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A Rat Model of Thyroid Hormone-Induced Bone Loss: Effect of Antiresorptive Agents on Regional Bone Density and Osteocalcin Gene Expression

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ABSTRACT

Thyroid hormone has been shown to stimulate bone resorption. Both endogenous hyperthyroidism and exogenous thyroxine suppressive therapy have been associated with reduction in bone mineral density (BMD), but the pathophysiology of thyroxine-induced bone loss is not well understood. First we studied the effect of L-T_4 (0.1–0.3 μg/g body weight ip/day) on bone turnover in rats by measuring regional BMDs and osteocalcin mRNA. Next we determined whether antiresorptive agents (calcitonin 1 μU/g ip/day or sodium etidronate given cyclically at 10 μg/g po for 3 consecutive days out of every week) could prevent bone loss. Groups of 10 male Sprague–Dawley rats each weighing 320–350 g were studied before and after 3 weeks of treatment. L-T_4 treatment resulted in reduction in BMDs in the lumbar spine, tail, and femur as measured by dual energy X-ray absorptiometry, but there was no correlation with the dosage of L-T_4 or the serum T_4 level. Treatment with sodium etidronate or calcitonin alone did not alter the regional BMD. Cyclical sodium etidronate, but not calcitonin, was able to prevent the bone loss induced by L-T_4 treatment. L-T_4 caused a dose-dependent increase in femur osteocalcin mRNA concentration. Treatment with calcitonin resulted in 50% reduction of osteocalcin mRNA, but sodium etidronate had no effect. In conclusion, cyclical sodium etidronate prevents bone loss induced by exogenous L-T_4 in rats and may be useful in preventing osteoporosis in patients given long term TSH-suppressive doses of thyroxine therapy.

INTRODUCTION

THYROID HORMONES have been shown to regulate normal bone maturation, as evidenced by the fact that hyperthyroidism or hypothyroidism in childhood is associated with abnormal growth and stature (1,2). In adults, recent evidence shows that an excess of thyroid hormones affects the remodeling system in cortical and trabecular bone and may contribute to the development of osteoporosis (3). Thyroid hormone increases calcium release from fetal rat long bone cultures and increases osteoblast number and activity (4). *In vivo*, thyroid hormone also stimulates osteoblast activity (5). In hyperthyroidism the activation frequency of bone remodeling is increased, which results in an accelerated bone resorption and a concomitant increase in bone formation, with a marked shortening of both resorptive and formative phases of the remodeling cycle (6). This results in a net reduction of bone mass. A number of studies confirmed that patients suffering from hyperthyroidism had lower bone mineral content at both cortical and trabecular bone (7). Furthermore, decrease in bone mineral densities (BMD) was similarly observed in subjects on physiologic or suppressive dosage of L-thyroxine treatment in some studies but not in others (8,9).

It is still debatable whether adjunctive therapy is warranted to prevent rapid bone loss during thyroxine therapy, especially in postmenopausal women and older individuals who present with symptomatic or severe bone loss. Antiresorptive agents such as calcitonin and the diphosphonates that act by inhibiting osteoclast activity have been shown to prevent bone loss in patients with postmenopausal osteoporosis (10–13). The present study describes an animal model of thyroxine-induced bone loss and investigates whether these antiresorptive agents could prevent the decrease in BMD.

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MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats of 8–10 weeks (320–350 g body weight) were chosen for study. They were housed in a room with controlled temperature (22 ± 1°C) and humidity (65–70%). They were fed with normal diet (Ralston Purina) and allowed free access to tap water.

Experimental design

Groups of 10 rats each were treated for 3 weeks accordingly. Control rats received daily intraperitoneal (ip) injection of normal saline. Hyperthyroidism was induced by giving levothyroxine (L-T_4, Sigma Chemical Co.) at doses of 0.1–0.3 mg/kg body weight ip daily, and hypothyroidism was induced by giving propylthiouracil (Sigma) as 0.1% (w/v) solution per os (po). As for antiresorptive therapy, salmon calcitonin (Miacalcic, Sandoz Pharmaceutical, Switzerland) was given at 1 U/kg ip daily, and disodium etidronate (Didronel, Norwich Eaten, Austria) was given cyclically at a dose of 10 mg/kg po for 3 consecutive days per week. The dosage and regimen of the antiresorptive therapy were similar to those applied to human studies.

Bone mineral density measurement

Animals were anesthetized with sodium pentobarbital (Abbott), 50 mg/kg injected ip and did not move during scanning. Total body and regional bone mineral densities (BMD) were measured by the small animal scan program of Norland XR-26 bone densitometer (Fort Atkinson, Wisconsin), which is operated by the principle of dual energy X-ray absorptiometry (14). The accuracy and precision of estimating BMD in total animal *in vivo* were found to be 1%. An ultrastable X-ray source, operated at 100 kV and 1 mA, was used to provide the X-ray beam that was then K-edged (46.8 keV) by the samarium filtering module. This resulted in production of monochromatic X-ray beams with two energies at approximately 45 and 80 keV. The dual energy beam was then scanned rectilinearly over the region of interest. The BMD was measured on a pixel-by-pixel basis and was calculated by a series of given iterations with an IBM PS-2 computer. The specially designed collimator and a special software were incorporated for the scanning of small animals. Bone scans were done on each animal for the total body, lumbar spine, tail, and right femoral region (Fig. 1). Regional BMDs were performed within a fixed area to assist in repeatable placement of cursors on follow-up scans. Bone densities were measured before and after 3 weeks of treatment. The whole set of experiment was carried out 3 times, each time with the same experimental design.

RNA extraction and hybridization

Rat femur was snap frozen in liquid nitrogen and RNA was extracted with guanidinium thiocyanate immediately after the muscle and periosteum were removed. The single step acid guanidinium-phenol-chloroform method was used to isolate the total cellular RNA (15). An oligonucleotide (5′-AGAGAGAGGGTCCTCATGGT-3′) was synthesized corresponding to the complementary sequences of the bases 1–20 of the rat osteocalcin cDNA (16). The oligonucleotide probe was 5′ end-labeled with [γ-^{32}-P]ATP and T_4 polynucleotide kinase (Promega Corporation, Madison, WI). Hybridization was carried out overnight in 5 × SSC (0.3 *M* sodium chloride–0.03 *M* sodium citrate), 20 m*M* phosphate pH 7.0, 10 × Denhardt solution, 7% sodium dodecyl sulfate (SDS), and 10% dextran sulfate at 62°C. Washing of the membrane was performed in 3 × SSC, 25 m*M* PO_4, pH 7.5, 10 × Denhardt solution, 5% SDS for 1 h at 57°C and then one wash in 1 × SSC, 1% SDS. The size of the rat osteocalcin mRNA was previously determined to be approximately 0.6 kb. The hybridization signal was ascertained by scanning densitometry in an LKB instrument (Rockville, MD). The amount of total RNA loaded per lane was normalized to the amount of 28 S RNA, which was determined by staining the membrane with methylene blue.

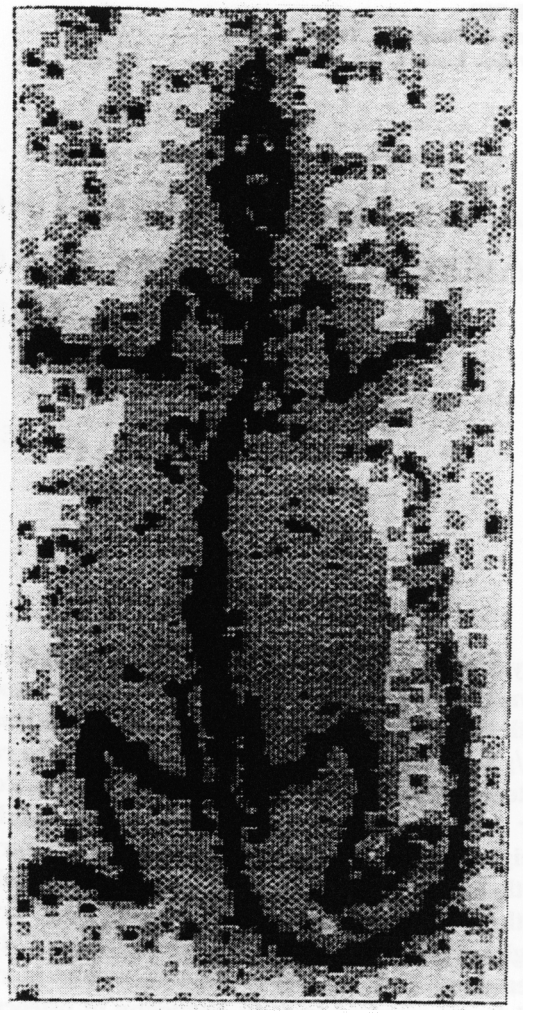

FIG. 1. Bone scan of the rat as determined by dual energy X-ray absorptiometry.

Thyroid function

Blood was collected at termination of the experiment by cardiac puncture, and sera were stored at −20°C until batch

TABLE 1. THYROID FUNCTION TESTS OF THE RATS[a]

Group	Serum T_4 (μg/dL)	TSH (ng/mL)
Control	5.5 ± 1.2	0.43 ± 0.26
T_4 0.1 mg/kg	8.7 ± 2.3*	<0.05*
T_4 0.2 mg/kg	16.2 ± 3.6*	<0.05*
T_4 0.3 mg/kg	20.8 ± 6.9*	<0.05*
Propylthiouracil	<1.0*	3.6 ± 0.65*

[a]Results are mean ± SD; $n = 10$ in each group.
*$p < 0.05$ vs control.

assay. Serum T_4 and TSH levels were measured by radioimmunoassay. Tracer and antibodies were obtained from ICN Biomedicals Inc. (Costa Mesa, CA).

Statistics

Comparison between groups was analyzed by one-way analysis of variance, and $p < 0.05$ was considered as significant. Spearman's rank correlation was used to determine the relation between the dose and duration of L-T_4 treatment with the regional BMDs.

RESULTS

Effect of L-T_4 on BMD

At baseline, all groups had similar body weight, total body bone mineral content (BMC), and regional BMDs. The mean total body BMC was 9.406 ± 0.216 (SD) g, femur BMD was 0.117 ± 0.002 g/cm^2, spine BMD was 0.113 ± 0.003 g/cm^2, and tail BMD was 0.106 ± 0.004 g/cm^2. After 3 weeks, body weight increased by 30% from 330 ± 15 g to 437 ± 95 g in the control rats. Total body BMC also increased by 11% to 10.478 ± 0.263 g. There was no significant change in linear growth (week 0, 24.69 ± 0.80 cm versus week 3, 25.80 ± 0.56 cm). L-T_4 treatment caused a dose-dependent elevation in the serum total T_4 level (Table 1). All hyperthyroid rats had unmeasurable TSH levels. L-T_4 treatment resulted in significant reduction in BMDs of the lumbar spine, tail, and femur (Table 2). However, there was no correlation between the regional BMDs and the dosage of L-T_4 treatment or the serum T_4 level. Propylthiouracil treatment reduced serum T_4 level and also caused a significant decrease in body weight. A reduction in total body mineral content was seen but there was no significant change in the regional BMDs in these hypothyroid rats. However, when the results were adjusted for the body weight in these hypothyroid rats, the total body BMC as well as the BMDs of the femoral and lumbar spine but not the tail were significantly higher than the control rats [total body BMC, 28.17 ± 2.73 vs 23.12 ± 0.72 g/kg body weight (BW), $p < 0.05$; femur BMD, 0.382 ± 0.028 vs 0.316 ± 0.020 g/cm^2/kg BW, $p < 0.05$; spine BMD, 0.455 ± 0.055 vs 0.332 ± 0.020 g/cm^2/kg BW, $p < 0.05$; tail BMD, 0.299 ± 0.014 vs 0.265 ± 0.029 g/cm^2/kg BW, p = NS, respectively].

Effects of antiresorptive agent in BMDs

L-T_4 0.3 mg/kg BW was chosen to study the effect of calcitonin and sodium etidronate on regional BMDs. Both sodium etidronate and calcitonin alone had no effect on regional BMDs. After treatment for 3 weeks, cyclical sodium etidronate treatment was able to prevent the reduction in BMDs induced by L-T_4 treatment (Table 3). Although rats treated with L-T_4 and calcitonin had higher regional BMDs compared with L-T_4 treatment alone (p = NS), they still had lower BMDs when compared with controls ($p < 0.05$).

Osteocalcin mRNA

L-T_4 treatment caused a dose-dependent increase in femur osteocalcin mRNA concentration when compared with controls

TABLE 2A. BONE DENSITY IN HYPERTHYROID AND HYPOTHYROID RATS[a]

Group	BW (g)	Total body BMC (g)	Femur BMD (g/cm^2)	Spine BMD (g/cm^2)	Tail BMD (g/cm^2)
Control	437 ± 95	10.478 ± 0.263	0.137 ± 0.007	0.144 ± 0.009	0.124 ± 0.007
T_4 0.1 mg/kg	427 ± 83	10.450 ± 0.395	0.124 ± 0.004*	0.126 ± 0.006*	0.118 ± 0.006*
T_4 0.2 mg/kg	431 ± 54	10.279 ± 0.299	0.121 ± 0.004*	0.123 ± 0.010*	0.114 ± 0.005*
T_4 0.3 mg/kg	420 ± 68	10.391 ± 0.278	0.119 ± 0.008*	0.122 ± 0.010*	0.115 ± 0.008*
Propylthiouracil	407 ± 88*	10.006 ± 0.069*	0.136 ± 0.009	0.148 ± 0.010	0.120 ± 0.010

[a]*Results are mean ± SD; n = 10 in each group.*
*$p < 0.05$ vs control.

TABLE 2B. BONE DENSITY OF HYPOTHYROID RATS AFTER ADJUSTMENT FOR BODY WEIGHT[a]

	TBMC[b]/weight (g/kg)	Femur BMD/weight (g/cm^2/kg)	Spine BMD/weight (g/cm^2/kg)	Tail BMD/weight (g/cm^2/kg)
Hypothyroidism	28.17 ± 2.73*	0.382 ± 0.028*	0.455 ± 0.055*	0.299 ± 0.014
Control	23.12 ± 0.72	0.316 ± 0.020	0.332 ± 0.020	0.265 ± 0.029

[a]Results are mean ± SD.
[b]TBMC, total body bone mineral content.
*$p < 0.05$.

TABLE 3. EFFECT OF ANTI RESPORTIVE AGENT OF REGIONAL BMDS[a]

Group	*Femur BMD (g/cm^2)*	*Spine BMD (g/cm^2)*	*Tail BMD (g/cm^2)*
Control	0.137 ± 0.007	0.144 ± 0.007	0.124 ± 0.007
T_4 0.3 mg/kg	0.119 ± 0.008*	0.122 ± 0.010*	0.115 ± 0.008*
T_4 + sodium etidronate	0.131 ± 0.006	0.143 ± 0.010	0.119 ± 0.008
T_4 + calcitonin	0.123 ± 0.005*	0.131 ± 0.007*	0.112 ± 0.007*
Sodium etidronate	0.140 ± 0.008	0.142 ± 0.009	0.126 ± 0.006
Calcitonin	0.138 ± 0.008	0.142 ± 0.006	0.123 ± 0.009

[a]Results are mean ± SD.
*$p < 0.05$ vs control.

(Fig. 2). The densitometric results for T_4 0.1, 0.2, and 0.3 mg/kg were 110 ± 6, 129 ± 15, and 175 ± 20% of the control, respectively. In the hypothyroid rat osteocalcin mRNA concentration was reduced to 40 ± 10% of the control. Treatment with calcitonin reduced osteocalcin mRNA to 50% of the control, whereas disodium etidronate did not affect osteocalcin gene expression. Treatment of the rats with both L-T_4 0.3 mg/kg and calcitonin resulted in a steady state osteocalcin mRNA concentration of about 70% of the control (Table 4).

DISCUSSION

Our present data demonstrated that in rats exogenous thyroid hormone produced bone loss in the spine and femoral bones simulating its action in humans. Using the rat model for studies of bone metabolism is well documented, and models of human pathologies like aging (17), estrogen depletion (18), immobilization (19), and dietary calcium deficiency (20) have been established. The use of a noninvasive technique such as DEXA for determination of BMD has also been varified in rats, with data correlating strongly with those obtained by ashing (21,22). The pattern of bone loss in this rat model is similar to that observed in humans. It has been reported that thyroid hormone induced bone loss in appendicular bone in premenopausal women (23), while in postmenopausal subjects bone loss is observed in both appendicular and axial bone (24). The overall pattern of bone loss induced by exogenous thyroid hormone therapy is similar to that observed in patients with endogenous hyperthyroidism (25). Ongphiphadhanakul et al performed similar study and demonstrated that excessive L-T_4 therapy induced bone loss in the rat femur, except that they did not observe any change in the rat spine (26).

The pharmacopathology of thyroid hormone-induced bone loss is not well understood. Although both T_3 and T_4 dose dependently stimulate ^{45}Ca release from cultured fetal rat long bones *in vitro* (4), the effect is biphasic in mouse; higher doses of T_3 inhibit rather than stimulate calcium release (27). We observed that in patients on physiologic dose of L-T_4 replacement with normal thyroid function, the *Z* score of the femoral neck correlated negatively with the serum-free thyroxine index (FTI) (8), whereas in patients on L-T_4 suppressive therapy with elevated serum FTI, no correlation was observed between the serum thyroid function and the regional BMD values or their *Z* scores. This was also observed in our present hyperthyroid rat model, but the degree of bone loss did not correlate with the dose of L-T_4 given or the serum thyroid hormone level. Whether thyroid hormone also affects other growth factors that modulate bone remodeling awaits further confirmation. In the present

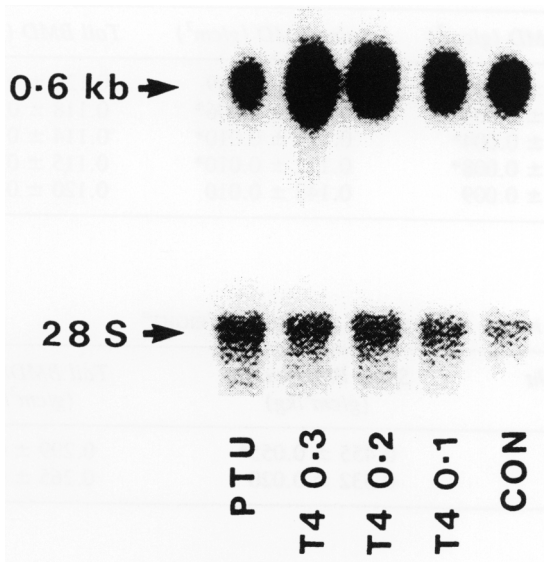

FIG. 2. Effect of thyroid hormone on osteocalcin mRNA level.

TABLE 4. EFFECT OF LT_4 AND ANTIRESORPTIVE AGENTS ON OSTEOCALCIN mRNA[a]

Group	*Densitometric unit (%)*
Control	100
T_4 0.1 mg/kg BW	110 ± 6
T_4 0.2 mg/kg BW	129 ± 15*
T_4 0.3 mg/kg BW	175 ± 20*
Calcitonin	50 ± 10*
T_4 0.3 mg/kg + calcitonin	70 ± 15
Sodium etidronate	90 ± 20
T_4 0.3 mg/kg + sodium etidronate	158 ± 16*

[a]Results are mean ± SD.
*$p < 0.05$ vs control.

study we also observed that in the hypothyroid rats, the adjusted regional BMDs were higher than controls. This finding has been reported in humans (28).

Thyroid hormone exerts its bone resorption activity via its action on the osteoblasts. Nuclear thyroid hormone receptors have been identified in rat osteosarcoma cell lines UMR-106 (29) and mouse osteoblast-like cell line MC3 T3-E1 (30). Also, human osteoblastic cell line MG63 has been shown to express T_3 receptor mRNA (31). Thyroid hormone receptors have not been identified in osteoclasts and there was no effect of T_3 on bone resorption by isolated osteoclasts (30). It is thus believed that the effect of thyroid hormone on osteoclastic bone resorption is indirectly mediated, and the osteoblast acts as the intermediary cell, thus resulting in a high turnover bone loss.

In this present rat model, we observed a positive relationship between serum T_4 level and osteocalcin mRNA expression. This finding has also been reported previously (32). Osteocalcin is a noncollagenous bone matrix protein synthesized by osteoblasts and has been a useful clinical marker for increased bone turnover (33). Serum osteocalcin is increased in hyperthyroid patients (34) and in patients with multinodular goitre with suppressed TSH levels (35). We also observed in patients on long-term L-T_4 suppressive therapy with elevated serum osteocalcin level, there was a negative correlation between the serum osteocalcin and the Z score of the regional BMDs (9). Thus estimation of osteocalcin may be a sensitive way of identifying which patient is at particular high risk of developing osteoporosis. However, our study has not addressed how thyroid hormone increases steady-state osteocalcin mRNA. Osteocalcin transcription is stimulated by 1,25-$(OH)_2$ D_3 (36), but thyroid hormone has not been shown to increase osteocalcin transcription in transfection studies (37). The action of thyroid hormone probably does not depend on vitamin D as hyperthyroidism is associated with a reduction in 1,25-$(OH)_2$ D_3 levels both in man (38) and rats (39).

In this study we also examined the protective effect of antiresorptive agents against L-T_4-induced bone loss. The diphosphonate agent, sodium etidronate, but not calcitonin, was able to prevent L-T_4-induced bone loss both in the femoral and spinal region. Interestingly, the increase in osteocalcin mRNA expression induced by L-T_4 was not altered by disodium etidronate treatment but was reversed by calcitonin. The suppressive effect of calcitonin is also reflected at the translational level as Ongphiphadhanakul et al. reported a decrease in serum osteocalcin level in rats treated with calcitonin (26). The diphosphonates are nonbiodegradable analogues of pyrophosphate with a high binding affinity for hydroxyapatite crystals (40). They inhibit bone resorption by binding to bone surfaces and cause injury to the osteoclasts. Calcitonin, on the other hand, inhibits osteoclastic activity and motility ad reduces their cell number (41). The effect of these agents on bone formation is complex and it has not yet been established for certain whether they exert any effect on the osteoblast. For example, studies of the long-term effect of calcitonin on bone remodeling suggest that it lengthens the formation phase at the expense of the reversal phase between resorption and formation (42). This might be due to an indirect effect via osteoclasts or to a direct effect via osteoblasts. So far calcitonin receptor has only been found in the osteoclast but not osteoblast (43). Thus the observed inhibitory effect of calcitonin on osteocalcin gene expression is probably via an intermediary effect on osteoclasts, thus decreasing bone turnover. Although treatment of the thyrotoxic rats with calcitonin negated the effect of thyroxine on steady-state osteocalcin mRNA, the actions of L-T_4 and calcitonin on osteocalcin gene transcription are probably at different levels and require further elucidation. It is also of interest that despite the effectiveness of sodium etidronate in preventing bone loss, it has no effect on L-T_4-induced elevation of osteocalcin mRNA concentration. Ongphiphadehanakul similarly reported that sodium etidronate was able to prevent the bone loss but not the increase in tartrate-resistant acid phosphatase induced by L-T_4 (44).

Both calcitonin and sodium etidronate have been proven to be effective in the treatment of postmenopausal osteoporosis (10–13). Thus it was surprising to observe in this study that calcitonin is ineffective in preventing bone loss induced by L-T_4, as calcitonin has in fact been demonstrated to be more effective in those osteoporotic patients with "high" turnover than in those with "low" turnover, as defined biochemically by an elevation of osteocalcin (45). The use of calcitonin in treatment high turnover bone loss is well illustrated by its effectiveness in treating Paget's disease (46). Thus, the role of antiresorptive agents in preventing thyroid hormone induced bone loss needs to be confirmed in human studies.

In conclusion, we have established a rat model of thyroid hormone induced bone loss. The diphosphonate agent, sodium etidronate, but not calcitonin, was able to prevent L-T_4-induced bone loss in the present model.

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