Osteoporotic Cytokines and Bone Metabolism on Rats with Induced Hyperthyroidism; Changes as a Result of Reversal to Euthyroidism

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Abstract

Hyperthyroidism is characterized by increased bone turnover and resorptive activity. Raised levels of serum osteoporotic cytokines, such as interleukin (IL) -1β, IL-6 and tumor necrosis factor (TNF)-α have been demonstrated previously in hyperthyroidism. These elevations are controversial and it is difficult to differentiate the contribution of thyroid hormones to the elevation of cytokines from that of the autoimmune inflammation in Graves' disease (GD) and follicular cell damage in thyroiditis. Therefore, we investigated the effect of thyroid hormones on serum IL-1β, IL-6, TNF-α levels and bone metabolism on L-thyroxine induced hyperthyroid rats and changes in cytokine levels and bone metabolism on the same rats after reversal to euthyroidism. Rats were treated with L-thyroxine for 5 weeks (0.4 mg/ 100 g food). Plasma T_3, T_4, TSH and serum IL-1β, IL-6, TNFα, Calcium (Ca), phosphorous (P), parathyroid hormone (PTH), alkaline phosphatase (ALP), bone alkaline phosphatase (B-ALP) levels were measured and differential leucocyte counts were made initially, at the 5th week of the experiment (hyperthyroid state) and 5 weeks after quitting the administration of L-thyroxine (euthyroid state). Significant rises in serum IL-1β, IL-6 and TNFα were noted in hyperthyroidism (P<0.001). In euthyroid state, IL-1β, IL-6 and TNFα decreased significantly, but IL-β and TNFα were significantly higher than the baseline values (P<0.05) while IL-6 levels turned back to the baseline values. Plasma T_3 and T_4 levels were significantly correlated with serum cytokines in hyperthyroid state while there was no correlation in euthyroid states. Ca and P levels did not differ significantly while PTH levels declined significantly in the hyperthyroid state (P<0.05). After the reversal to the euthyroidism, there was no significant change in Ca, P and PTH levels. ALP and B-ALP increased significantly in hyperthyroidism (P< 0.001, P<0.01) and they did not decrease in euthyroid state. The lymphocyte number and ratio in differentials increased significantly in the hyperthyroid state (P<0.001). In euthyroidism they decreased significantly (P<0.001) but it was significantly higher than the baseline value (P<0.05). Our findings showed that the deleterious effect on bone metabolism in hyperthyroidism might be mediated by cytokines and the increased bone turnover in hyperthyroidism failed to decrease despite euthyroidism.

Key Words: hyperthyroidism, interleukin-1β, interleukin 6, tumor necrosis factor-α, bone metabolism, bone turnover

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Introduction

Thyroid hormones are necessary for normal skeletal growth. However, their excess may lead to bone resorption. Ultimately hyperthyroidism is accompained by osteoporosis (24, 34). Although there is ample evidence for increased bone turnover in hyperthyroidism (10), the precise mechanisms for this action of thyroid hormone on bone remain unclear.

Locally produced factors are important in maintaining normal bone metabolism (19). IL-6 in particular, has a major influence on bone turnover and stimulates differentiation and proliferation of osteoclasts (16). IL-1β and TNFα are also implicated in bone resorption, particularly in high turnover states (25). Raised levels of serum osteoporotic cytokines such as IL-1β, IL-6 and TNFα have been reported in hyperthyroidism with different etiologies like Graves' disease (GD), toxic nodular goiter (TNG) and subacute thyroiditis (4, 7, 17). Conversely there exists studies reporting that IL-1β and TNFα have failed to rise in GD and TNG (28, 29). Similarly, in the postpartum thyroiditis (1) and interferon-γ thyroiditis, IL-6 (20) failed to increase.

Serum IL-6 has a number of sources, including blood mononuclear cells and bone tissue. IL-6 mRNA is also present in thyroid follicles (13). In thyroid hyperfunction like TNG, intrathyroidal production of IL-6 is one source of elevated IL-6 levels in serum (17). In GD although follicular cells are also able to express certain cytokines, intrathyroidal lymphocytes are the main source of IL-6 production (36). Serum IL-6 normalizes on remission of the subacute thyroiditis, indicating that it is related to follicular cell damage (17). The data about the levels of cytokines in hyperthyroidism are controversial and it has remained difficult to differentiate the contribution of thyroid hormones to these elevations from that of the autoimmune inflammatory process in GD, hyperfunction of the gland in TNG and follicular cell damage in thyroiditis. To address this question, we investigated the effects of thyroid hormones on serum IL-1β, IL-6 and TNFα levels and on bone metabolism on L-thyroxine-induced hyperthyroid rats intraindividually. As discrepancy exist in the results of studies to determine whether euthyroidism after medical therapy can completely normalize bone metabolism (18, 23), the changes in cytokines and bone metabolism were also investigated on the same rats that were taken back to euthyroidism.

Materials and Methods

Experimental Design

Fifteen female adult Wistar Albino rats of 250-300 g body weight were supplied from the Laboratory Animal Service of the University of Istanbul. The animals were permitted ad libitum access to standard laboratory chow and tap water. Hyperthyroidism was induced by administration of L-thyroxine (Organon Inc, Istanbul, Turkey) 0.4 mg/100 g food for 5 weeks (15, 30, 31, 35).

Blood samples were taken from tail vein initially, 5 weeks after the administration of L-thyroxine (hyperthyroid state) and 5 weeks after quitting L-thyroxine administration (euthyroid state).

Biochemical Measurements

The plasma T_3,T_4,TSH and serum IL-1β, IL-6 and TNFα calcium (Ca), phosphorous (P), Parathyroid hormone (PTH), alkaline phosphatase (ALP) and bone alkaline phosphatase (B-ALP) were analyzed. Additionally differential leucocyte counts were made.

T_3, T_4 and TSH analyses were performed by chemiluminescent enzyme immunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA). IL-1β, IL-6 and TNFα were measured by ELISA (Quantikine, R&D Systems, Minneapolis, MN, USA). The sensitivity for IL-1β, IL-6 and TNFα were 5, 10, 5 pg/mL respectively. Ca, P were measured by autoanalyser (Hitachi 717, Boehringer Mannheim, Germany). PTH was analysed by immuno radiometric assay (Diagnostic Systems Laboratories. Inc., Texac, USA). ALP and B-ALP were determined by a kinetic-enzymatic method (Boehringer, Mannheim, Germany). For differential leucocyte counts, blood smears were stained with May-Grunwald and Giemsa Solutions (Merc, Darmstad, Germany). At the same time the total leucocyte number and the total numbers of subgroubs in mm^3 were determined by hematology analyzer (Beckman Coulter, HMX, USA).

Statistical Analysis

Data were expressed as mean(SD) and analyzed by repeated measures of ANOVA. For adjustment of multiple comparisons Least Significant Difference (LSD) was used. Correlations between plasma T_3/T_4 and serum cytokines in different stages were tested by Pearson-Bravis test and *P* less than 0.05 was accepted as significant.

Results

After 5 weeks of L-thyroxine administration the measured values of significantly increased T_3, T_4 and significantly decreased TSH served to confirm the establishment of hyperthyroidism in the rats. Five weeks after quitting L-thyroxine administration, T_3, T_4 and TSH values reversed to the baseline values

(Table 1).

Significant rises in serum IL-1β, IL-6 and TNFα were noted in hyperthyroidism (P<0.001). In euthyroid state IL-1β and TNFα decreased (P<0.01), but they did not arrive at the baseline values and they were significantly higher than the baseline values (P <0.05). Meanwhile IL-6 levels reversed to the baseline values (Table 2). Plasma T_3 and T_4 levels were significantly correlated with serum cytokines (IL-1β r=0.59, P<0.01 and r=0.69, P<0.01; IL-6 r=0.75, P<0.01 and r=0.67, P<0.01; TNFα r=0.64, P<0.05 and r=0.54, P<0.05 respectively) in hyperthyroid state (Fig. 1). However T_3 and T_4 levels did not correlate with serum cytokines in euthyroid states.

Serum Ca and P levels did not differ significantly compared to baseline values whereas PTH levels declined significantly in the hyperthyroid state (P< 0.05). After reversal to the euthyroidism there was no significant change in Ca, P and PTH levels (Table 2).

ALP and B-ALP increased significantly in hyperthyroidism compared to baseline values (P< 0.001 and P<0.01 respectively) and they did not decrease in euthyroid state (Table 2).

The lymphocyte number in differentials increased significantly from 7400±788/mm^3 (61.0± 2.94%) to 9893±775/mm^3 (80.4±3.97%) in the hyperthyroid state (P<0.001). In euthyroidism the number of the lymphocyte decreased significantly to 8211±1517/mm^3 (70.8±4.73%) (P<0.001), but it was significantly higher than the baseline values (P< 0.05).

Table 1. The plasma levels of T_3, T_4 and TSH in baseline, hyperthyroid and euthyroid state of rats (means±SD; n=15)

State	T_3 (ng/dL)	T_4 (μg/dL)	TSH (μIU/mL)
Baseline	75.45±7.18	4.66±1.25	2.24±0.44
Hyperthyroid	178.91±7.68	12.32±3.23	1.94±0.21
Euthyroid	71.81±5.87	4.59±1.09	2.26±0.43
P^a	<0.001	<0.001	<0.01
P^b	<0.001	<0.001	<0.01
P^c	NS	NS	NS

P^a, difference of hyperthyroid state from baseline; P^b, difference of euthyroid state from hyperthyroid state; P^c, difference of euthyroid state from baseline; NS, not significant

Discussion

In our study, the levels of cytokines increased due to administration of L-thyroxine. Plasma T_3 and T_4 levels were significantly correlated with serum cytokines in hyperthyroid state. The increases in the level of IL-6 were explained with autoimmune inflammation, thyroid hyperfunction and follicular cell damage in the hyperthyroid patients with different etiology like GD, TNG, and thyroiditis (17, 21). Watson *et al*. (36) reported that intrathyroidal lymphocytes were the main source of IL-6 production in GD although follicular cells were also able to express certain cytokines. As the thyroid hormones were administrated externally and there were correlations between plasma T_3/T_4 and serum cytokines in hyperthyroid state the origin of elevated levels of IL-1β, IL-6 and TNF-α were due to thyroid hormones in our study and we showed that osteoporotic cytokine levels increased in the circulation in hyperthyroidism independent of the etiology.

Osteoblasts and blood mononuclear cells express thyroid hormone receptors (5, 6, 26). For this reason in hyperthyroidism the secretion of osteoporotic cytokines from the osteoblast and/or blood mononuclear cells may increase by the effect of increased amount of thyroid hormones.

Hyperthyroidism is a well documented cause of impaired bone turnover characterized by increased osteoblastic and osteoclastic activity, resulting in predominance of bone resorption and in decreased bone mass (5, 24). There is no knowledge on the direct stimulating effects of thyroid hormones on osteoclastic activity. Besides stimulating stromal cell progenitors, these hormones are reported to have important functions in osteoblastic maturation (8). Therefore, the cytokine secretion from stromal cells and osteoblasts may increase by the effect of increased thyroid hormones in hyperthyroidism.

IL-6 is produced by both stromal cells and osteoblastic cells in response to stimulation by the bone-resorbing cytokines IL-1 and TNF (11, 22). IL-6 stimulates bone resorption by enhancing osteoclast proliferation and differentiation (16). *In vitro* thyroid hormones do not stimulate IL-6 production directly in fetal rat limb bones (33). However in the presence of physiological concentrations of thyroid hormones, the IL-1 stimulated IL-6 response and bone resorption is greatly increased in fetal rat limb bone cultures (33). In our study it was seen that the levels of IL-1β, IL-6 and TNFα increased in the hyperthyroid state. After the reversal of the rats to euthyroidism, we saw that the level of IL-6 arrived at the baseline values while the levels of IL-1β and TNFα decreased significantly, but not to the baseline values (Table 2). This may suggest the stimulation of IL-6 by IL-1 at certain concentrations. As the concentration of IL-1 decreases to the values below these concentrations, it will not stimulate IL-6 any more.

Blood mononuclear cells produce IL-6 under normal basal conditions (11). Lakatos *et al*. (17)

Table 2. Serum levels of measured parameters in baseline, hyperthyroid and euthyroid states of rats (means ± SD; n=15)

Parameter	Baseline	Hyperthyroid	Euthyroid	P^a	P^b	P^c
IL-1β (pg/mL)	807.08±46.87	967.50±40.92	860.58±81.23	<0.001	<0.01	<0.05
IL-6 (pg/mL)	1007.50±69.16	1111.91±67.34	1001.66±75.17	<0.001	<0.001	NS
TNFα (pg/mL)	510.00±29.07	559.16±30.80	525.41±31.43	<0.001	<0.01	<0.05
Ca (mg/dL)	9.19±0.85	9.46±0.98	9.20±0.90	NS	NS	NS
P (mg/dL)	7.45±0.87	7.70±0.66	7.62±0.66	NS	NS	NS
PTH (mg/dL)	2.91±0.39	2.73±0.37	2.80±0.37	<0.05	NS	NS
ALP (U/L)	189.75±39.18	219.0±42.16	217.50±42.24	<0.001	NS	<0.001
B-ALP (U/L)	27.50±4.48	37.25±8.41	36.91±8.40	<0.01	NS	<0.01

P^a, difference of hyperthyroid state from baseline; P^b, difference of euthyroid state from hyperthyroid state; P^c, difference of euthyroid state from baseline; NS, not significant

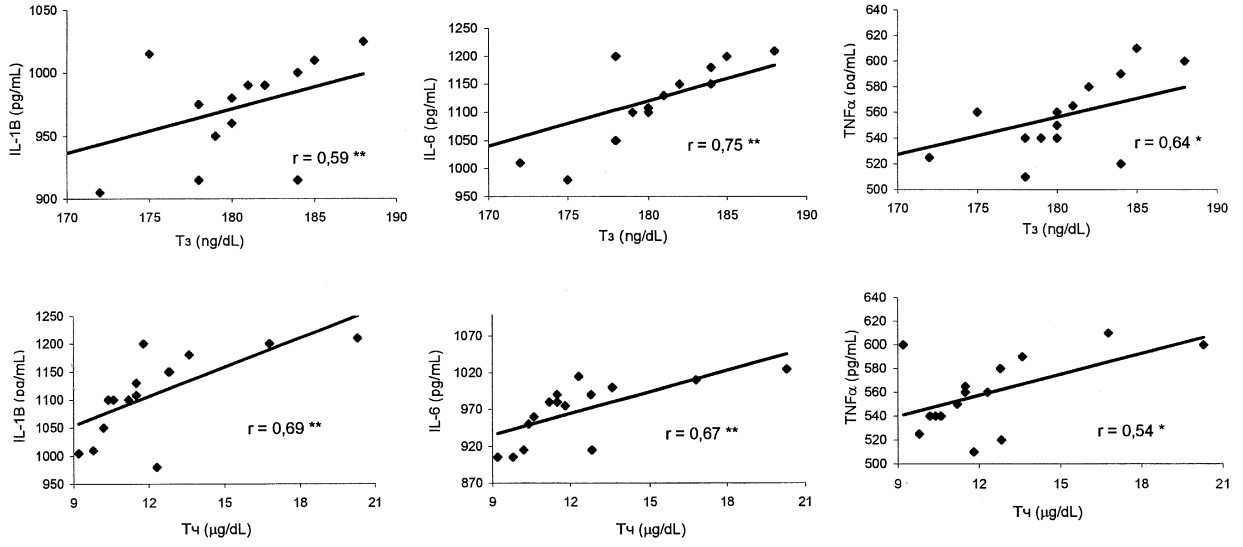

Fig. 1. The correlations between the levels of plasma T_3/T_4 and serum cytokines in hyperthyroid state. *P<0.05, **P<0.01

showed the increasing of basal IL-6 secretion from mononuclear cells in GD and TNG. Horowitz *et al.* (14) reported that IL-1β and TNFα secreted from peripheral monocytes induced the secretion of IL-6 by stimulating stromal cells and osteoblast. In our study lymphocytosis was observed in hyperthyroid rats while there was no change in monocyte number and ratio. We also showed lymphocytosis in hyperthyroidism in our previous study (31). IL-1β is also secreted from lymphocytes (9). Increased numbers of lymphocytes encountered in hyperthyroidism may be responsible for high IL-1β levels. This may lead to elevated IL-6 levels released from activated osteoblasts. After the reversal of the rats to euthyroidism the number of lymphocyte decreased significantly, but it was noticable that these values were significantly greater than the baseline values.

Barnefalk *et al.* (3) found out that high plasma Ca concentration increased the secretion of IL-6 from mononuclear blood cells, but in our study hyperthyroid rats showed no significant increase in serum Ca levels while there were significant increases in IL-1β, IL-6 and TNFα levels. We also demonstrated that thyroid hormones did not cause significant changes in the plasma level of Ca in our previous studies (30, 31, 32).

Thyroid hormones given in supraphysiological doses result in a reduction in net bone tissue mass by an accentuation in remodelling, causing increased resorptive activity (2). Enhanced bone destruction results in a slight rise in serum Ca, which in turn causes a compensatory reduction in the PTH level and augmentation of renal P retention (27). Our findings of insignificant increases in serum Ca and P levels and a significant decline in PTH are consistent with these data. Reversal to euthyroidism did not cause any change in Ca, P and PTH levels (Table 2).

The levels of serum ALP and B-ALP were found to increase significantly in hyperthyroidism in our study. These findings are consistent with the activation of the osteoblasts in cohort with the increasing of osteoclastic activity. However, we did not observe any significant decrease in ALP and B-ALP levels after reversal to euthyroidism (Table 2). Garnero *et al*. (10) concluded that B-ALP is an important biochemical marker demonstrating the increase in bone turnover. Our findings showed that increased bone turnover in hyperthyroidism failed to decrease despite euthyroidism.

The deleterious effects on bone tissue in hyperthyroidism may be mediated by IL-1β, IL-6 and TNFα. Despite the high bone turnover, the reversal of IL-6 to the baseline values suggests that IL-6, may not a sensitive indicator for high bone turnover. It will be important to investigate for the mechanism responsible for the ongoing high bone turnover despite euthyroidism.

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