly suggest that zinc induces changes in the extracellular matrix synthesized by proliferating chondrocytes. The matrix contains smaller PG with a higher degree of sulfation than those synthesized in the absence of zinc. Synthesis of PG with a high degree of sulfation would favor the persistence of unmineralized matrix and as a result, the growth of tissue. The role of PG in endochondral calcification has been object of considerable controversy. It has been suggested recently that PG interact with calcium, inhibiting calcium phosphate formation in vitro [Dziewiatkowski and Majznerski, 1985] and the growth of hydroxyapatite crystals [Chen et al., 1984]. Together, these

observations have led to the conclusions that a decrease in PG concentration is a prerequisite for calcification to occur.

The observation that PG decrease at the onset of the mineralization process supports this concept [Lohmander and Hjerpe, 1975]. However, other studies have shown preservation of PG during calcification [Poole et al., 1982]. A likely explanation for these observations are that PG are structurally modified rather than removed before and/or during calcification [Hagiwara et al., 1995]. Alternatively, since PG are highly negative-charged molecules, it has been proposed that the sulfate groups of PG may provide the anionic groups responsible for localized calcium ion binding which initiates nucleation [Addadi et al., 1987]. It has also been reported that PG may be responsible for changes in the morphology of calcite crystals [Wu et al., 1994].

The extracellular matrix produced by cells of the growth plate is in a constant state of remodeling. Thus, it requires the rapid synthesis of its components, incorporation into the matrix and breakdown. At present, little is known about the metabolism of PG in the growth plate. The growth of long bones occurs after proliferation, differentiation, hypertrophy, and calcification of the cartilage matrix in the epiphyseal growth plate, followed by replacement of the calcified cartilage by bone [Tian et al., 1986]. It has been postulated that changes of PG sulfation may play an important role in inducing and/or promoting calcification in growth plate cartilage [Hagiwara et al., 1995].

Numerous evidence shows that an impairment in enzymatic activity of the sulfation pathway, or of sulfate transport determines undersulfation of proteoglycans in the cartilage and an increased rate of matrix calcification [Schwartz et al., 1978; Sugahara and Schwartz, 1979; Sugahara and Schwartz, 1982a, 1982b; Rossi et al., 1996]. It has been recently demonstrated that addition of Zn to cultures of growth plate chondrocytes decreases mineralization in a dose-dependent manner [Litchfield et al., 1998; Kirsch et al., 2000]. Our results like the observations just mentioned allow us to speculate that the effect of Zn in promoting longitudinal growth may occur through stimulation of proliferation of the cells responsible for longitudinal growth, proliferating chondrocytes, and of stimulation of the synthesis of non-mineralizing extracellular matrix, with a higher content of PG with a high degree of sulfation.

On the other hand, the results of this study also show that zinc at concentrations higher than 10 μM interrupts almost completely the incorporation of ^{3}H-thymidine on both proliferating and hypertrophic chondrocytes. These results may be interpreted as meaning that zinc interrupts the proliferation of these cells or that it may have potential cytotoxic effects. Whatever be the explanation, these results indicate the need for conducting human studies to determine the potential adverse effects of excess zinc on linear growth in children. It is very important to define the optimal amounts of zinc that must be used as supplementation at early stages of development to promote linear growth.

In summary, the results presented here strongly suggest that the direct activity that zinc exerts on the cells of the growth plate may explain, in part, its effects on longitudinal growth. The response to Zn is greater in proliferating chondrocytes that cause longitudinal growth, as opposed to its effect on hypertrophic chondrocytes. The results presented in this study provide further explanation about the stimulatory effect of Zn on longitudinal growth, and suggest a direct stimulatory regulation at the cellular level in the target tissue.

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REFERENCES

Addadi L, Moradian G, Shay E, Maroudas NG, Weiner SA. 1987. Chemical model for cooperation of sulfate and carboxilates in calcite crystal nucleation: relevance to biomineralization. Proc Natl Acad Sci USA 84:2732–2736.

Allen LH. 1994. Nutritional influences on linear growth: a general review. Eur J Clin Nutr 48:S75–S89.

Barnard R, Haynes KM, Werther GA, Waters MJ. 1988. The ontogeny of growth hormone receptors in the rabbit tibia. Endocrinology 122:2562–2569.

Boden SD, Kaplan FS, Fallon MD, Ruddy R, Belik J, Anday E, Zackai E, Ellis J. 1987. Metatropic dwarfism: uncoupling of endochondral and perichondral growth. J Bone Joint Surg Am 69:174–184.

Boyd A, Shapiro IM. 1980. Energy dispersive X-ray elemental analysis of isolated epiphyseal growth chondrocyte fragments. Histochemistry 69:85–94.

Canalis E. 1983. The hormonal and local regulation of bone formation. Endocrinol. Rev 4:62–75.
Carney SL, Muir H. 1988. The structure and function of cartilage proteoglycans. Physiol Rev 68:858–891.
Castillo-Duran C, Cassorla F. 1999. Trace minerals in human growth and development. J Pediatr Endocrinol Metab 12:589–601.
Castillo-Durán C, Heresi G, Fisberg M, Uauy R. 1987. Controlled trial of zinc supplementation during recovery from malnutrition: effects on growth and immune function. Am J Clin Nutr 45:602–608.
Chen CC, Boskey AL, Rosenberg LC. 1984. The inhibitory effect of cartilage proteoglycans on hydroxyapatite growth. Calcif Tissue Int 36:285–290.
Chesters JK. 1989. Biochemistry of Zinc in cell division and tissue growth. Mills CF, editor. Zinc in human biology London, Berlin: Springer-Verlag. pp 109–118.
Chesters JK. 1992. Trace elements-gene interactions. Nutr Rev 50:217–223.
Dean DD, Schwartz Z, Bonewald L, Muniz OE, Morales S, Gómez R, Brooks BP, Qiao M, Howell DS, Boyan BD. 1994. Matrix vesicles produced by osteoblasts-like cells in culture becomes significantly enriched in proteoglycans-degrading metalloproteinases after addition of b-glycerophosphate and ascorbic acid. Calcif Tissue Int 54:399–408.
Dorup I, Clausen T. 1991. Effects of magnesium and zinc deficiencies on growth and protein synthesis in skeletal muscle and the heart. Br J Nutr 66:493–504.
Dorup I, Flyvbjerg A, Everts ME, Clausen T. 1991. Role of insulin-like growth factor-1 and growth hormone in growth inhibition induced by magnesium and zinc deficiencies. Br J Nutr 66:505–521.
Droke EA, Spears JW, Armstrong JD, Kegley EB, Simpson RB. 1993. Dietary zinc affects serum concentrations of insulin and insulin-like growth factor I in growing lambs. J Nutr 123:13–19.
Dziewiatkowski DD, Majznerski LL. 1985. Role of proteoglycans in endochondral ossification. Calcif Tissue Int 37:560–567.
Escarot-Charrier B, Glorieux FA, van der Rest M, Pereira G. 1983. Osteoblasts isolated from mouse calvaria initiate matrix mineralization in culture. J Cell Biol 96:639–643.
Farquharson C, Whitehead CC, Loveridge N. 1994. Alterations in glycosaminoglycan concentration and sulfation during chondrocyte maturation. Calcif Tisssue Int 54:296–303.
Favier AE. 1992. Hormonal effects of zinc on growth in children. Biol Trace Elem Res 32:383–398.
Futami T, Ototani N, Nagatsuka Y, Yosizawa Z. 1979. Comparison of carbohydrate-containing substances from non-calcified and calcified portions of bovine costal cartilage. J Biochem 85:1067–1073.
Gibson RS, Vanderkooy PDS, MacDonald AC, Goldman A, Ryan BA, Berry M. 1989. A growth-limiting, mild zinc-deficiency syndrome in some Southern Ontario boys with low height percentiles. Am J Clin Nutr 49:1266–1273.
Giugliano R, Milward DJ. 1984. Growth and zinc homeostasis in the severely Zn-deficient rat. Br J Nutr 52:545–560.
Hagiwara H, Aoki T, Yoshimi T. 1995. Immunoelectron microscopic analysis of chondroitin sulfates during calcification in the rat growth plate cartilage. Histochemistry 103:213–220.
Hambidge KM, Casey CE, Krebs NF. 1986. Zinc. Trace elements in human and animal nutrition. Vol 2. New York: Academic Press. p 1–137.
Ianotti JP. 1990. Growth plate physiology and pathology. Orthop Clin N Am 21:1–17.
Isaksson OGP, Lindahl A, Nilsson A, Isgaard J. 1987. Mechanism of the stimulatory effect of growth hormone on longitudinal bone growth. Endocr Rev 8:426–438.
Isaksson OG, Lindahl A, Nilsson A, Isgaard J. 1988. Action of growth hormone: current views. Acta Pediatr Scand (Suppl) 343:12–18.
Isaksson OG, Ohlsson C, Nilsson A, Isgaard J, Lindahl A. 1991. Regulation of cartilage growth by growth hormone and insulin-like growth factor I. Pediatr Nephrol 5:451–453.
Keller KM, Brauer PR, Keller JM. 1989. Modulation of cell surface structure by growth of cells in the presence of chlorate. Biochem 28:8100–8107.
Kirsch T, Harrison G, Worch KP, Golub EE. 2000. Regulatory roles of zinc in matrix vesicle-mediated mineralization of growth plate cartilage. J Bone Miner Res 15:261–270.
Koyano Y, Hejna M, Flechtenmacher J, Schmid TM, Thonar EJ-MA, Mollenhauer J. 1996. Collagen and proteoglycan production by bovine fetal and adult chondrocytes under low levels of calcium and zinc ions. Conn Tissue Res 34:213–225.
Leach RM, Nesheim MC. 1965. Nutritional, genetic, and morphological studies of an abnormal cartilage formation in young chicks. J Nutr 86:236–244.
Litchfield TM, Ishikawa Y, Wu LNY, Wuthier RE, Sauer GR. 1998. Effect of metal ions on calcifying growth plate cartilage chondrocytes. Calcif Tissue Int 62:341–349.
Lohmander S, Hjerpe A. 1975. Proteoglycans of mineralizing rib and epiphyseal cartilage. Biochim Biophys Acta 404:93–109.
Luan Y, Praul CA, Gay CV, Leach RM Jr. 1996. Basic fibroblast growth factor: an autocrine growth factor for epiphyseal growth plate chondrocytes. J Cell Biochem 62:372–382.
Maroteaux P, Stanescu V, Stanescu R. 1983. Hypochondrogenesis. Eur J Pediatr 141:14–22.
Minguell JJ, Tavassoli M. 1989. Proteoglycan synthesis by hemopoietic progenitor cells. Blood 73:1821–1827.
Nilsson A, Ohlsson C, Isaksson OGP, Lindahl A, Isgaard J. 1994. Hormonal regulation of longitudinal bone growth. Eur J Clin Nutr 48:S150–S160.
Ninh NX, Thissen JP, Maiter D, Adam E, Mulumba N, Ketelslegers JM. Reduced liver insulin-like growth factor-1 gene expression in young zinc-deprived rats is associated with a decrease in liver growth hormone (GH) receptors and serum GH-binding protein. J Endocrinol 144:449–456.
O'Dell BL, Reeves PO. 1988. Zinc status and food intake. In: Mills CF, editor. Zinc in human biology. Berlin: Springer-Verlag. pp 173–181.
Poole AR, Pidoux I, Rosenberg L. 1982. Role of proteoglycans in endochondral ossification: immunofluorescent localization of link protein and proteoglycan monomer in bovine fetal epiphyseal growth plate. J Cell Biol 92:249–260.

Price JS, Oyajobi BO, Russell RGG. 1994. The cell biology of bone growth. Eur J Clin Nutr 48:S131–S149.

Rivera JA, Ruel MT, Santizo MC, Lonnerdal B, Brown KH. 1998. Zinc supplementation improves the growth of stunted rural Guatemalan infants. J Nutr 128:556–562.

Rodríguez JP. 1995. Changes in sulfation extent of membrane-associated proteoglycans produced by Sertoli cells in culture. J Cell Biochem 57:22–29.

Rodríguez JP, Conget P, Minguell JJ. 1995. The sulfation degree of membrane-associated proteoglycan from a hemopoietic cell line is determined by changes in the growth state of the cell. Eur J Cell Biol 67:261–266.

Rodríguez JP, Santibañez JF, Martínez J. 1998. Soluble factors secreted by PC-3 cells induce structural changes in proteoglycans produced by fetal rat osteoblasts. Tumour Biol 19:19–29.

Ronaghy H, Reinhold JG, Mahloudji M, Ghavami P, Fox MR, Halsted JA. 1974. Zinc supplementation of malnourished schoolboys in Iran: increased growth and other effects. Am J Clin Nutr 27:112–121.

Rosselot G, Reginato AM, Leach RM. 1992. Development of a serum-free system to study the effect of growth hormone and insulin-like growth factor-I on cultured postembryonic growth plate chondrocytes. Cell Dev Biol 28A:235–244.

Rosselot G, Vasilatos-Younken R, Leach RM. 1994. Effect of growth hormone, insulin-like growth factor I, basic fibroblast growth factor, and transforming growth factor β on cell proliferation and proteoglycan synthesis by avian postembryonic growth plate chondrocytes. J Bone Min Res 9:431–439.

Rossi A, Bonaventure J, Delezoide AL, Cetta G, Superti-Furga A. 1996. Undersulfation of proteoglycans synthesized by chondrocytes from a patient with achondrogenesis type 1B homozygous for an L483P substitution in the diastrophic dysplasia sulfate transporter. J Biol Chem 271:18456–18464.

Ruz M, Castillo-Durán C, Lara X, Codoceo J, Rebolledo A, Atalah EA. 1997. 14-mo zinc-supplementation trial in apparently healthy Chilean preschool children. Am J Clin Nutr 66:1406–1413.

Schelchter N, Russel S, Spencer E, Nicoll C. 1986. Evidence suggesting that the direct growth-promoting effect of growth hormone on cartilage in vivo is mediated by local production of somatomedin. Proc Natl Acad Sci USA 83:7032–7934.

Schwartz NB, Ostrowski V, Brown KS, Pratt RM. 1978. Defective PAPS-synthesis in epiphyseal cartilage from brachymorphic mice. Biochem Biophys Res Commun 82:173–178.

Sugahara K, Schwartz NB. 1979. Defect in 3′-Phosphoadenosine 5′-phosphosulfate formation in brachymorphic mice. Proc Natl Acad Sci USA 76:6615–6618.

Sugahara K, Schwartz NB. 1982a. Defect in 3′-Phosphoadenosine 5′-phosphosulfate synthesis in brachymorphic mice. I Characterization of the defect. Arch Biochem Biophys 214:589–602.

Sugahara K, Schwartz NB. 1982b. Defect in 3′-Phosphoadenosine 5′-phosphosulfate synthesis in brachymorphic mice. II Tissue distribution of the defect. Arch Biochem Biophys 214:602–609.

Tian M-Y, Yanagishita M, Hascall VC, Reddi AH. 1986. Biosynthesis and fate of proteoglycans in cartilage and bone during development and mineralization. Arch Biochem Biophys 247:221–232.

Trippel SB, Corvol MT, Dumontier MF, Rappaport R, Hung HH, Mankin HJ. 1989. Effect of somatomedin-C/insulin-like growth factor I and growth hormone on cultured growth plate and articular chondrocytes. Pediatr Res 25:76–82.

Walravens PA, Chakar A, Mokni R, Denise J, Lemonnier D. 1992. Zinc supplements in breastfed infants. Lancet 340:683–685.

Westmoreland N, Hoekstra WG. 1969. Pathological defects in the epiphyseal cartilage of zinc-deficient chicks. J. Nutr 98:76–82.

Williams RJP. 1989. An introduction to the biochemistry of zinc. In: Mills CF, editor. Zinc in human biology. London: Springer-Verlag. pp 15–31.

Wrobleski J, Engstrom M, Skottner A, Madsen K, Friberg U. 1987. Subcellular location of IGF-1 in chondrocytes from rat rib growth plate. Acta Endocrinol 115:37–43.

Wu T-M, Rodríguez JP, Fink DI, Carrino DA, Blackwell J, Caplan AI, Heuer AH. 1994. Crystallization studies on avian eggshell membranes: implications for the molecular factors controlling eggshell formation. Matrix Biol 14:507–513.