Role of Cholesterol in the Regulation of Growth Plate Chondrogenesis and Longitudinal Bone Growth*

Received for publication, May 27, 2003, and in revised form, November 5, 2003
Published, JBC Papers in Press, November 11, 2003, DOI 10.1074/jbc.M305518200

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Inborn errors of cholesterol synthesis are associated with multiple systemic abnormalities, including skeletal malformations. The regulatory role of cholesterol during embryogenesis appears to be mediated by Shh, a signaling molecule in which activity depends on molecular events involving cholesterol. Based on this evidence, we hypothesized that cholesterol, by modifying the activity of Ihh (another of the Hedgehog family proteins) in the growth plate, regulates longitudinal bone growth. To test this hypothesis, we treated rats with AY 9944, an inhibitor of the final reaction of cholesterol synthesis. After 3 weeks, AY 9944 reduced the cumulative growth, tibia growth, and the tibia growth plate height of the rats. To determine whether cholesterol deficiency affects bone growth directly at the growth plate, we cultured fetal rat metatarsal bones in the presence of AY 9944. After 4 days, AY 9944 suppressed metatarsal growth and growth plate chondrocyte proliferation and hypertrophy. The inhibitory effect on chondrocyte hypertrophy was confirmed by the AY 9944-mediated decreased expression of collagen X. Lastly, AY 9944 decreased the expression of Ihh in the metatarsal growth plate. We conclude that reduced cholesterol synthesis in the growth plate, possibly by altering the normal activity of Ihh, results in suppressed longitudinal bone growth and growth plate chondrogenesis.

A large of body of evidence supports the notion that cholesterol is essential for normal vertebrate embryogenesis (1, 2). The identification of Smith-Lemli-Opitz syndrome (SLOS) (3, 4), a classic autosomal recessive syndrome, as a congenital defect of cholesterol biosynthesis has subsequently led to the discovery of other dysmorphic syndromes, in humans and in mutant mice, caused by inborn errors of the cholesterol synthetic pathway (5). These syndromes are characterized by multiple malformations, including central nervous system and skeletal anomalies. The current hypothesis is that cholesterol plays its role in normal embryogenesis by modifying embryonic signaling proteins. The normal expression of Sonic hedgehog (Shh), one of the Hedgehog family proteins, is involved in the patterning of several tissues, including brain and spinal cord, axial skeleton, and limbs (6). To be activated, Shh undergoes an auto-processing reaction that entails internal cleavage and leads to an amino-terminal product that is responsible for the molecule function (7, 8). This process requires cholesterol, which binds covalently to the amino-terminal domain of Shh and allows it to anchor to the membrane of the secreting cells (9, 10).

Along with skeletal malformations, individuals with inborn errors of cholesterol synthesis typically experience post-natal growth failure (2), which raises the question whether cholesterol deficiency adversely affects longitudinal bone growth after embryogenesis is completed. In mammals, longitudinal bone growth occurs at the growth plate of the long bone by endochondral ossification, a two-step process in which cartilage is first formed and then remodeled into bone (11, 12). Growth plate chondrocyte proliferation and chondrocyte hypertrophy lead to formation of new cartilage, chondrogenesis (13). Simultaneously, the growth plate is invaded from the bony metaphysis by blood vessels and bone cell precursors, which remodel the cartilage into bone (14). Indian hedgehog (Ihh), another member of the Hedgehog family, is expressed in the growth plate where it controls chondrocyte proliferation and differentiation (15–19). Based on this evidence, we hypothesized that cholesterol has an important functional role during longitudinal bone growth and that such role is mediated by the activation of Ihh in the growth plate. To test our hypothesis, we first treated rats with AY 9944, an inhibitor of 7-dehydrocholesterol reductase, which is the enzyme that catalyzes the final reaction of cholesterol synthesis. After 3 weeks, we analyzed the effects of AY 9944 on the rat body and tibia growth. To determine whether cholesterol deficiency directly affects longitudinal bone growth, we cultured whole rat metatarsal bones in the presence of AY 9944 and evaluated its effects on metatarsal linear growth, on metatarsal chondrocyte proliferation and chondrocyte hypertrophy/differentiation, and on the expression of Ihh in the metatarsal growth plate.

EXPERIMENTAL PROCEDURES

Animal Care—For the in vivo study, Sprague-Dawley rats (4-week-old) were housed under standard conditions with a 12-h light/dark cycle (darkness from 7:00 p.m. to 7:00 a.m.). The animals (n = 12/group) received a daily dose of AY 9944 (25 mg/kg, dissolved in distilled water) for 3 weeks by gavage. The control rats received vehicle only (distilled water). Weight and whole body length (from nose to anus) were measured weekly after the animals were sedated with an injection of ketamine hydrochloride (0.02 mg/100 g body weight) (Phoenix Pharmaceutical, Inc.) and acepromazine (0.1 mg/100 g body weight) (Roche Applied Science). Serial radiographs of the tibiae were obtained weekly, with each animal placed prone on an x-ray cassette. The lengths of both tibiae were measured on the radiograph, and the average value was calculated. After 21 days (3 weeks) of treatment, blood was collected by ventricular puncture from each animal before sacrificing them by CO2.

Organ Culture—The second, third, and fourth metatarsal bone rudiments were isolated from Sprague-Dawley rat embryos (20 dpc) and
cultured individually in 24-well plates (20, 21). Each well contained 0.5 ml of minimum essential medium (MEM) (Invitrogen) supplemented with 0.05 mg/ml ascorbic acid (Invitrogen), 1 mM sodium glycerophosphate (Sigma), 0.2% bovine serum albumin (Sigma), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen), and AY 9944 (1–5 μg/ml) orLovastatin (10 μM) (an inhibitor of hydroxymethylglutaryl-coenzyme A reductase, the rate-limiting enzyme of cholesterol synthesis). The plates were incubated in a humidified incubator with 5% CO2 in air at 37 °C. The medium was changed every other day.

**Measurement of Longitudinal Growth**—The length of each bone rudiment was measured every day under a dissecting microscope using an eyepiece micrometer. The culture medium was briefly removed immediately before each measurement.

**[^3H]Thymidine Incorporation**—To assess cell proliferation, we measured[^3H]thymidine incorporation into newly synthesized DNA (22). After 4 days of culture,[^3H]thymidine was added to the culture medium at a concentration of 5 μCi/ml (specific activity, 25 Ci/mmol) (Amer- sham Biosciences), and the rudiments were incubated for an additional 3 h.

To analyze the sites of the growth plate where DNA synthesis occurred, we incubated the control and the AY 9944-treated bones with[^3H]thymidine as described above. At the end of the incubation, all bones were fixed in buffered formalin. Longitudinal sections (5 μm thick) were hybridized to

**In Situ Hybridization**—In situ hybridization was performed as described by Pelton, et al. (23). Metatarsals were fixed overnight in 4% paraformaldehyde at 4 °C and then dehydrated in ethanol and embedded in paraffin. Longitudinal sections (5 μm thick) were hybridized to

** Autoradiography**—Bone rudiments were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Three 5-μm sections were obtained and cleaned of paraffin in xylene and rehydrated in graded ethanol. The sections were incubated in 1% H2O2 for 10 min to inactivate endogenous peroxidase. After pre-incubation with 0.1% trypsin for 12 min was used at room temperature followed by a triple wash in phosphate-buffered saline. After pre-incubation with 1.5% blocking serum for 30 min at room temperature, the sections were incubated for 30 min at room temperature with goat IgH polyclonal antibody (1:50) (Santa Cruz Biotechnology II, Santa Cruz, CA) or rabbit polyclonal antisera raised against mouse type X collagen (Abcam, Cambridge, MA) following by an incubation for 30 min at room temperature with goat anti-rabbit or goat anti-goat conjugated with horseradish peroxidase. The sections were then visualized with peroxi- dase substrate for 5 min. After counterstaining in hematoxylin solution for 30 s, the sections were mounted with Permount medium. Control experiments were done by omission of the primary antibody.

**Quantitative Histology**—For the in vivo study, tibiae were removed and fixed with 4% paraformaldehyde overnight, decalcified with Decal- cifier II (Surgipath, Richmond, IL) for 1 h, and paraffin-embedded.

**Fig. 1.** Effects of systemic administration of AY 9944 on body size. 4-week-old rats were treated with either vehicle (left) or oral AY 9944 (right) for 3 weeks.

**Fig. 2.** Effects of systemic administration of AY 9944 on cumulative body and tibial growth in rats. 4-week-old rats (n = 12/group) were treated with either vehicle or oral AY 9944 for 3 weeks. A, body length (from nose to anus) was measured weekly, and cumulative growth was calculated at the end of the 3-week period. B, radiographs of the tibiae were obtained weekly, with the lengths of both tibiae measured on the radiograph and the average value calculated. Cumulative growth was calculated at the end of the 3-week period.
Three 5–7-μm thick longitudinal sections were obtained and stained with hematoxylin/phloxine/saffron. The effects of AY 9944 on the tibial growth plate were evaluated by measuring the heights (μm) of the whole growth plate, the proliferative zone, and the hypertrophic zone of the growth plate. All measurements were performed by a single observer blinded to the treatment regimen.

For the organ culture study, at the end of the culture period, metatarsals were fixed in 4% paraformaldehyde overnight. After routine processing, three longitudinal 5-μm sections were obtained from each metatarsal bone and stained with toluidine blue. From each of the three sections, the heights of the epiphyseal zone, proliferative zone, hypertrophic zone, and of the ossification center were measured, and the average value was calculated. In the 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 long axis of the bone. In the hypertrophic zone, large cells (defined by a height of ≥9 μm) form a layer adjacent to the calcified region of the metatarsal bone, the ossification center. All quantitative histology was performed by a single observer blinded to the treatment category.

Statistics—All data are expressed as the mean ± S.E. Statistical significance was determined by Student’s t test or by analysis of variance.

RESULTS

Effects of AY 9944 on Animal Body Growth and Tibial Growth—Rats given 25 mg/kg AY 9944 daily for 21 days gained significantly less weight than the control rats (121.0 ± 7.25 versus 75.5 ± 7.52 g; control versus AY 9944, mean ± S.E., p < 0.001). At the beginning of the experimental period, the mean body and tibial lengths of the control and treated groups were not statistically different.

After 21 days, AY 9944 caused a significant reduction of the cumulative body growth (3.33 ± 0.22 versus 3.96 ± 0.22 cm; control versus AY 9944, mean ± S.E., p < 0.001) (Figs. 1 and 2A) and of the tibial growth (0.92 ± 0.04 versus 0.72 ± 0.03 cm; control versus AY 9944, mean ± S.E., p < 0.001) (Fig. 2B). The inhibitory effect on the tibial growth was already significant after 7 days of AY 9944 administration (Fig. 2B). When examined histologically, the growth plates of the AY 9944-treated rats appeared thinner than those of the control rats (Fig. 3). AY 9944 caused a significant decrease in the height of the growth plate hypertrophic zone (311 ± 25 versus 184 ± 20 μm; control versus AY 9944, mean ± S.E., p < 0.001) and of the whole growth plate (576 ± 29 versus 403 ± 38 μm; control versus AY 9944, mean ± S.E., p < 0.001). The height of the proliferative zone was not significantly affected by the AY 9944 treatment. Lastly, at the end of the experimental period, the mean plasma cholesterol level of the AY 9944-treated group was significantly lower than that of the control group (68.4 ± 2.85 versus 20.2 ± 3.86 mg/dl, control versus AY 9944, mean ± S.E., p < 0.001).

Effects of AY9944 and Lovastatin on Metatarsal Bone Growth—Metatarsal bones from rat embryos (20 dpc) were cultured in serum-free medium containing graded concentrations of AY 9944 for 4 days. At the beginning of the experimental period, the mean lengths of the control and treated groups were not statistically different. After 4 days, AY 9944 caused a significant concentration-dependent inhibition of longitudinal bone growth (n = 35–54/group) (A) or lovastatin (0 control or 10 μM) (n = 30/group) (B). Bone length was measured daily using an eyepiece micrometer in a dissecting microscope. p < 0.001 (versus control).

Effects of AY9944 on Growth Plate Chondrogenesis—Because the rate of longitudinal bone growth depends primarily on the rate of growth plate chondrogenesis, we evaluated the effects of AY 9944 on chondrocyte proliferation and chondrocyte hypertrophy/differentiation, the two main components of growth plate chondrogenesis. To prevent the occurrence of toxic effects, we cultured the bone rudiments in the presence of the lowest inhibitory concentration of AY 9944 on metatarsal growth, 2 μg/ml. To determine the site of the growth plate in which chondrocyte proliferation occurred, we performed auto-
radiography of [3H]thymidine-labeled bones. 2 μg/ml AY 9944 caused a significant decrease of the labeling index (number of labeled cells/total cells) in the epiphyseal and the proliferative zones (n = 5–6/group, p < 0.01) (Figs. 5 and 6). Consistent with the reduced number of proliferating chondrocytes, treatment with AY 9944 significantly decreased the height of both the epiphyseal (n = 8–11/group, p < 0.01) (Fig. 7) and proliferative (n = 8–11/group, p < 0.001) (Fig. 7) zones. We then evaluated the effects of AY 9944 on chondrocyte hypertrophy/differentiation by assessing the expression of collagen X (a marker of chondrocyte differentiation) in the growth plate by in situ hybridization and immunohistochemistry. In the control metatarsal bones, type X collagen protein and mRNA expression were primarily observed in the hypertrophic zone of the growth plate (Fig. 8, A–C). Treatment with AY 9944 resulted in a dramatic decrease in the expression of both protein and mRNA (Fig. 8, B–D). When quantitative histology was performed, the height of the hypertrophic zone in the AY 9944-treated bones was significantly lower than that of the control (n = 8–11/group, p < 0.01) (Fig. 7). Lastly, we measured the length of the ossification center of the metatarsal bones at the end of the 4-day culture period. In contrast to the effects exerted on the growth plate, AY 9944 did not significantly affect the length of the metatarsal ossification center (data not shown).

Effects of AY 9944 on the Expression of Ihh Protein—To evaluate the effects of AY 9944 on Ihh expression, immunohistochemical studies were performed on the metatarsal bones. In the control bones, the expression of Ihh protein was mainly detected in the lower proliferative and hypertrophic zones (Fig. 9A). In the AY 9944-treated bone rudiments, such expression was markedly reduced (Fig. 9B).

DISCUSSION

Systemic administration of AY 9944 (a distal inhibitor of cholesterol synthesis) to rats induced a significant reduction of their cumulative body and tibial growth. Previous studies have shown that AY 9944 is highly teratogenic in rats. In fetuses of treated dams, AY 9944 caused holoprosencephaly, growth retardation, and multiple skeletal malformations. SLOS, a genetic syndrome caused by the deficiency of 7-dehydrocholesterol reductase (the enzyme inhibited by AY 9944), is also characterized by variable degrees of holoprosencephaly, intrauterine growth retardation, cranio-facial anomalies, and limb malformations. Interestingly, most of the malformations observed in individuals with SLOS and in AY 9944-treated rats have been described in mice in which Sonic hedgehog (Shh), a key developmental gene, has been knocked out. Subsequent experiments have shown that AY 9944-induced malformations in rodent embryos are indeed caused by disruption of the Shh signaling cascade. It has been recently demonstrated that the activity of Shh depends on post-translational events through which the amino-terminal signaling part of the Shh protein is covalently linked to cholesterol, and such binding gives Shh a
strong affinity for cell membranes (26). Based on this evidence, it is likely that most of the embryonic anomalies associated with cholesterol deficiency (AY 9944-mediated or because of inborn errors of cholesterol synthesis) are secondary to altered Shh activity.

Our finding of reduced body and tibial growth in rats given AY 9944 would suggest that cholesterol deficiency affects skeletal growth even after embryogenesis is completed. However, systemic suppression of cholesterol synthesis may impair longitudinal bone growth either directly (cholesterol deficiency in the growth plate) or indirectly via the reduced synthesis of cholesterol-derived systemic factors such as steroid hormones.

To determine whether cholesterol regulates longitudinal bone growth locally at the growth plate, we used an organ culture model, the fetal rat metatarsal bone. The metatarsal bone, which comprises two cartilaginous regions resembling the mature growth plate, is a model that enabled us to assess the effects of AY 9944 on longitudinal bone growth as well as on the two main processes responsible for such growth, growth plate chondrocyte proliferation and chondrocyte hypertrophy/differentiation. In this model, AY 9944 inhibited metatarsal longitudinal growth. Similar growth inhibition was observed in metatarsal bones cultured with lovastatin, which, unlike AY 9944, inhibits an early step of cholesterol synthesis.

These findings, which are consistent with the effects elicited by AY 9944 in vivo, suggest that impaired cholesterol synthesis in the growth plate is responsible for suppressed longitudinal bone growth. AY 9944 suppressed growth plate chondrocyte proliferation as assessed by autoradiography and histology. In addition, AY 9944 reduced the height of the growth plate hypertrophic zone as assessed by histological studies. The cellular enlargement of the growth plate chondrocytes adjacent to the bone ossification center is critical for longitudinal bone growth (17, 18). In the AY 9944-treated bone rudiments, the decreased expression of collagen X (a marker for terminally differentiated chondrocytes) (16) confirmed the AY 9944-mediated inhibition of growth plate chondrocyte hypertrophy/differentiation. A narrowed hypertrophic zone can be due to either a decreased number of proliferating cells undergoing hypertrophy/differentiation or to an increased number of hypertrophic cells being replaced by bone tissue. In our study, the lack of any effect of AY 9944 on the length of the metatarsal ossification center renders the latter possibility unlikely.

To determine the molecular mechanisms by which cholesterol deficiency leads to reduced longitudinal bone growth and growth plate chondrogenesis, we evaluated the expression of Ihh in the AY 9944-treated metatarsal bones. In our system, AY 9944 inhibited Ihh expression. Ihh, along with Shh and Dhh, belongs to a family of morphogens involved in embryonic patterning, limb bud development, and endochondral bone formation (17). During a post-embryonic phase, Ihh is localized in the growth plate where it regulates chondrocyte proliferation

**FIG. 8. Expression of collagen X in control (A–C) and AY 9944-treated (B–D) metatarsal bones.** Upper panels show immunolocalization of collagen X protein using a rabbit polyclonal antibody directed against mouse collagen X at a dilution of 1:200. Brown staining (arrow) shows the collagen X protein. Lower panels show in situ hybridization for collagen X mRNA. Black staining (arrow) indicates collagen X mRNA expression in the growth plate hypertrophic zone.

**FIG. 9. Expression of Ihh protein in control (A) and AY 9944-treated (B) metatarsal bones.** At the end of the culture period (4 days), the metatarsal bones were embedded in paraffin. After routine processing, immunohistochemistry was performed using a goat Ihh polyclonal antibody at a dilution of 1:50. Brown staining (arrow) shows the Ihh protein.
and hypertrophy. Ihh null mice display markedly reduced chondrocyte proliferation and delayed and abnormal chondrocyte hypertrophy/differentiation (17). Based on this evidence, we speculate that reduced cholesterol synthesis in the metatarsal bones because of AY 9944 treatment suppresses Ihh expression and, in turn, chondrocyte proliferation and hypertrophy/differentiation. In light of the known role of cholesterol in Shh activation, it is conceivable that cholesterol may also be essential for the activation of Ihh and its anchorage to the cell membrane. Along with decreased cholesterol synthesis, decreased activity of different enzymes involved in cholesterol deficiency, rather than the accumulation of precursor(s), is the primary cause of the inhibitory action of AY 9944 on embryonic development and longitudinal bone growth. Second, experimental evidence indicates that, in pregnant rats treated with AY 9944, accumulation of 7-dehydrocholesterol in the presence of normal levels of cholesterol is compatible with normal development (1) and normal Shh signaling (27). Third, in individuals with SLOS there is a poor correlation of clinical dysplasia with normal Shh signaling (27). Third, in individuals with SLOS (Supplemental Longitudinal Osteodysplasia Syndrome) leads to accumulation of 7-dehydrocholesterol, the immediate precursor of cholesterol in its synthetic pathway. Thus, the increased levels of this precursor could potentially be responsible for the effects elicited by AY 9944. On the other hand, there are several arguments in favor of the theory that decreased activity of different enzymes involved in cholesterol synthesis (because of either chemical inhibitors like lovastatin or inborn errors of metabolism) (1, 5) have similar effects on embryonic development and bone growth. First, decreased activity of different enzymes involved in cholesterol synthesis (because of either chemical inhibitors like lovastatin or inborn errors of metabolism) (1, 5) have similar effects on embryonic development and bone growth. Second, experimental evidence indicates that, in pregnant rats treated with AY 9944, accumulation of 7-dehydrocholesterol in the presence of normal levels of cholesterol is compatible with normal development (1) and normal Shh signaling (27). Third, in individuals with SLOS there is a poor correlation of clinical severity with the levels of 7-dehydrocholesterol but a strong inverse correlation with the level of cholesterol (2).

In conclusion, our findings indicate that cholesterol, possibly mediated precursor of cholesterol in its synthetic pathway. Thus, the increased levels of this precursor could potentially be responsible for the effects elicited by AY 9944. On the other hand, there are several arguments in favor of the theory that decreased activity of different enzymes involved in cholesterol synthesis (because of either chemical inhibitors like lovastatin or inborn errors of metabolism) (1, 5) have similar effects on embryonic development and bone growth. Second, experimental evidence indicates that, in pregnant rats treated with AY 9944, accumulation of 7-dehydrocholesterol in the presence of normal levels of cholesterol is compatible with normal development (1) and normal Shh signaling (27). Third, in individuals with SLOS there is a poor correlation of clinical severity with the levels of 7-dehydrocholesterol but a strong inverse correlation with the level of cholesterol (2).

Acknowledgments—We thank Dr. Olsen for providing the mouse type X collagen mRNA probe, Dr. Lunstrum for the rabbit polyclonal anti-

sera raised against mouse type X collagen peptides, and Wyeth-Ayerst Laboratories for AY 9944.

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