Stanniocalcin 1 Acts as a Paracrine Regulator of Growth Plate Chondrogenesis*

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During embryogenesis, the expression of mammalian stanniocalcin (STC1) in the appendicular skeleton suggests its involvement in the regulation of longitudinal bone growth. Such a role is further supported by the presence of dwarfism in mice overexpressing STC1. Yet, the STC1 inhibitory effect on growth may be related to both postnatal metabolic abnormalities and prenatal defective bone formation. In our study, we used an organ culture system to evaluate the effects of STC on growth plate chondrogenesis, which is the primary determinant of longitudinal bone growth. Fetal rat metatarsal bones were cultured in the presence of recombinant human STC (rhSTC). After 3 days, rhSTC suppressed metatarsal growth, growth plate chondrocyte proliferation and hypertrophy/differentiation, and extracellular matrix synthesis. In addition, rhSTC increased the number of apoptotic chondrocytes in the growth plate. In cultured chondrocytes, rhSTC increased phosphate uptake, reduced chondrocyte proliferation and matrix synthesis, and induced apoptosis. All these effects were reversed by culturing chondrocytes with rhSTC and phosphonoformic acid, an inhibitor of phosphate transport. The rhSTC-mediated inhibition of metatarsal growth and growth plate chondrocyte proliferation and hypertrophy/differentiation was abolished by culturing metatarsals with rhSTC and phosphonoformic acid. Taken together, our findings indicate that STC1 inhibits longitudinal bone growth directly at the growth plate. Such growth inhibition, likely mediated by an increased chondrocyte phosphate uptake, results from suppressed chondrocyte proliferation, hypertrophy/differentiation, and matrix synthesis and by increased apoptosis. Last, the expression of both STC1 and its binding site in the growth plate would support an autocrine/paracrine role for this growth factor in the regulation of growth plate chondrogenesis.

Stanniocalcin (STC)2 is a glycoprotein first identified as a secretory product of the corpuscles of Stannius, an endocrine gland unique to bony fish (1, 2). The primary function of STC in fish is to prevent hypercalcemia (3) by inhibiting calcium uptake by the gills and gut and stimulating phosphate reabsorption by the kidneys (1, 4).

The mammalian homolog of STC (STC1) has been found in humans, rats, and mice (5–7). Human and mouse STC1 proteins are closely related to each other (98% amino acid identity) and share 80% amino acid identity with fish STC (5). Compared with fish STC, STC1 seems to have a preferential effect on phosphate metabolism than on calcium metabolism (8, 9), with evidence suggesting that sodium-dependent Pi (NaPi) transporter(s) may be a target of STC1 activity (10). Although fish STC is primarily expressed in one organ (the corpuscle of Stannius), mammalian STC1 is virtually ubiquitous. The distribution of STC protein and/or mRNA in multiple organs such as kidney, intestine, heart, thyroid, lung, placenta, brain (11–15), and bone (with virtually undetectable serum levels) (11) would suggest a paracrine rather than an endocrine role for STC1 in mammals.

With respect to its expression in bone, STC1 mRNA and protein have been detected in the mouse appendicular skeleton from early in embryogenesis (16, 17). In addition, STC1 mRNA expression has been found in osteoblasts and articular cartilage of 30-day-old mouse femora (10, 18, 19). Transgenic mice overexpressing STC1 exhibit significant reduction in birth weight and reduced adult body size, which would suggest a regulatory role for STC1 in mammalian bone growth (20, 21).

In mammals, linear growth occurs at the long bone growth plates by endochondral ossification, a process by which cartilage is formed and then remodeled into bone (22, 23). Growth plate chondrocyte proliferation and hypertrophy/differentiation, and extracellular matrix secretion all lead to formation of new cartilage, chondrogenesis (24). As new cartilage is continuously formed, calcified cartilage at the metaphyseal border of the growth plate is replaced by bone tissue (25). Thus, the rate of long bone growth depends primarily on the rate of chondrogenesis in the growth plate.

To determine whether STC1 regulates longitudinal bone growth, we first studied STC1 and STC binding site expression in the rat metatarsal growth plate. Then, we cultured rat metatarsal bone rudiments in the presence of recombinant human STC (rhSTC) and assessed its effects on metatarsal linear growth, growth plate chondrocyte proliferation and hypertrophy/differentiation, cartilage matrix synthesis, and apoptosis. Last, we evaluated the effects of rhSTC on growth plate chondrocyte phosphate uptake and the relationship between phosphate uptake modifications and growth plate chondrogenesis.

EXPERIMENTAL PROCEDURES

Organ Culture—The second, third, and fourth metatarsal bone rudiments were isolated from Sprague-Dawley rat fetuses at 20 days postconception and cultured individually in 24-well plates (26, 27). Each well contained 0.5 ml minimum essential medium (MEM) (Invitrogen) supplemented with 0.05 mg/ml ascorbic acid (Sigma), 1 mM sodium glycerophosphate (Sigma), 0.2% bovine serum albumin (BSA, Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin (In vitrogen). Bone rudiments were cultured for 3 days in a humidified incubator with 5% CO2 in air at 37 °C. The medium was changed on day 2. In the first set of experiments, metatarsals were cultured for 3 days in serum-free
medium in the absence or presence of rhSTC (0–100 ng/ml). For rhSTC production, the open reading frame of human STC without Met was amplified by PCR and cloned into the bacterial expression vector pQE-30 that contained an N-terminal His tag for subsequent affinity purification (10). In the second set of experiments, the bone rudiments were cultured in the presence of rhSTC (100 ng/ml) with or without phosphonoformic acid (PFA, 0.3 mM), a known inhibitor of phosphate transport. Control bones were treated with equal volumes of vehicle (PBS plus 0.1% BSA). Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (DHETE Publication (National Institutes of Health) 85-23, revised 1988).

**Chondrocyte Culture**—Metatarsal rudiments were rinsed in PBS, then incubated in 0.2% trypsin for 1 h and 0.2% collagenase for 3 h. Cell suspension was aspirated repeatedly and filtered through a 70-μm cell strainer, rinsed first in PBS then in serum-free Dulbecco’s modified Eagle’s medium, and counted. Chondrocytes were seeded in 100-mm dishes at a density of 4×10⁶ cells/10 ml in Dulbecco’s modified Eagle’s medium with 100 units/ml penicillin and 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, and 10% FBS. The culture medium was changed at 72-h intervals. Confluent cells were trypsinized and grown in 24-well plates at the density of 1×10⁵/ml in the same medium and allowed to return to 70–80% confluence.

**In Situ Ligand Binding Assay**—To detect the expression of STC binding site in the growth plate, in situ ligand binding was performed using an STC-alkaline phosphatase (STC-AP) fusion protein (a gift from Dr. Graham Wagner, University of Western Ontario, Canada) (28). Paraformaldehyde-fixed, paraffin-embedded tissue sections were dehydrated, rehydrated, and equilibrated in Hanks’ balanced salt solution (containing 0.1% BSA, pH 7.5) (HBHA) before incubation with 0.5 nM STC-AP fusion protein, AP, or STC-AP fusion protein plus 1 μM rhSTC in HBHA for 90 min at room temperature. Thereafter, the tissue sections were washed in HBHA with 0.1% Tween and fixed for 30 s in 60% acetone, 3% formaldehyde in 20 mM HEPES, pH 7.5. Endogenous alkaline phosphatase activity was inactivated by a 60-min incubation in HBHA at 65 °C. Color development of ligand-associated AP activity was then initiated at room temperature by the addition of AP liquid substrate (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate). Finally, slides were washed in distilled water to stop the reaction and mounted. A negative control slide was obtained by preparing the slide without AP-TC1 fusion protein addition.

**Measurement of Longitudinal Growth**—The length of each bone rudiment was measured under a dissecting microscope using an eyepiece micrometer. To calculate the metatarsal growth rate, length measurements were performed daily during the culture period.

**5-Bromo-2′-deoxyuridine (BrdUrd) in Situ Incorporation**—After 3 days in culture, BrdUrd was added to the culture medium at a final concentration of 10 μM (29). Bone rudiments were incubated for an additional 5 h. At the end of the incubation, all bones were fixed in 4% phosphate-buffered paraformaldehyde, embedded in paraffin, and cut in 5–7-μm-thick longitudinal sections. Bone sections were stained for BrdUrd according to the manufacturer’s protocol (Roche Applied Science). The BrdUrd-labeling index was calculated as the number of BrdUrd-labeled cells per grid divided by the total number of cells per grid. The grid circumscribed a portion of the growth plate zone (epiphyseal or proliferative) analyzed through a 40× objective and generally contained an average of 50 cells. For each growth plate zone, the fraction of labeled cells in three distinct grid locations was calculated and averaged. For each treatment group, 10 bones were sampled, and 3 growth plate sections of each bone were analyzed. The labeling index (number of labeled cells/total cells) was determined separately for the epiphyseal zone and for the proliferative zone. All determinations were made by the same observer blinded to the treatment category.

**Quantitative Histology**—At the end of the culture period, metatarsals were fixed in 4% phosphate-buffered paraformaldehyde overnight. After routine processing, three longitudinal, 5–7-μm-thick sections were obtained from each metatarsal bone and stained with toluidine blue. From each of the three sections, we measured the height of the epiphyseal zone, proliferative zone, hypertrophic zone, and of the ossification center and calculated the average value. In the metatarsal growth plate, the epiphyseal zone is characterized by small and rounded cells irregularly arranged in the cartilage matrix. The proliferative zone comprises cells with a flattened shape arranged in columns parallel to the longitudinal axis of the bone. In the hypertrophic zone, large cells (defined by a height ≥9 μm) form a layer adjacent to the calcified region of the metatarsal bone, the primary ossification center. All quantitative histology was performed by a single observer blinded to the treatment category.

**In Situ Hybridization**—Metatarsals were fixed overnight in 4% paraformaldehyde at 4 °C, then dehydrated in ethanol and embedded in paraffin. Sections were hybridized to 35S-labeled antisense riboprobes. Slides were exposed to photographic emulsion at 4 °C for 4 days, then developed, fixed, and cleared. Sections were counterstained with hematoxylin and viewed using a light microscope. Sections hybridized with a labeled-sense riboprobe were used as negative controls. The mouse type X collagen (Col10a1) probe (a gift from Dr. Bjorn Olsen, Harvard Medical School, Boston, MA) was a 650-bp HindIII fragment containing 400 bp of non-collagenous (NC1) domain and 250 bp of 3′-untranslated sequence of the mouse Col10a1 gene in pBluescript (30).

**Immunohistochemistry**—Bone rudiments were fixed overnight in 4% paraformaldehyde and embedded in paraffin. 5–7-μm-thick longitudinal sections were obtained and deparaffinized in xylene and rehydrated in graded ethanol. Sections were incubated in 1% H2O2 for 10 min followed by 3 rinses with PBS. For digestion, 0.1% trypsin for 12 min was used at room temperature followed by a triple wash in PBS. After pre-incubation with 1.5% blocking serum for 30 min at room temperature, sections were incubated for 30 min at room temperature with rabbit anti-STC polyclonal antibody (1:200) (6), rabbit polyclonal antiserum raised against mouse type X collagen peptides, anti-NC2 domain (1:2000) (31), or goat anti-collagen II antibody (1:200) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The secondary antibody was an anti-rabbit or anti-goat antibody conjugated with biotin (ABC staining system, Santa Cruz Biotechnology), applied for 30 min at a dilution of 1:200. This step was followed by incubation for 30 min with avidin and biotinylated horseradish peroxidase. The sections were then visualized with peroxide substrate for 5 min and mounted with Permount medium. Control experiments were performed using normal rabbit or goat serum instead of the primary antibody.

Cultured primary chondrocytes grown on sterile glass coverslips were treated with or without STC (100 ng/ml) for 24 h before being fixed in cold methanol for 5 min at −10 °C and dried. Cells were blocked with 1.5% normal blocking serum in PBS for 1 h and then incubated in goat anti-collagen II antibody (1:50) for 30 min followed by an incubation for 30 min with avidin and biotinylated horseradish peroxidase. Cells were then visualized with peroxide substrate for 5 min, counterstained with hematoxylin, and mounted with Permount medium. Control experiments were performed using normal goat serum instead of the primary antibody.

**In Situ Cell Death**—At the end of the culture period, metatarsals were fixed in 4% phosphate-buffered paraformaldehyde, embedded in paraffin, and cut in 5–7-μm-thick longitudinal sections. From each bone,
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three sections parallel to the long axis of the bone were obtained. Apoptotic cells in the growth plate were identified by terminal deoxyuridine triphosphate-mediated deoxyuridine triphosphate nick end labeling, according to the manufacturer’s instructions (TDT-FragEL kit; Oncogene Research Products, Boston, MA) with slight modifications (deparaffinized and rehydrated sections were treated with proteinase K for 10 min instead of 20 min) (32). A positive control was generated by covering the entire tissue section with 1 μg/μl DNase I in 1 × TBS/1 mM MgSO₄, after proteinase K treatment, whereas a negative control was generated by substituting distilled H₂O for the terminal deoxyuridine-yltransferase in the reaction mixture. All other steps were performed as described above (data not shown).

Apoptosis was quantitated by determining the apoptotic index (calculated as the number of apoptotic cells per grid divided by the total number of cells per grid). The grid circumscribed a portion of the growth plate analyzed through a 40× objective and generally contained an average of 50 cells. For each growth plate, the fraction of labeled cells in three distinct grid locations was calculated and averaged. For each treatment group 10 bones were sampled and 3 growth plate sections of each bone were analyzed.

Measurement of [35S]Sulfate Incorporation into Proteoglycans (PGs)—As a measure of cartilage matrix synthesis, we assessed [35S]sulfate incorporation into PGs (26). Na₂[35S]SO₄ (Amersham Biosciences, specific activity up to 100 mCi/mmol), at a concentration of 5 μCi/ml, was added to the culture medium 3 h before the end of the 3-day culture period. The metatarsal rudiments were washed 3 times for 10 min with Puck’s saline culture medium 3 h before the end of the 3-day culture period. The apoptotic cells in the growth plate were identified by terminal deoxynucleotidyltransferase in the reaction mixture. All other steps were performed as described above (data not shown).

Measurement of [35S]SO₄ Incorporation into Proteoglycans (PGs)—As a measure of cartilage matrix synthesis, we assessed [35S]sulfate incorporation into PGs (26). Na₂[35S]SO₄ (Amersham Biosciences, specific activity up to 100 mCi/mmol), at a concentration of 5 μCi/ml, was added to the culture medium 3 h before the end of the 3-day culture period. The metatarsal rudiments were washed 3 times for 10 min with Puck’s saline solution and then digested in 1.5 ml of MEM with 0.3% papain at 60 °C for 16 h. They were then incubated with 0.5 ml of 10% cetyl pyridinium chloride (Sigma) in 0.2M NaCl, and dissolved in 0.5 ml of 23 N formic acid. The amount of radioactivity incorporated into PG expressed in percent of control.

The incorporation of [35S]sulfate into PG by cultured chondrocytes was assessed according to the method of Venkatesan et al. (33). Cells were labeled with 5 μCi/ml Na₂[35S]SO₄ for the last 6 h of the 24-h experimental period. PGs were extracted from the cell layer and culture medium with 3 M guanidine hydrochloride and precipitated by using 5% cetyl pyridinium chloride. The amount of radioactivity incorporated into PG was measured by liquid scintillation. Results are presented as total radio-labelled PG expressed in percent of control.

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Western Blot—Whole cell lysates from cultured chondrocytes treated with/without 100 ng/ml STC for 24 h were solubilized with 1% SDS sample buffer and electrophoresed on a 4–15% SDS-PAGE gel (Bio-Rad). Proteins were transferred onto a nitrocellulose membrane and were probed with rabbit polyclonal antibodies against caspase 3 and caspase 9 (Santa Cruz Biotechnology). The blots were developed using a horseradish-peroxidase-conjugated polyclonal goat-anti rabbit IgG antibody and enhanced chemiluminescence system (Amersham Biosciences). The protein size was confirmed by molecular weight standards (Invitrogen).

P, Chondrocyte Uptake—Chondrocytes in 70–80% confluence were washed with fresh serum-free medium containing 0.1% BSA and incubated with or without rhSTC (100 ng/ml) and/or PFA (0.1 mM). Before performing the P uptake assay, the cells were washed 3 times with 500 μl of washing buffer (150 mM choline chloride, 1.8 mM MgCl₂, 1 mM CaCl₂, and 10 mM HEPES buffered with Tris-HCl, pH 7.4). For each treatment group, chondrocytes from three wells were used to determine P uptake, whereas chondrocytes from 3 more wells were trypsinized and counted with hemacytometer. P uptake was measured in 200 μl of washing buffer (150 mM NaCl instead of choline chloride) containing 1 μCi/ml NaPi (Amersham Biosciences) at 37 °C for 30 min. Cells were then washed 3 times with 500 μl of ice-cold washing buffer followed by solubilization in 0.2 N NaOH. P uptake was determined by liquid scintillation counting. The data were expressed as cpm.

Flow Cytometry Assay—Apoptotic cell death was quantified by a flow cytometric assay based on the number of cells with fragmented DNA. Cultured chondrocytes were treated with/without STC 100 ng/ml for 24 h before being harvested by centrifugation and fixated in 80% ethanol that had been precooled to −20 °C. The cells were re-suspended in phosphate-buffered saline containing 50 μg/ml propidium iodide, 0.1% Nonidet P-40, and 100 μg/ml RNase (Sigma) and incubated for 1 h. The number of cells with fragmented DNA was then quantified using 1–2×10⁴ cells on a FACSort flow cytometer and the CellQuest analysis program (BD Biosciences).

Reverse Transcript-PCR—At the end of the culture period, total RNA was extracted from the growth plates of 18 rat metatarsal bones using the Qiagen RNeasy mini kit (Qiagen Inc., Valencia CA). Primers specific for rat Pit-1 (5′ primer, 5′-TAGCTGTGTAATAATAGCCAG-GTTTC-3′; 3′ primer, 5′-AGAAGTTGTCACTGACACGAC-3′; product size 342 bp) and rat FGF23 (5′ primer, 5′-CAGCTGTAG-AGGCTATTCAAGACT-3′; 3′ primer, 5′-CTATTTACACTCAGGG-TAGCCAGACATC-3′; product size 200 bp) were used. The housekeeping gene β-actin (5′ primer, 5′-GTGACAGACTACCTCATGGAAGTCC-3′; 3′ primer, 5′-CATAGGATCTCTTTACGGATACCA-3′; product size 330 bp) was used as normalization control. The recovered RNA was further processed using 1st Strand cDNA synthesis kit for reverse transcription-PCR (avian myeloblastosis virus) (Roche Diagnostics) to produce cDNA. One microgram of total RNA and 1.6 μg of oligo-p(dT)₁₅ primer were incubated for 10 min at 25 °C followed by incubation for 60 min at 42 °C in the presence of 20 units of avian myeloblastosis virus reverse transcriptase and 50 units of RNase inhibitor in a total 20-μl reaction. The cDNA products were directly used for PCR or stored at −80 °C for later analysis. The reaction (100 μl total volume) was performed using a PerkinElmer Life Sciences GeneAmp PCR system 9600 in the presence of 20 pmol of primers, 200 nmol of dNTP, 150 nmol of MgCl₂, 1× PCR buffer (Expand High Fidelity PCR buffer; Roche Applied Science), and 2.5 units of Expand High Fidelity DNA polymerase (Roche Applied Science). The conditions for amplification were 2 min 30 s at 96 °C followed by 35 cycles of denaturation for 45 s at 96 °C, annealing for 1 min at 55 °C, elongation for 1 min 30 s at 72 °C, and finally, extension for 10 min at 72 °C. PCR products were separated by electrophoresis in a 2% agarose gel with ethidium bromide (1.5 μg/ml).

Statistics—All data are expressed as the mean ± S.E. Statistical significance was determined by t test or by analysis of variance.

RESULTS
Expression of STC1 and STC Binding Sites in the Growth Plate—To determine whether STC1 regulates growth plate chondrogenesis in a paracrine/autocrine fashion, we first evaluated its protein expression by immunohistochemistry. STC1 was predominantly localized in the lower proliferative zone and in the hypertrophic zone of the growth plate (Fig. 1A). To detect the expression of the STC binding site, we then performed a ligand binding assay by employing a STC-AP fusion protein. The expression of the STC binding site was mainly detected in the lower proliferative and hypertrophic zones (Fig. 1C), with a pattern
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Effects of rhSTC on metatarsal longitudinal growth and growth plate histology. A, fetal rat metatarsals (days post-coitus 20) were cultured for 3 days in serum-free MEM containing graded concentrations of rhSTC (0–100 ng/ml, n = 63–86/group). Bone length was measured daily using an eyepiece micrometer in a dissecting microscope. B, fetal rat metatarsals were cultured for 3 days in serum-free MEM with or without 100 ng/ml rhSTC (n = 8/group) or with 100 ng/ml rhSTC plus 0.3 mM PFA (n = 6–9/group). After routine histological processing, bones were embedded in paraffin, and 5–7-μm longitudinal sections were obtained. The heights of the growth plate epiphyseal, proliferative and hypertrophic zones and the length of the ossification center (OC) were measured by a single observer blinded to the treatment regimen.

Effects of rhSTC on collagen X expression. Upper panels show immunolocalization of collagen X protein in control (A) and STC-treated (B) metatarsal bones using a rabbit polyclonal antibody directed against mouse collagen X at a dilution of 1:200. Brown staining (arrow) shows stanniocalcin protein. Lower panels show in situ hybridization for collagen X mRNA in control (C) and STC-treated (D) bones. Black punctuate staining (arrow) indicates collagen X mRNA expression. HZ, hypertrophic zone.

Similar to that of the STC protein. Nonspecific binding was ruled out by using either AP alone or STC-AP plus 1 μM rhSTC (data not shown). No staining was observed in the growth plate of negative controls when either STC1 antibody or STC-AP fusion protein was omitted (Fig. 1, B and D, respectively).

Effects of rhSTC on longitudinal bone growth and growth plate chondrogenesis—During the 3 days of the culture period, rhSTC induced a significant, concentration-dependent suppression of the metatarsal longitudinal growth (n = 63–86/group, p < 0.001, Fig. 2A). Because the rate of longitudinal bone growth depends primarily on the rate of growth plate chondrogenesis, we evaluated the effects of rhSTC on chondrocyte hypertrophy/differentiation and chondrocyte proliferation. To assess chondrocyte hypertrophy, we examined the bone rudiments histologically. After 3 days in culture, treatment with 100 ng/ml rhSTC reduced the height of the growth plate hypertrophic zone (n = 8/group, p < 0.05, Fig. 2B). We then evaluated the effects of rhSTC on chondrocyte differentiation by assessing the expression of collagen X (a marker of chondrocyte differentiation) in the growth plate by immunohistochemistry and in situ hybridization. Compared with control metatarsal bones, STC-treated metatarsals exhibited a marked reduction of type X collagen protein and mRNA expression (Fig. 3, A–B and C–D, respectively). To study chondrocyte proliferation, we examined the in situ BrdUrd incorporation into the bone rudiments at the end of the culture period. 100 ng/ml rhSTC significantly decreased BrdUrd incorporation into the growth plate epiphyseal and proliferative zones (representative sections of control and STC-treated bones, Fig. 4, A–B; BrdUrd labeling index: n = 30/group, p < 0.01, Fig. 4C). Consistent with these findings, treatment with rhSTC significantly reduced the height of the growth plate proliferative zone (n = 8/group, p < 0.05, Fig. 2B). To determine whether the STC-mediated inhibition of the metatarsal growth was also due to chondrocyte apoptosis, we evaluated in situ cell death in the metatarsal growth plates by terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling. 100 ng/ml STC increased the number of apoptotic chondrocytes throughout the metatarsal growth plate (representative sections of control and STC-treated bones, Fig. 4, D–E; apoptotic index: n = 30/group, p < 0.05, Fig. 4F). To confirm the STC-mediated induction of apoptosis in the metatarsal bones, we next quantified apoptosis in cultured chondrocytes by flow cytometry. 24 h of treatment with 100 ng/ml STC significantly increased the number of apo-
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FIGURE 4. Effects of rhSTC on growth plate chondrocyte proliferation and cell death. A and B, metatarsal bones cultured for 3 days without (A) or with 100 ng/ml rhSTC (B) were labeled with BrdUrd and prepared for staining as described below. A representative BrdUrd-labeled cell is indicated by the arrow. C, BrdUrd labeling index. Fetal rat metatarsals were cultured for 3 days in serum-free with or without 100 ng/ml rhSTC. At the end of the culture period, BrdUrd was added to the culture medium at a final concentration of 10 μM. Bone rudiments were incubated for an additional 5 h. At the end of the incubation all bones were fixed in 4% phosphate-buffered paraformaldehyde, embedded in paraffin, and cut in 5–7-μm-thick longitudinal sections. Staining was performed according to the manufacturer’s protocol (Roche Applied Science). The BrdUrd-labeling index was calculated as the number of BrdUrd-labeled cells per grid divided by the total number of cells per grid. The grid circumscribed a portion of the growth plate zone (epiphyseal or proliferative) analyzed through a 40× objective and generally contained an average of 50 cells. For each growth plate zone, the fraction of labeled cells in three distinct grid locations was calculated and averaged. For each treatment group, 10 bones were sampled, and 3 growth plate sections of each bone were analyzed (n = 30/group). D and E, representative photomicrograph showing apoptosis in the metatarsal growth plate. Fetal rat metatarsal bones were cultured for 3 days without (D) or with 100 ng/ml rhSTC (E). Apoptotic cells in the growth plate were identified by terminal deoxynucleotidyltransferase-mediated deoxy-UTP nick end labeling. The arrow indicates an apoptotic chondrocyte. F, apoptotic index. Fetal rat metatarsals were cultured for 3 days in serum-free MEM with/without 100 ng/ml rhSTC or with 100 ng/ml rhSTC plus 0.3 mM PFA. At the end of the culture period metatarsal bones underwent routine histological processing. 5–7-μm-thick longitudinal sections were obtained and treated with terminal deoxynucleotidyltransferase-mediated deoxy-UTP nick end labeling assay. The apoptotic index was calculated as the number of apoptotic cells per grid divided by the total number of cells per grid. The grid circumscribed a portion of the growth plate analyzed through a 40× objective and generally contained an average of 50 cells. For each growth plate the fraction of labeled cells in three distinct grid locations was calculated and averaged. The index was calculated by a single observer blinded to the treatment regimen (n = 30/group). For each treatment, 10 bones were sampled, and 3 sections of each bone were analyzed.

To evaluate the effect of STC on extracellular matrix synthesis, we studied the expression of collagen II by immunohistochemistry and proteoglycan synthesis by [35S]sulfate incorporation into metatarsal bones. Treatment of the metatarsal bones with 100 ng/ml rhSTC caused a dramatic decrease of collagen II expression throughout the metatarsal growth plate (Fig. 6, A–B) and reduced [35S]sulfate incorporation compared with control (p < 0.01, Fig. 6C). To confirm the effects observed in the organ culture model, we studied the STC effects on extracellular matrix synthesis in cultured chondrocytes. As in the whole metatarsal bones, STC significantly reduced collagen II expression (Fig. 6, D–E) and [35S]sulfate incorporation into cultured chondrocytes isolated from the metatarsal growth plates (p < 0.01, Fig. 6F).

Effects of rhSTC on Chondrocyte P, Uptake—In light of the stimulatory role of STC on phosphate transport in the kidney and in osteoblasts, apoptotic chondrocytes (p < 0.01, Fig. 5A). Consistent with the STC-mediated induced apoptosis, treatment of cultured chondrocytes with rhSTC increased the expression of caspase-3 and caspase-9, as assessed by Western blot (Fig. 5B).

FIGURE 5. Apoptosis and caspases 3 and 9 expression in cultured chondrocytes treated with rhSTC. A, cultured chondrocytes were treated with or without STC 100 ng/ml or with STC 100 ng/ml plus 0.1 mM PFA for 24 h before being harvested by centrifugation and fixed in 80% ethanol that had been precooled to −20 °C. The number of cells with fragmented DNA was then quantified by flow cytometry. Data represent the means ± S.E. from three independent experiments. B, cultured chondrocytes were treated with or without 100 ng/ml rhSTC for 24 h, harvested, lysed, electrophoresed, and immunoblotted for caspases 3 and 9 and the loading control, actin. A blot from a representative experiment is presented. More than three independent experiments were performed with similar results.
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FIGURE 6. Effects of rhSTC on cartilage matrix synthesis. Shown is expression of collagen II in metatarsal bones treated without (A) or with rhSTC (B), assessed by immunohistochemistry using a goat polyclonal anti-collagen II antibody (1:200). Brown staining (arrow) shows collagen II protein in the metatarsal growth plate. C, [35S]sulfate incorporation into metatarsal bones. Metatarsal bones cultured with or without 100 ng/ml rhSTC or with 100 ng/ml rhSTC plus 0.3 mM PFA were incubated with [35S]sulfate at the concentration of 5 μCi/ml 3 h before the end of the 3-day culture period. The amount of radioactivity incorporated into proteoglycans was measured by liquid scintillation. Results expressed as total radiolabeled proteoglycans in percent of control (n = 19/group). D and E, expression of collagen II in cultured chondrocytes isolated from metatarsal growth plates and treated without (D) or with rhSTC (E) by immunocytochemistry. F, [35S]sulfate incorporation in cultured chondrocytes. Chondrocytes cultured with or without 100 ng/ml rhSTC or with 100 ng/ml rhSTC plus 0.1 mM PFA were incubated with [35S]sulfate at the concentration of 5 μCi/ml 6 h before the end of the 24-h culture period. The amount of radioactivity incorporated into proteoglycans was measured by liquid scintillation. Results are expressed as the mean ± S.D. of cellular and medium cpm/μg of protein (n = 12/group).

and of the role played by phosphate on chondrocyte function, we hypothesized that rhSTC may inhibit metatarsal growth by stimulating phosphate uptake by the growth plate chondrocytes. To test our hypothesis, we first treated chondrocytes isolated from metatarsal bones with 100 ng/ml rhSTC. rhSTC induced a nearly 2-fold increase in the Pi cellular uptake (n = 6 samples obtained from 2 experiments, p < 0.01, Fig. 7A). Such stimulatory effect on phosphate uptake was reversed by co-culturing chondrocytes with rhSTC and PFA (0.1 mM), an inhibitor of phosphate (Pit) transport (p < 0.01, Fig. 7A). Furthermore, the addition of STC markedly reduced the number of cultured chondrocytes, with such effect reversed by cotreatment with 0.1 mM PFA (data not shown).

Effects of PFA on rhSTC-induced Inhibition of Metatarsal Growth and Growth Plate Chondrogenesis—To determine whether the rhSTC-mediated inhibition of metatarsal growth and growth plate chondrogenesis were caused by an increased chondrocyte Pi uptake, we cultured the metatarsal rudiments in the presence of rhSTC with or without PFA. Co-treatment with 100 ng/ml rhSTC and 0.3 mM PFA abolished the inhibitory effects of rhSTC on longitudinal bone growth (n = 60/group, p < 0.001, Fig. 7B), on chondrocyte hypertrophic and proliferative zone heights (n = 6–9/group, p < 0.05, Fig. 2B), on in situ cell death in the growth plate (apoptotic index: n = 30/group, p < 0.05, Fig. 4F), and on the [35S]sulfate incorporation into metatarsal bones (p < 0.05, Fig. 6C). Similar reversal of the STC-mediated inhibition of apoptosis and [35S]sulfate incorporation was observed in cultured chondrocytes co-treated with 100 ng/ml rhSTC and 0.1 mM PFA (p < 0.05, Fig. 5A; p < 0.05, Fig. 6F, respectively).

Effects of rhSTC on NaPi Transporter (Pit-1) and FGF23 mRNA Expression in the Metatarsal Growth Plate—To shed light on the mechanism under which rhSTC stimulated chondrocytes Pi uptake, we performed reverse transcription-PCR to detect the expression of Pit-1 and FGF23 mRNA in metatarsal bones treated with STC. 100 ng/ml rhSTC significantly increased Pit-1 mRNA expression in the metatarsal growth plate, whereas it decreased mRNA expression of FGF23 (Fig. 7C).

DISCUSSION

The addition of rhSTC to the culture medium of rat metatarsal bones caused a concentration-dependent suppression of their linear growth, suggesting a direct inhibitory effect of STC1 on mammalian longitudinal bone growth. Detection of STC1 and STC binding site expression in the metatarsal growth plate would also suggest that STC1 inhibits bone growth by acting on an autocrine/paracrine fashion in the growth plate.

Previous experimental evidence in transgenic mice overexpressing human STC1 had shown an STC-mediated growth inhibition in mammals (20, 21). When compared with control mice, STC transgenic mice exhibited a 30–50% postnatal growth reduction. However, hyperphosphatemia and the reduced serum alkaline phosphatase levels in these mice confounded the potential direct effects of STC in the growth plate. Because the rate of longitudinal bone growth depends primarily on the rate of growth plate chondrogenesis, we evaluated the effects of rhSTC on the main processes responsible for chondrogenesis, growth plate chondrocyte hypertrophy/differentiation and chondrocyte proliferation, and cartilage matrix synthesis. The addition of rhSTC decreased the height of hypertrophic zone and the expression of ColX, a marker of
terminally differentiated chondrocytes. 100 ng/ml rhSTC also suppressed growth plate chondrocyte proliferation, as assessed by BrdUrd incorporation and histology. In addition, STC-treated metatarsal bones and cultured chondrocytes exhibited increased apoptosis, implying a role for STC1 in the regulation of programmed cell death. The STC-mediated increased expression of caspase 3 and caspase 9 (two proapoptotic molecules) in cultured chondrocytes confirmed STC as a modulator of chondrocyte apoptosis. Last, collagen II expression and [35S]sulfate incorporation (both in whole metatarsals and cultured chondrocytes) was decreased by rhSTC, indicating that the STC-mediated decreased growth plate formation is in part due to suppressed cartilage matrix synthesis.

To our knowledge, our study is the first one to have addressed the functional role of STC1 in growth plate chondrogenesis and longitudinal bone growth. In a previous study, rhSTC was shown to accelerate osteoblast differentiation in fetal rat calvaria cell cultures (10). These apparently discordant results may depend on the cell type and stage of differentiation as well as on the culture conditions. In addition, calvarial bones form by intramembranous ossification, a process not involved in longitudinal bone growth. Our organ culture model (the whole metatarsal bone) maintains the same spatial organization seen in the intact growth plate, with chondrocytes at different developmental stages interacting with one another and with the extracellular matrix as they do during growth plate chondrogenesis. Thus, our findings relative to the rhSTC effects on metatarsal longitudinal growth and growth plate chondrogenesis may closely reflect the activity of endogenous STC1 within the growth plate.

STC was originally isolated from holostean and teleostean fishes, where it prevents calcium entry from the gills and intestine and, thus, functions as a very potent antihypercalcemic hormone. In mammals, STC1 seems to have a preferential effect on phosphate metabolism. When injected into rats, rhSTC reduced renal phosphate excretion and had no effect on calcium excretion (8). In vitro studies showed that rhSTC increased phosphate uptake by the swine and rodent serosal intestinal surface (9) and by a neural crest-derived cell line (34). In addition, transgenic mice overexpressing STC1 exhibited elevated serum phosphate levels more consistently than elevated calcium levels (20). In light of these lines of evidence, we hypothesized that the STC1-mediated inhibition of metatarsal longitudinal growth and growth plate chondrogenesis were due to modifications of chondrocyte phosphate transport. Indeed, isolated metatarsal chondrocytes exposed to rhSTC increased Pi uptake. Co-treatment of isolated chondrocytes with rhSTC and PFA (an inhibitor of phosphate transport) reversed the rhSTC stimulatory effect on phosphate uptake and apoptosis and the rhSTC inhibitory effect on chondrocyte proliferation and extracellular matrix synthesis. More importantly, PFA reversed the STC-mediated suppression of metatarsal longitudinal growth, growth plate chondrocyte proliferation and hypertrophy, and extracellular matrix synthesis.

**FIGURE 7. Effects of rhSTC on chondrocyte Pi uptake and Pit1 and FGF23 mRNA expression.**

A, chondrocytes in 70–80% confluence were washed with fresh serum-free medium containing 0.1% BSA incubated with/without rhSTC (100 ng/ml) or rhSTC plus PFA (0.1 mM) and cultured for additional 1 or 2 days. Pi uptake was measured as described under “Experimental Procedures.” Pi uptake was determined by liquid scintillation counting. The data are expressed as cpm/10³ cells (n = 6 samples obtained from 2 experiments; * p < 0.01, ** p < 0.01). B, effects of rhSTC and PFA on metatarsal longitudinal growth. Fetal rat metatarsals (days post-coitus 20) were cultured for 3 days in serum-free MEM with or without 100 ng/ml rhSTC or with 100 ng/ml rhSTC plus 0.3 mM PFA (n = 6/group). Bone length was measured daily using an eyepiece micrometer in a dissecting microscope. C, reverse transcription-PCR analysis of Pit-1 and FGF23 mRNA expression in the metatarsal bones. Total RNA was extracted from the growth plates of 18 metatarsal bones treated with or without rhSTC and then reverse-transcribed to cDNA. The housekeeping gene β-actin was used as normalization control. PCR products were separated by electrophoresis in a 2% agarose gel with ethidium bromide.
and the STC-mediated induction of chondrocyte apoptosis. All these findings indicate that the mechanisms through which STC1 inhibits longitudinal bone growth and growth plate chondrogenesis depend on the increased chondrocyte phosphate uptake and, in turn, intracellular phosphate concentration. It has been previously reported that inorganic phosphate induces apoptosis of osteoblast-like cells (35) and chondrocytes (36–40). All the studies conducted in cultured chondrocytes, with one exception (40), have consistently shown that elevated intracellular phosphate induces apoptosis. In a study on ADTC5 cells (a chondrogenic cell line), treatment with P, and Ca\(^{2+}\) led to a decrease in the Bcl-2/Bax ratio, which is believed to disrupt the mitochondrial membrane and promote release of mitochondrial components, irreversibly engaging the cell toward apoptosis (39). In support of the central role of an altered mitochondrial permeability in the phosphate-mediated chondrocyte apoptosis (and consistent with our findings), Sabbagh et al. (41) have recently demonstrated that treatment of primary mouse chondrocytes with high concentrations of phosphate induced the expression of caspase 9, a key enzyme in the mitochondrial apoptotic pathway. The negative regulation of Chiba (a transcription factor known to promote chondrocyte proliferation and differentiation) (42, 43) by elevated phosphate in cultured ATDC5 cells (44) would implicate a similar mechanism underlying the STC-mediated inhibition of growth plate chondrocyte proliferation and differentiation observed by us.

Our findings also indicate that the increased chondrocyte phosphate uptake caused by rStSTC is associated with the increased expression of Pit-1 (type III Na\(^{+}\), co-transporter) in the metatarsal growth plate. Consistent with our finding, Yoshiko et al. (10) have previously demonstrated that rSTC induces the expression of Pit-1 in rat osteoblasts. Along with STC1, FGF23 plays an important role in the regulation of phosphate metabolism in mammals. FGF23 has been shown to induce phosphaturia, likely by inhibiting renal Na\(^{+}\), K co-transporter activity (45). Initially identified in most tumors from patients with tumor-induced osteomalacia, the expression of FGF23 has been subsequently detected in many tissues including heart, liver, parathyroid, brain, thymus, and bone (46, 47). Our study also demonstrates the expression of FGF23 in growth plate chondrocytes. In light of its effects in the renal epithelial cells, it is plausible that FGF23 in the growth plate may reduce the activity of Pit-1 and, in turn, inhibit chondrocyte phosphate uptake. Thus, our findings support the hypothesis of a local regulation of phosphate metabolism in growth plate chondrocytes by STC1 and FGF23. STC1 may induce chondrocyte phosphate uptake directly by increasing the expression of Pit-1 and indirectly by reducing the expression of FGF23. In conclusion, our study indicates that STC1 inhibits longitudinal bone growth by suppressing growth plate chondrocyte proliferation, hypertrophy, cartilage matrix synthesis, and by inducing chondrocyte apoptosis. Such effects on chondrocyte function are likely due to an STC-mediated increased intracellular phosphate content. The expression of both STC1 and its binding site in the growth plate would support an autocrine/paracrine role for this growth factor in the regulation of growth plate chondrogenesis. Future studies regarding the regulation of STC1 expression and its interaction with other growth factors in the growth plate will help to further elucidate the physiological role of STC1 in bone physiology.

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