의학석사 학위논문

The Effect of Thyroid Stimulating Antibody on the Bone Remodeling in Graves' Disease

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서울대학교 대학원

의학과 분자유전체의학전공

배 재 현

The Effect of Thyroid Stimulating Antibody on the Bone Remodeling in Graves' Disease

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이 논문을 의학석사 학위논문으로 제출함

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Abstract

Background: Thyroid hormone is known as a main contributor to bone loss in hyperthyroidism. However, recent studies reported that thyroid stimulating hormone (TSH) acts directly on the bone through TSH receptor (TSHR), independent of the effects of thyroid hormone. As serum thyroid stimulating antibody (TSAb) binds to TSHR in Graves' disease, serum TSAb may produce a direct effect on bone metabolism.

Aim of the study: We conducted this study to identify the effects of serum TSAb on bone metabolism in patients with Graves' disease.

Materials and Methods: In patients with newly diagnosed Graves' disease, the association between serum TSAb levels and biochemical parameters of bone metabolism are evaluated. To identify the effect of TSAb on osteoblasts, we examined alkaline phosphatase (ALP) activity assay and the expression of osteoblastogenic genes in murine mesenchymal C3H10T1/2 cells.

Results: One hundred and thirty nine patients were included in the study. Patients with serum TSAb were 127; men were 25, premenopausal women 83, and postmenopausal women 19. Patients with serum thyroid blocking antibody (TBAb) were 12; all females. In patients with serum TSAb, the levels of serum triiodothyronine (T_3), free T_4 (FT_4), TSAb, and parathyroid hormone was significantly correlated with the levels of serum bone-specific alkaline phosphatase (BAP), osteocalcin (OC), and C-terminal telopeptide of type Ⅰ collagen (CTx). After multivariate regression analysis, serum TSAb was an independent variable for serum OC in men (P=0.019) and for serum BAP and OC in premenopausal women (P=0.043 and P=0.043). Treatment with TSH and TSAb did not show significant changes in ALP activity assay and the expression of BAP, OC and CTx in murine C3H10T1/2 cells

Conclusion: The levels of serum TSAb were associated with biochemical parameters of osteoblastogenesis and bone coupling in patients with Graves' disease, independent of the

effects of T_3 and FT_4. Serum TSAb might have a protective role in osteoporosis in men and premenopausal women with Graves' disease.

Key words: Thyroid stimulating antibody; Bone-specific alkaline phosphatase; Osteocalcin; C-terminal telopeptide of type Ⅰ collagen

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Introduction

Hyperthyroidism is an important cause of secondary osteoporosis and increases a risk of fragility fracture, especially in postmenopausal women (1-5). Although this condition is a high-bone-turnover state with accelerated bone resorption and formation, a net increase of bone resorption results in bone loss (6). Traditionally, high levels of serum thyroid hormones are considered major contributors to bone loss in hyperthyroidism (7). However, recent studies reported that low levels of thyroid stimulating hormone (TSH) play an important role in bone remodeling.

Role of thyroid hormone in bone metabolism in hyperthyroidism

Thyroid hormone receptors (TRs) are found in osteoblast, osteoclast, and their bone marrow-derived precursor cells (8, 9). Thyroxine (T_4), a predominant form of circulating thyroid hormone, is converted to triiodothyronine (T_3) by type 2 deiodinase, which controls local concentration and availability of active thyroid hormone in the bone (6). Thyroid hormones enter bone cells through specific transporter, monocarboxylate transporter 8, and regulate the expression of this transporter (10, 11). Intracellular T_3 acts directly on bone cells through TRα1, mainly expressed form of TRs in the bone, and TRβ1 (12, 13). T_3 stimulates differentiation of osteoblast (14-16) and induces synthesis, degradation, modification, mineralization, and remodeling of bone matrix (14, 15, 17, 18). In addition, T_3 increases the number and activity of osteoclast (19, 20). However, as the expression of TR on osteoclast is not identified (21), the mechanism of T_3 action on osteoclast is not clear. The indirect action of T_3 on osteoclast through the action of osteoblast was demonstrated; both in receptor activator of nuclear factor-κB ligand (RANKL)-dependent and independent pathways (22-24). The effect of T_3 on osteoblast regulates expression of osteoprotegerin (OPG), an osteoclastogenesis inhibitory factor, and decoy receptors, which are involved in RANKL-dependent osteoclastogenesis (24). Whereas,

the effect of T_3 on osteoclast with absence of osteoblast was reported (25, 26). Although not completely understood, Indian hedgehog homolog and parathyroid hormone-related peptide feedback loop (27), growth hormone/insulin-like growth factor 1 signaling pathway (12), fibroblast growth factor receptor signaling pathway (28, 29), and Wnt-β-catenin signaling pathway (30) are involved in the response of bone to thyroid hormone.

Hyperthyroidism accelerates bone resorption and formation. Duration of each phase is reduced by 60% and 30% respectively, and frequency of initiation of bone turnover is increased (3). This process causes uncoupling of osteoblast and osteoclast activities, and results in a net increase of bone resorption; a 10% net loss of bone per remodeling cycle (3). An expansion of remodeling space leads to decreased cortical bone thickness (31), increased trabecular bone perforations (3), and then increases the risk of fracture. Previous cross-sectional and case-control studies reported that untreated hyperthyroidism decreased bone mineral density (BMD) (32-34) and increased the risk of vertebral (35-37), non-vertebral fractures (36-40). Treatment of hyperthyroidism with antithyroid drugs returned reduced BMD up to normal range (36, 37, 40-43), while revealed inconsistent effects on the risk of hip fracture; persisted (40) or reduced (37). Surgical treatments restored decreased BMD and reduced the risk of fracture in hyperthyroidism (36, 44). However, radioactive iodine therapy was associated with the increased risk of fracture (36). The rate of decline in elevated thyroid hormone levels might account for different effects on the bone between antithyroid drugs, surgical treatment and radioactive iodine therapy (36, 45).

Role of thyroid stimulating hormone in bone metabolism in hyperthyroidism

The studies of TR knockout mice revealed predominance and functional differences between TR isoforms in bone cell, and a critical role of thyroid hormones in skeletal morphogenesis and growth plate development. However, these mice did not display skeletal remodeling phenotype.

Mice devoid of both TRα and TRβ showed underdevelopment, undergrowth, and debilitating phenotype of hypothyroidism. Although adipogenesis was increased and trabecular BMD was decreased in bone marrow of these mice, there was no impairment in bone remodeling (46, 47).

Subclinical hyperthyroidism, defined as a suppressed TSH levels in the presence of normal circulating T_3 or T_4, was associated decreased BMD and the increased risk of fracture in cross-sectional studies with postmenopausal women (48, 49). In addition, serum TSH levels was positively correlated with BMD regardless of the levels of thyroid hormone, and negatively correlated with biochemical parameters of bone resorption in postmenopausal women (50, 51). In spite of thyroid hormone replacement and euthyroid state, individuals with TSHR mutations showed high-born-turnover phenotype; reduced BMD and elevated levels of serum OC and N-terminal telopeptide of type Ⅰ collagen (52, 53).

There were evidences that TSH acts directly on the bone through TSH receptor (TSHR), independent of its effects on circulating thyroid hormone. TSHRs are found in osteoblast and precursor cells of osteoblast and osteoclast (54, 55). TSHR heterozygote mice manifest a 50% reduction in TSHR with normal thyroid follicle, euthyroid state, and normal growth. However, these mice showed reduced BMD at all sites, and bone loss was not corrected by replacement of thyroid extract. TSHR heterozygote mice demonstrated increase in osteoclast formation and osteoblast differentiation, resulting in pronounced osteoporosis. However, these mice also showed focal osteosclerosis, which was associated with uncoupling between bone resorption and formation. In this study, TSH regulated osteoclastogenesis and osteoblastogenesis negatively through distinct and unrelated mechanisms; by down-regulating *c-jun* N-terminal kinases (JNK)/*c-jun* and NFκB signaling pathway and by attenuating low-density lipoprotein receptor-related proten-5 (LRP-5) and fetal liver kinase -1 (Flk-1) expression, respectively (55). Osteoporosis exacerbated by the loss of TSH signaling was also reported in the study with TSHR knockout mice (56). In the study with murine embryonic stem (ES) cells, TSH inhibited osteoclastogenesis and bone resorption (57). Recombinant human TSH (rhTSH) reduced the

levels of serum CTx (50) and urinary secretion of CTx and N-terminal telopeptide of type Ⅰ collagen (NTx) in patients performed thyroidectomy for differentiated thyroid carcinoma (38). Furthermore, TSH induced osteoblastogenesis through Wnt-dependent feed-forward loop in murine ES cells (58). In aged ovariectomized rats, TSH administration, at doses insufficient to alter thyroid hormone, prevents bone loss and restores bone mass through both antiresorptive and anabolic effects (59). However, the role of TSH on osteoblastogenesis and osteoblast activity is less clear than on osteoclastogenesis and osteoclast activity. Although there is a debate about TSH action on the bone, previous preclinical and epidemiologic studies showed that TSH had a protective effect on bone loss.

Possibility of the effect of thyroid stimulating antibody on bone metabolism in hyperthyroidism

Graves' disease is an autoimmune thyroid disease and the most common cause of primary hyperthyroidism. Hyperthyroidism is caused by thyroid stimulating antibody (TSAb) (32, 60-62), or TSHR antibody with stimulating activity, which is detected by thyrotropin binding inhibitor immunoglobulin (TBII) assays (63-68). The increased serum T_4 and T_3 levels, caused by the action of serum TSAb, are involved in the high-bone-turnover osteoporosis in hyperthyroidism (3, 5). The levels of serum TSH are decreased with inverse relationship with the levels of thyroid hormone in Graves' disease. However, as serum TSAb binds to TSHR, serum TSAb might produce a beneficial effect on high-bone-turnover osteoporosis in Graves' disease.

In Japanese male patients with untreated Graves' disease, the levels serum TSAb were negatively correlated with BMD at distal radius and positively correlated with urinary CTx (69). However, after multiple regression analysis, age and the levels of FT_4 remained as independent risk factors for reduced BMD at the DR (69). It is possible that the action of serum TSAb might be related to overproduction of intracellular T_3 as the action of TSH (70) or competitive

suppression of serum TSAb on TSH signaling pathway (71). Although the results from these studies indicate deleterious effects of TSAb on the bone, they do not exclude beneficial effects of serum TSAb on bone loss in Graves' disease. In the study with murine ES cells, both TSH and TSAb decreased the number of osteoclasts and inhibited biochemical parameters of osteoclastogenesis, such as calcitonin receptor, tartrate resistant acid phosphatase, cathepsin K, matrix metalloproteinase-9, and carbonic anhydrase Ⅱ) (57). Moreover, TSAb suppressed production of tumor necrosis factor-α, an osteoclastogenic cytokine, and increased the expression of OPG, in murine ES cells (57). Treatment of murine osteoblasts and osteoclasts with TSAb failed to induces a cyclic adenosine monophosphate (cAMP) response, but TSH also failed to induce a cAMP response (72). There were limited evidences about the effect of serum TSAb on bone metabolism in Graves' disease.

Therefore, we examined 1) the association between serum TSAb and biochemical parameters of bone metabolism, 2) the association between serum thyroid blocking antibody (TBAb), or TSHR antibody with blocking activity, and biochemical parameters of bone metabolism, and 3) the effect of TSH and TSAb on osteoblastogenesis in murine mesenchymal C3H10T1/2 cells.

Materials and Methods

Patients

One hundred and thirty nine patients with newly diagnosed Graves' disease were included in the study; 114 were women, aged 17-69 years, mean ± standard deviation (SD) was 38 ± 13 years, and 24 were postmenopausal; 25 were men, aged 20-67 years, mean ± SD was 40 ± 12 years, and 5 were above 50 years-old. Patients have been taking antithyroid drug (methimazole or propylthiouracil) within 2 weeks or never. And all patients did not have been taking any medications that might affect calcium and mineral metabolism. Patients with underlying bone disease, malignancy with bone metastasis, renal insufficiency, hepatic dysfunction and who treated with radioactive iodine or surgery prior to the study were excluded. Patients were divided into two groups owing to the activity of serum TSHR antibody; TSAb and TBAb (73-76). TBAb is determined by clinical courses, defined as spontaneously developed hypothyroid status with persistently high levels of serum TBII.

Biochemical analyses

All samples were stored at -70℃ until measurements. The levels serum TSH were measured by immunoradiometric assay (Daiichi Radioisotope Laboratories, Tokyo, Japan). The serum FT_4 and T_3 levels were measured by radioimmunoassay (Dainabot Radioisotope Laboratories, Tokyo, Japan). The levels of TSHR antibody were measured using first generation TBII assay (RSR, Cardiff, UK). Serum concentrations of calcium, phosphorous, total protein, and albumin were measured on Hitachi 7600 chemistry autoanalyzer (Hitachi, Tokyo, Japan). The levels of serum corrected calcium were calculated by the equation; corrected calcium = 0.8 × (4.1 - patients' albumin) + patient's calcium. Serum parathyroid hormone (PTH) levels were measured by immunoradiometric assay (Cisbio Bioassays, Parc Marcel Boiteux, Codolet, France), and serum

25-hydroxyvitamin D3 (25-OHD3) levels were measured using Diels-Alder derivatization and ultraperformance liquid chromatography-tandem mass spectrometry (Waters, Milford, MA, USA). The levels of serum bone-specific alkaline phosphatase (BAP) were measured by enzyme immunoassay (Quidel, San Diego, CA, USA). Serum osteocalcin (OC) levels were measured by immunoradiometric assay (Cisbio Bioassays, Parc Marcel Boiteux, Codolet, France). Serum C-terminal telopeptide of type Ⅰ collagen (CTx) levels were measured using electrochemiluminescence immunoassay (Roche, Indianapolis, IN, USA).

Cell cultures and induction of osteoblastogenic differentiation

Murine mesenchymal cells C3H10T1/2 (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). For osteoblastogenic differentiation, C3H10T1/2 cells were cultured in DMEM with 10% FBS supplemented with osteogenic medium (OM; 50 μg/mL L-ascorbic acid and 10 mM β-glycerophosphage), as previously described (77).

Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) activity assays were performed, as previously described (77). Differentiated osteoblasts were treated with bovine TSH (100 mUI/mL) and M22 (200 ng/mL), a synthetic monoclonal human TSAb, every other day for two weeks. And then, cells were washed three times with ice-cold Tris-buffered saline (TBS), pH 7.4 and scraped immediately after adding 0.5 mL of ice-cold 50 mM TBS. The collected lysates were sonicated for 20 sec at 4℃. Enzyme activity assays were performed in buffer (10 mM $MgCl_2$ and 0.2 M alkaline buffer, pH 10.3) containing 10 mM p-nitrophenylphosphate as substrate. The reaction was stopped by adding 0.3 M NaOH solution, and absorbance was read at OD (optical density) 405.

Real-time quantitative polymerase chain reaction measurements of gene expression

The mRNA expression of ALP and OC were determined by real-time quantitative polymerase chain reaction (RT-qPCR), as previously described (77). Differentiated osteoblasts were treated with bovine TSH (100 mUI/mL) and M22 (200 ng/mL) every other day for two weeks. Complementary DNA synthesis was performed with 1 μg of total RNA. Quantitative PCR was performed by applying the real-time SYBR Green PCR technology with the use of an ABI PRISM 7900HT sequence detection system (Applied Biosystems). The sequences of primers were as follows; ALP forward 5'-CTTGCTGGTGGAAGGAGGCAGG-3', ALP reverse 5'-CACGTCTTCTCCACCGTGGGTC-3'; OC forward 5'-CCACCCGGGAGCAGTGT-3', OC reverse 5'-CTAAATAGTGATACCGTAGATGCGTTTG-3'; CTx forward 5'-GCGAAGGCAACAGTCGCT-3', CTx reverse 5'-CTTCCTCCTTTTCTATTCGATGCC-3'; GAPDH forward 5'-GTGGACATTGTTGCCATCAACG-3', GAPDH reverse 5'-GAGGGAGTTGTCATATTTCTCG-3' (Bioneer, Daejeon, Korea). Amplification reaction was performed in a 20 μL volume, using 2 × Universal SYBR PCR Master Mix (PerkinElmer Life Sciences). Thermal cycling conditions were as follows: 40 cycles of 30 sec at 95℃, 30 sec at 56℃, and 30 sec at 72℃. All PCR reactions were performed at least in duplicate and the expression levels were normalized to GAPDH signal in the same reaction.

Statistical analyses

The results of continuous variables are reported as mean ± standard deviation, and coefficient intervals were computed as two-tailed using 95% coverage. Categorical variables are presented as frequencies. The comparisons between groups were performed by one-way analysis of variance. Pearson's correlations were performed for parametric variables and Spearman's

correlations for nonparametric variables. Linear regression modes were used in univariate and multivariate analyses. All statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). A *P*-value below 0.05 was accepted as statistically significant.

Results

Baseline characteristics of patients with Graves' disease

Clinical characteristics and biochemical parameters of the participants are shown in Table 1. The number of patients with serum TSAb was 127; men were 25, premenopausal women 83, and postmenopausal women 19. Patients with serum TBAb were 12; premenopausal women were 7, postmenopausal women 5. There was no significant difference in biochemical parameters between groups. The levels of serum TSH were undetectable (<0.05 uIU/mL), except one patient (0.98 uIU/mL), in patients with serum TSAb.

Serum TSAb in patients with Graves' disease is associated with biochemical parameters of osteoblastogenesis and bone coupling

From the correlation analysis, serum T_3, FT_4, TSAb, and PTH was significantly correlated with serum BAP, OC, and CTx. Serum PTH showed positive correlation with serum BAP in men (r=0.533, P=0.006), premenopausal women (r=0.277, P=0.011), and in postmenopausal women (r=0.552, P=0.014). Age is negatively correlated with serum OC in men (r=-0.579, P=0.002). Serum TSAb showed positive correlation with OC in men (r=0.466, P=0.019) and premenopausal women (r=0.296, P=0.007). Serum T_3 is positively correlated with serum OC in premenopausal women (r=0.410, P=0.001). Serum T_3 and FT_4 is positively correlated with serum CTx in premenopausal women (r=0.388, P=0.001 and r=0.246, P=0.026) (Table 2). After adjusted for all variables, which were significant in univariate analyses, serum TSAb showed persistent positive correlation with serum BAP (r=0.200, P=0.039) and OC (r=0.161, P=0.046) in premenopausal women. Serum T_3 was not associated with BAP and positively correlated with serum OC (r=0.317, P=0.005) and CTx (r=0.289, P=0.010) in premenopausal women (Table 3).

Table 1. Baseline clinical characteristics and biochemical parameters of the participants

	TBII with stimulating activity (TSAb)			TBII with blocking activity (TBAb)
	Men	Premenopausal women	Postmenopausal women	
Number	25	83	19	12 (all female)
Age (years)	40 ± 12	32 ± 9	56 ± 6	46 ± 14
T_3 (ng/dL)	395.9 ± 205.1	430.9 ± 183.0	343.4 ± 123.8	209.7 ± 122.7
FT_4 (ng/dL)	3.17 ± 1.09	3.63 ± 1.54	3.08 ± 1.02	1.56 ± 0.93
TBII (%)	48.2 ± 25.6	53.7 ± 23.5	41.3 ± 24.6	75.0 ± 22.0
Ca (mg/dL)	9.7 ± 0.4	9.6 ± 0.4	9.5 ± 0.4	9.6 ± 0.7
P (mg/dL)	4.3 ± 1.1	4.4 ± 1.0	4.6 ± 0.5	4.2 ± 1.2
25-OHD3 (ng/mL)	18.4 ± 10.8	16.7 ± 8.7	17.3 ± 9.0	17.0 ± 6.9
PTH (pg/mL)	14.9 ± 8.2	12.8 ± 7.9	15.8 ± 6.7	14.3 ± 7.5
BAP (U/L)	59.4 ± 28.7	58.2 ± 27.2	67.7 ± 33.7	41.2 ± 15.7
OC (ng/ml)	54.0 ± 29.5	45.9 ± 19.6	41.1 ± 27.4	31.8 ± 18.5
CTx (ng/mL)	0.87 ± 0.44	0.78 ± 0.35	0.85 ± 0.42	4.22 ± 13.29

The data is reported as mean ± standard deviation.

T_3; triiodothyronine, FT_4; free thyroxine, TBII; thyrotropin binding inhibitor immunoglobulin, Ca; calcium, P; phosphorous, 25-OHD3; 25-hydroxyvitamin D3, PTH; parathyroid hormone, BAP; bone-specific alkaline phosphatase, OC; osteocalcin, CTx; C-terminal telopeptide of type Ⅰ collagen

Table 2. Reference values for laboratory tests

	Men		Premenopausal women	Postmenopausal women
TSH (uIU/mL)	0.4 – 4.1		0.4 – 4.1	0.4 – 4.1
T_3 (ng/dL)	87 – 184		87 – 184	87 – 184
FT_4 (ng/dL)	0.93 – 2.13		0.93 – 2.13	0.93 – 2.13
TBII (%)	<15		<15	<15
Ca (mg/dL)	8.8 - 10.5		8.8 - 10.5	8.8 - 10.5
P (mg/dL)	2.5 - 4.5		2.5 - 4.5	2.5 - 4.5
25-OHD3 (ng/mL)	9.0 – 37.6		9.0 – 37.6	9.0 – 37.6
PTH (pg/mL)	10 – 65		10 – 65	10 – 65
BAP (U/L)	15.0 – 41.3		11.6 – 29.6	14.2 – 42.7
OC (mg/mL)	18 – 30 years	24 – 70	11 – 43	15 - 46
	>30 years	14 - 46		
CTx (ng/mL)	30 – 50 years	<0.584	<0.573	<1.008
	50 – 70 years	<0.704		
	>70 years	<0.854		

TSH; thyroid stimulating hormone, T_3; triiodothyronine, FT_4; free thyroxine, TBII: thyrotropin binding inhibitor immunoglobulin, Ca; calcium, P; phosphorous, 25-OHD3; 25-hydroxyvitamin D3, PTH; parathyroid hormone, BAP; bone-specific alkaline phosphatase, OC; osteocalcin, CTx; C-terminal telopeptide of type Ⅰ collagen

Table 3. Correlations between the levels of serum BAP, OC, and CTx with age and biochemical parameters in patients with TSAb (univariate analysis)

	Age	T_3	FT_4	TSAb	Ca	P	PTH	25-OHD3
Men (n=25)								
BAP†	-0.293	0.384	0.126	0.360	0.042	0.362	0.533**	-0.314
OC†	-0.579**	0.381	0.100	0.466*	0.068	0.400	0.342	-0.276
CTx	-0.089	0.279	0.177	0.348	0.203	0.497	0.010	-0.295
Premenopausal women (n=83)								
BAP†	0.002	0.277*	0.084	0.313**	-0.054	-0.018	0.261*	0.161
OC†	-0.153	0.410**	0.177	0.296**	0.134	0.100	0.153	0.050
CTx	-0.048	0.388**	0.246*	0.107	0.311	0.337	-0.039	-0.025
Postmenopausal women (n=19)								
BAP†	-0.302	-0.218	-0.079	0.259	0.026	0.034	0.552*	-0.258
OC†	-0.141	0.027	0.200	0.380	0.085	0.253	0.221	-0.022
CTx	-0.016	0.072	0.236	0.082	0.475	0.332	-0.248	-0.049

The correlation coefficients are presented. †Log-transformed values were used for analysis.

*P<0.05, **P<0.01

T_3; triiodothyronine, FT_4; free thyroxine, TSAb; thyroid stimulating antibody, Ca; calcium, P; phosphorous, PTH; parathyroid hormone, 25-OHD3; 25-hydroxyvitamin D3, BAP; bone-specific alkaline phosphatase, OC; osteocalcin, CTx; C-terminal telopeptide of type Ⅰ collagen

We investigated independent variables for serum BAP, OC, and CTx by multivariate analysis using linear regression (Table 4). Age, serum T_3, FT_4, TSAb, and PTH were included as variables and all variables are adjusted in each analysis. Serum T_3 and PTH was independent variables for serum BAP in men (r=0.666, P=0.020 and 0.002). Age, serum TSAb, and PTH were independent variables for serum OC in men (r=0.754, P=0.003, 0.019, and 0.034). Serum TSAb showed positive correlation with serum CTx, but statistically insignificant in men (r=0.348, P=0.095). In premenopausal women, serum TSAb and PTH were independent variables for serum BAP (r=0.435, P=0.043 and 0.021), and serum T_3 and TSAb were independent variables for serum OC (r=0.443, P=0.002 and 0.043). Serum T_3 was also independent variable for serum CTx in premenopausal women (r=0.388, P=0.001). In postmenopausal women, serum PTH was independent variable for serum BAP (r=0.552, P=0.014). There was no significant finding for serum OC and CTx in postmenopausal women.

Serum TSAb was an independent variable for biochemical parameters of osteoblastogenesis, regardless of the levels of serum T_3 and FT_4 (Table 6). Moreover, there was no association between the levels of serum TSAb and PTH (Table 7).

Serum TBAb in patient with Graves' disease is not associated with biochemical parameters of bone metabolism

From the correlation analysis, serum T_3 and FT_4 showed strong positive correlation with serum BAP (r=0.729, P=0.007 and r=0.669, P=0.017). Serum TBAb, T_3, and FT_4 showed strong negative correlation with serum OC (r=-0.658, P=0.020, r=0.921, P=0.001 and r=0.907, P=0.001). Serum TBAb, T_3, and FT_4 also showed strong negative correlation with serum CTx (r=-0.581, P=0.047, r=0.816, P=0.001 and r=0.718, P=0.009). Although multivariate analysis using linear regression revealed that serum TBAb was not independent variable for serum BAP, OC, and CTx, serum TBAb seemed to have inverse relationship with serum TSAb in association

Table 4. Correlations between the levels of serum TSAb, T_3, and FT_4 with biochemical parameters of bone metabolism (multivariate analysis)

	Men		Premenopausal women		Postmenopausal women	
	r	$r^{\ddagger}$	*r*	$r^{\ddagger}$	*r*	$r^{\ddagger}$
TSAb						
BAP†	0.360	0.274	0.313**	0.200*	0.259	0.396
OC†	0.466*	0.410	0.296**	0.161*	0.380	0.463
CTx	0.348	0.267	0.107	-0.014	0.082	0.121
T_3						
BAP†	0.384	0.361	0.277*	0.199	-0.218	-0.414
OC†	0.381	0.211	0.410**	0.317**	0.027	-0.478
CTx	0.279	0.112	0.388**	0.289*	0.072	-0.380
FT_4						
BAP†	0.126	-0.211	0.084	-0.075	-0.079	0.271
OC†	0.100	-0.144	0.177	-0.075	0.200	0.484
CTx	0.177	-0.005	0.246*	0.016	0.236	0.446

†Log-transformed values were used for analysis.

r = correlation coefficient, $r^{\ddagger}$= partial correlation coefficient adjusted by age, T_3, FT_4, TSAb, and PTH

*P<0.05, **P<0.01

TSAb; thyroid stimulating antibody, T_3; triiodothyronine, FT_4; free throxine, PTH; parathyroid hormone, BAP; bone-specific alkaline phosphatase, OC; osteocalcin, CTx; C-terminal telopeptide of type Ⅰ collagen

Table 5. Linear regression from age and biochemical parameters in patients with TSAb (multivariate analysis)

	Men		Premenopausal women		Postmenopausal women	
	β ± SE	*P*	β ± SE	*P*	β ± SE	*P*
BAP†						
T_3	0.001 ± 0.001	0.020	0.001 ± 0.001	0.079	-	-
TSAb	-	-	0.004 ± 0.002	0.043	-	-
PTH	0.027 ± 0.008	0.002	0.012 ± 0.005	0.021	0.038 ± 0.014	0.014
OC†						
Age	-0.020 ± 0.006	0.003	-	-	-	-
T_3	-	-	0.001 ± 0.001	0.002	-	-
TSAb	0.007 ± 0.003	0.019	0.003 ± 0.002	0.043	0.008 ± 0.005	0.109
PTH	0.019 ± 0.008	0.034	-	-	-	-
CTx						
T_3	-	-	0.001 ± 0.001	0.001	-	-
TSAb	0.006 ± 0.003	0.095	-	-	-	-

Independent variables and their values for serum BAP, OC, and CTx in linear regression models are presented. †Log-transformed values were used for analysis.

β; coefficient of regression, SE; standard error of β coefficient

A $P<0.05$ was considered statistically significant.

T_3; triiodothyronine, T_4; thyroxine, TSAb; thyroid stimulating antibody, PTH; parathyroid hormone, BAP; bone-specific alkaline phosphatase, OC; osteocalcin, CTx; C-terminal telopeptide of type Ⅰ collagen

Table 6. Correlation between the levels of serum TSAb and T_3 or FT_4 (multivariate analysis)

	Men		Premenopausal women		Postmenopausal women	
	r	$r^{\ddagger}$	*r*	$r^{\ddagger}$	*R*	$r^{\ddagger}$
T_3	0.392	0.380	0.339*	0.427*	0.167	0.081
FT_4	0.192	-0.161	-0.006	-0.276	0.037	-0.001

r = correlation coefficient, $r^{\ddagger}$= partial correlation coefficient adjusted by T_3, FT_4, TSAb

*$P<0.05$

TSAb; thyroid stimulating antibody, T3; triiodothyronine, FT_4; free thyroxine

Table 7. Correlation between the levels of serum TSAb and PTH (univariate analysis)

	PTH	Ca	Corrected Ca	25-OHD3
Men	0.100	-0.189	-0.015	0.037
Premenopausal women	0.060	0.090	0.099	0.211
Postmenopausal women	-0.179	-0.103	0.062	0.230

TSAb; thyroid stimulating antibody, PTH; parathyroid hormone, Ca; calcium, Corrected Ca; corrected calcium, 25-OHD3; 25-hydroxyvitamin D3

Corrected calcium is calculated by the equation; corrected calcium = 0.8 × (4.1 - patients' albumin) + patient's calcium.

with biochemical parameters of bone metabolism in patients with Graves' disease. Serum T_3 was significant for serum BAP (P=0.005) and serum FT_4 for serum OC (P=0.004), persistently.

TSH and TSAb does not induce osteoblastogenic activity or the expression of osteoblastogenic genes

Treatment with bovine TSH on osteoblasts, differentiated from C3H10T1/2 cells, reduced ALP activity (0.23 mmoL/min/mg protein), compared to treatment with vehicle (0.52 mmoL/min/mg protein). When osteoblasts were cultured without OM supplement, treatment with bovine TSH did not lead to significant change of ALP activity (TSH 0.32, vehicle 0.28 mmoL/min/mg protein). Treatment with synthetic TSAb reduced ALP activity with or without OM (0.37 or 0.15 mmoL/min/mg protein). However, these changes were not statistically significant (Figure 1).

The expressions of ALP, OC, and CTx are higher in osteoblasts cultured with OM. However, treatment with bovine TSH did not induce significant alterations in the expressions of ALP, PC, and CTx compared to treatment with vehicle (Figure 2). Treatment with synthetic TSAb showed the same results as with TSH.

Figure 1. The effect of TSH and TSAb on ALP activity in murine C3H10T1/2 cells. Treatment with TSH on cells, cultured with OM, reduced ALP activity. However, treatment with TSAb on cells did not cause significant change of ALP activity. Cells were treated with vehicle, TSH or TSAb. ALP activity was determined and normalized to protein content. Each bar represents mean ± standard deviation. $^{*}P<0.05$ was accepted as statistically significant. ALP; alkaline phosphatase, VEH; vehicle, TSH; thyroid stimulating hormone, TSAb; thyroid stimulating antibody, OM; osteogenic medium (50 μg/mL L-ascorbic acid and 10 mM β-glycerophosphage)

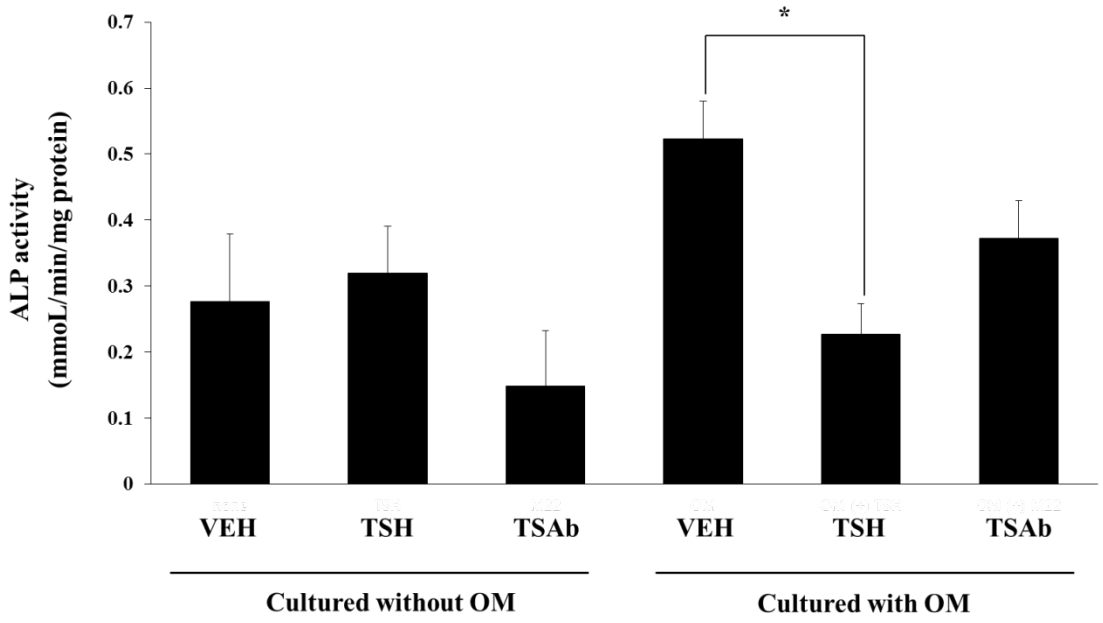
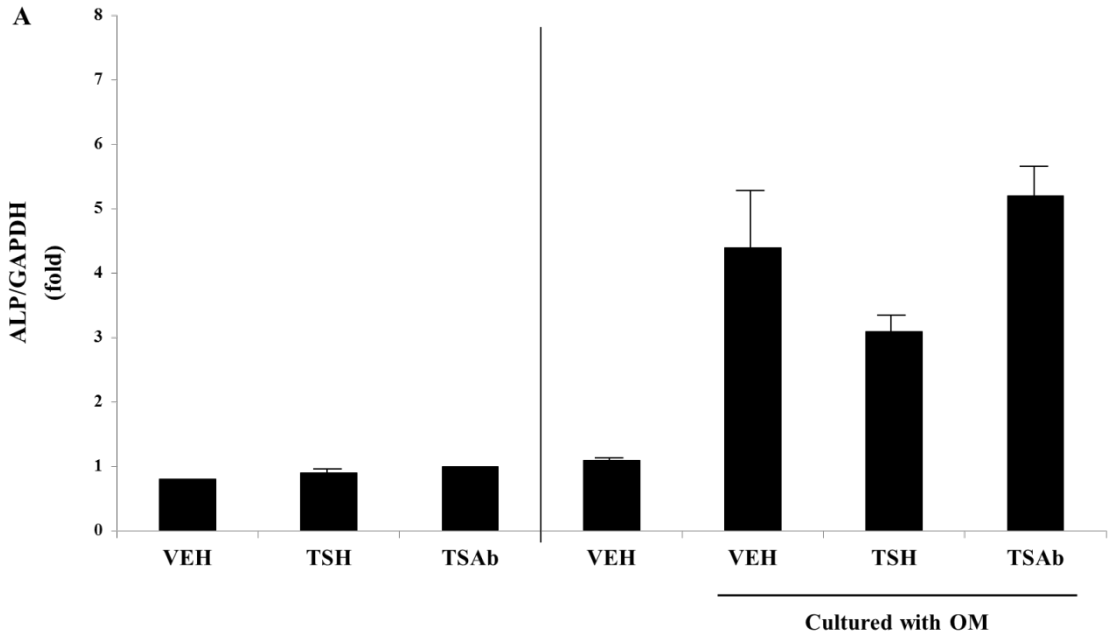
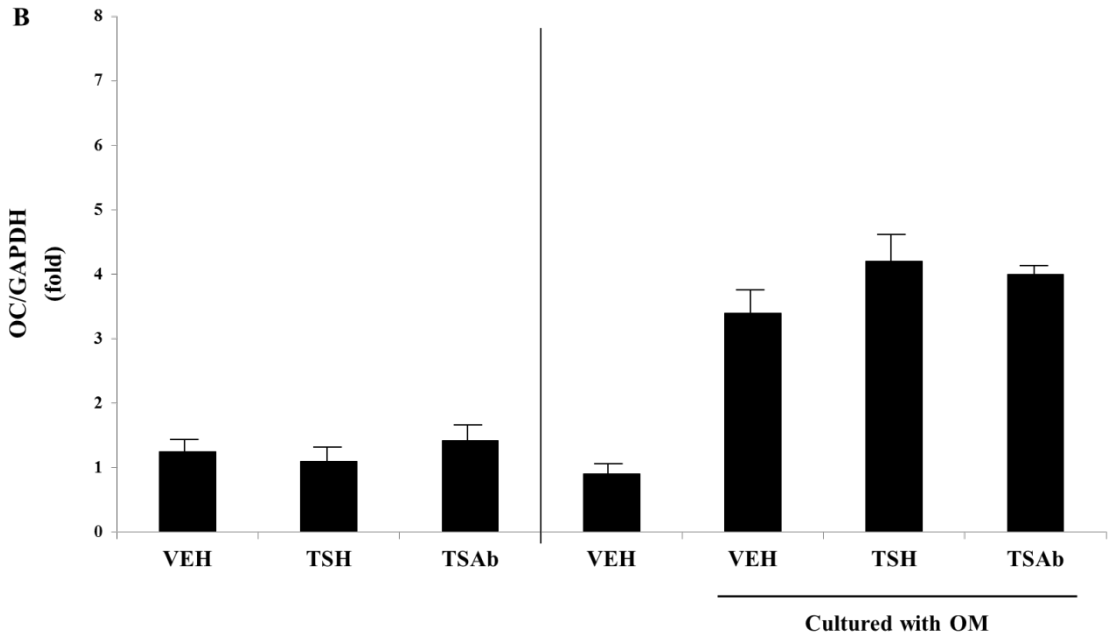
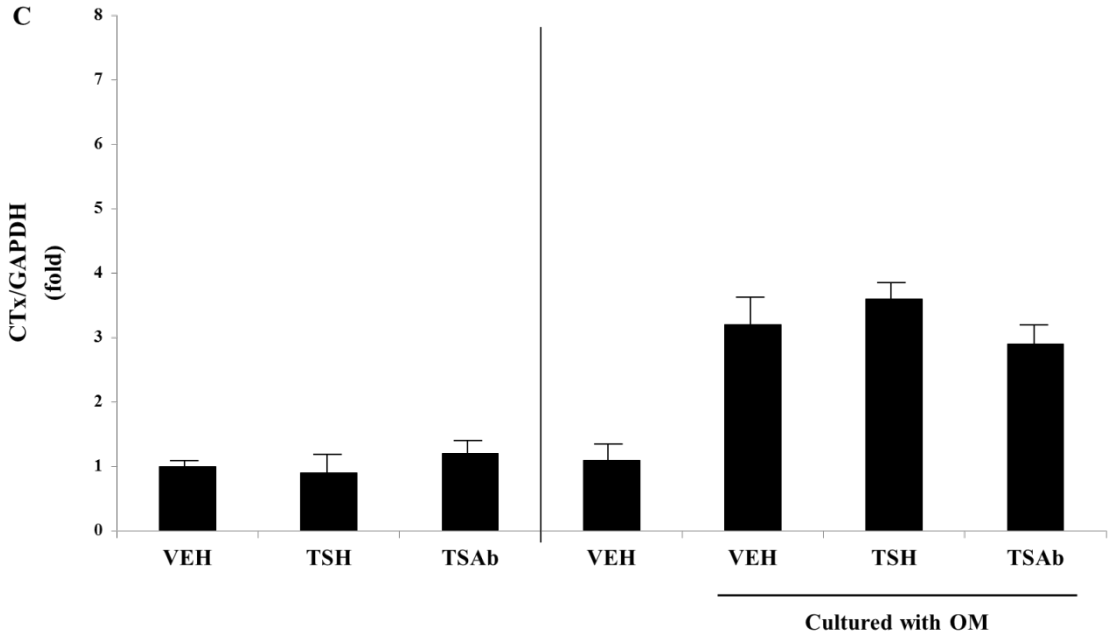


Figure 2. The expression of osteoblastogenic genes in murine C3H10T1/2 cells after treatment with TSH and TSAb. (A) The expression of ALP did not altered significantly after treatment with TSH or TSAb. (B) The expression of OC did not changed significantly after treatment with TSH or TSAb. (C) The expression of CTx did not show significant difference after treatment with TSH or TSAb. Cells were treated with vehicle, TSH or TSAb. All expression levels were normalized to the expression of GAPDH in the same reaction and presented with folds. Each bar represents mean ± standard deviation. ALP; alkaline phosphatase, OC; osteocalcin, CTx; C-terminal telopeptide of type Ⅰ collagen, GAPDH; glyceride 3-phosphate dehydrogenase, VEH; vehicle, TSH; thyroid stimulating hormone, TSAb; thyroid stimulating antibody, OM; osteogenic medium (50 μg/mL L-ascorbic acid and 10 mM β-glycerophosphage)




B
OC/GAPDH (fold)
8
7
6
5
4
3
2
1
0
VEH
TSH
TSAb
VEH
VEH
TSH
TSAb
Cultured with OM


C
CTx/GAPDH (fold)
8
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TSH
TSAb
VEH
VEH
TSH
TSAb
Cultured with OM

Discussion

In our study, serum TSAb was positively correlated with the biochemical parameters of osteoblastogenesis and bone coupling in patients with Graves' disease. The levels of serum BAP reflect osteoblastogenesis, bone formation and those of serum OC indicate osteoblastogenesis, bone formation, and bone coupling. In addition, serum TSAb was an independent variable for serum OC in men, and for serum BAP and OC in premenopausal women. As the serum TSH levels were undetectable (<0.05 uIU/mL), except one patient (0.98 uIU/mL), in patients with serum TSAb, the effect of serum TSH could be excluded. Although serum TSAb had a positive correlation with serum T_3, serum TSAb was an independent variable for serum OC and CTx in all groups, for serum OC in men, and for serum BAP and OC in premenopausal women, regardless of the serum T_3 and FT_4 levels.

Serum CTx, a biochemical marker of osteoclastogenesis and bone resorption, was not associated with serum TSAb. However, serum OC, which is secreted solely by osteoblast, showed a positive correlation with serum TSAb in this study. As serum OC is involved in bone coupling and promotes differentiation of osteoclast (78, 79), it is possible that serum TSAb might be also associated with osteoclastogenesis and bone resorption.

Correlation analysis showed that the levels of serum PTH were positively correlated with serum BAP in all groups, and with serum OC in men. In addition, serum PTH was an independent variable for serum BAP and OC in the same groups. It is well known that PTH binds to the cells of osteoblast lineage (80), and produces both anabolic and catabolic effects on the bone. In the aspect of anabolic effect, PTH increases proliferation, differentiation of osteoblasts (81-86) and decreases apoptosis of osteoblasts (87, 88). However, in multivariate analysis, the effect of serum TSAb on biochemical parameters of bone remodeling was independent of serum PTH. According to this result, serum TSAb may acts through a distinct mechanism from PTH signaling pathway.

These findings suggest that serum TSAb might be involved in bone remodeling, especially in osteoblastogenesis and bone formation. Considering that the low serum TSH levels are associated with bone loss in hyperthyroidism, serum TSAb can produce a positive effect on bone remodeling by restoration of deficient TSH signaling pathway in patients with Graves' disease. Although the number of patients is small and the result is not statistically significant, the analysis of the patients with serum TBAb supports this hypothesis. In contrast with serum TSAb, serum TBAb showed negative tendency in association with the biochemical parameters of osteoblastogenesis, bone formation, and bone coupling.

However, most patients with Graves' disease had bone loss in spite of the presence of serum TSAb. The possible explanation is that the levels of serum TSAb are not sufficient to compensate the deficient TSH signaling pathway in patients with Graves' disease, especially in controlled disease status. In our study, serum TSAb was positively correlated with the biochemical parameters of bone formation in men and premenopausal women, but not in postmenopausal women. The results were persistent after being adjusted by age to eliminate the effect of aging on bone metabolism. Partially, this finding might explain the higher rates of osteoporosis and fracture risk in postmenopausal women with Graves' disease.

To investigate the effects of TSAb on osteoblastogenesis and bone formation, we examined the ALP activity and the expression of osteoblastogenic genes in murine osteoblasts, differentiated from mesenchymal C3H10T1/2 cells. The effects of TSH were evaluated in the same manner. However, unlike the result of statistical analysis, treatment with TSAb revealed no significant change in ALP activity. Treatment with TSH decreased ALP activity significantly compared to negative control. The expressions of ALP, OC, and CTx did not reveal significant differences from negative control after treatment with both TSAb and TSH. We did not evaluate cAMP response to TSAb and TSH.

It is not well understood about TSHR signaling pathways in osteoblast and osteoclast (55, 59, 70, 72, 89). TSHR is a seven-transmembrane, glycosylated G protein coupled receptor on

thyrocytes and other cell lines. Most TSHR signals are transduced by Gs/cAMP/protein kinase A pathway. However, several studies failed to elicit cAMP response to TSH in bone cells (72, 89). TSH is reported to mediate signal transduction via Gq/phospholipase C (PLC)/protein kinase C (PKC) pathway in preadipocytes, and thyrocytes (90-92). In addition, TSH transduces signals through Gq/PLC/PKC pathway, insulin-like growth factor (IGF)/phosphoinositide-3-kinase pathway, and non-canonical Wnt 5a/frizzled coreceptor pathway in murine ES cells (58). In human osteoblast-like (SaOS2) cells, TSH regulates signal transduction by IGF/IGF binding protein pathways (93). These are cAMP-independent pathways, and TSH or TSAb might activate TSHR signaling pathways through any of these mechanisms. Some authors insisted that the levels of TSHR in bone cells might be insufficient to generate detectable cAMP response (94).

There is little evidence about the effect of TSAb on bone metabolism (57). In the two studies with primary bone marrow cells, TSH and monoclonal TSHR agonist antibody produce inhibitory effect on osteoclastogenesis (57, 95, 96).

Several limitations are present in this study. First, we only evaluate biochemical parameters of bone turnover as surrogate markers of bone loss. Second, patients with serum TBAb was too small to evaluate its clinical relevance. The association of serum TSAb, TBAb with BMD or risk of fracture should be investigated in the large number of patients. Third, we could not show the effects of TSH and TSAb on osteoblastogenesis and bone formation. The possibility of cAMP-independent TSHR signaling pathway should be considered in subsequent experiments.

In conclusion, serum TSAb is associated with biochemical parameters of osteoblastogenesis and bone coupling, independent of the effects of circulating thyroid hormone in patients with Graves' disease. Serum TSAb might have a protective role to osteoporosis in men and premenopausal women with Graves' disease by restoration of deficient TSH signaling pathway. TSHR agonist,

such as rhTSH, monoclonal TSHR antibodies, or synthetic small molecules (97) might be used for therapeutic options to maintain bone homeostasis. Further investigations are needed to identify the effects of TSH and TSAb on bone remodeling and to find clinical applications.

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요약 (국문초록)

배경: 갑상선기능항진증에서 발생하는 골다공증은 전통적으로 갑상선호르몬의 효과에 의해 발생하는 것으로 알려져 왔다. 하지만 최근 연구들에서 갑상선자극호르몬이 갑상선호르몬의 효과와는 별개로 골에 직접적으로 작용한다는 결과들을 보고하고 있다. 그레이브스병에서 갑상선자극항체는 갑상선자극호르몬 수용체를 통해 작용하기 때문에, 갑상선자극항체 역시 골 대사에 직접적인 효과를 보일 수 있다.

목적: 그레이브스병 환자에서 혈청 갑상선자극항체의 골 대사 대한 보호효과를 평가하기 위해 연구를 수행하였다.

재료 및 방법: 그레이브스병을 새로 진단받은 환자들을 대상으로 혈청 갑상선자극항체와골 대사와 관련된 생화학적 지표들의 연관성에 대해 평가하였다. 혈청 갑상선자극항체의 조골세포에 대한 효과를 확인하기 위해 생쥐의 조골세포에서 알칼리성 인산가수분해효소의 활성과 조골세포의 형성과 관련된 유전자의 발현을 평가하였다.

결과: 139명의 환자가 연구에 포함되었다. 이 중 혈청 갑상선자극항체를 가지고 있는 환자는 127명(남자 25명, 폐경 전 여자 83명, 그리고 폐경 후 여자 19명)이었고 혈청 갑상선억제항체를 가지고 있는 환자는 12명이었다. 혈청 갑상선자극항체를 가지고 있는 환자에서 혈청 트리요오드티로닌과 유리 티록신, 갑상선자극항체 및 부갑상선호르몬 등이 g혈청 골 특이적 알칼리성 가수분해효소(BAP), osteocalcin (OC) 및 C-terminal telopeptide of type Ⅰ collagen (CTx) 등과 의미 있는 상관관계가 있었다. 다변량 회귀분석에서는 남자에서 혈청 갑상선자극항체가 혈청 OC의 독립적인 인자였고(P=0.019), 폐경 전 여자에서 혈청 갑상선자극항체가 혈청 BAP 및 OC의 독립적인 인자였다 (P=0.043, 및 P=0.043). 생쥐의 조골세포를 이용한 *in vitro* 실험에서 갑

상선자극호르몬과 갑상선자극항체를 처리하였을 때 알칼리성 인산가수분해효소의 활성도와 BAP, OC 및 CTx 등의 발현에는 의미 있는 변화가 없었다.

결론: 그레이브스병 환자에서 혈청 갑상선자극항체는 갑상선호르몬의 효과와 독립적으로 조골세포의 형성 및 골 coupling을 반영하는 생화학적 지표들과 관련이 있었다. 그레이브스병이 있는 남자와 폐경 전 여자 환자에서 혈청 갑상선자극항체는 골다공증에 대해 보호 역할을 할 수 있다.

주요어: 갑상선자극항체; 골 특이적 알칼리성 인산가수분해효소; Osteocalcin; C-terminal telopeptide of type Ⅰ collagen

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