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Interleukin-6 is not essential for bone turnover in hypothyroid mice

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Abstract: Interleukin-6 (IL-6) has been shown to be involved in the pathogenesis of several bone diseases characterized by an imbalance between bone resorption and formation. The aim of the study was to estimate serum markers of bone turnover: osteoclast-derived tartrate-resistant acid phosphatase form 5a (TRACP 5b) and osteocalcin in IL-6-deficient mice to assess the role of IL-6 in bone metabolism in hypothyroidism in mice. C57BL/6J (wild-type; WT) and C57BL/6J$^{IL6-/-Kopf}$ (IL-6 knock-out; IL6KO) mice randomly divided into 4 groups with 10 in each one: 1/ WT mice in hypothyroidism (WT-ht), 2/ WT controls, 3/ IL6KO mice with hypothyroidism (IL6KO-ht) and 4/ IL6KO controls. Experimental model of hypothyroidism was induced by intraperitoneal injection of propylthiouracyl. The serum levels of TRACP 5b and osteocalcin were determined by ELISA. Serum concentrations of TRACP 5b (median and interquartile ranges) were significantly decreased in both groups of mice with hypothyroidism: WT (3.2 (2.5-4.7) U/l) and IL6KO (2.6 (1.8-3.5) U/l) as compared to the respective controls. Similarly, serum osteocalcin levels were significantly reduced in both groups of mice in experimental hypothyroidism: WT (25.8 (23.0-28.2) ng/ml) and IL6KO (21.5(19.0-24.6) ng/ml) in comparison to the respective controls. There were no significant differences in bone turnover markers between IL6KO and WT mice both in hypothyroid and control animals. The results of the present study suggest that IL-6 does not play an important role in bone turnover in both euthyroid and hypothyroid mice.

Key words: IL-6 - Hypothyroidism - IL-6 knockout mice - Bone turnover

Introduction

Activation of the signaling pathway mediated by glycoprotein(gp)-130 by interleukin-6 (IL-6) and its soluble receptor (sIL-6R) was regarded as a pivotal mechanism for the regulation of osteoclastogenesis [1,2]. Nevertheless, in IL-6 knockout (IL6KO) mice no specific changes in bone structure were found [3,4]. Moreover, in gp-130 deficient mice the amount of osteoclasts was highly elevated [5]. These data suggest that IL-6 does not influence resorption and normal bone turnover. However, IL6KO mice were protected against increased bone resorption after acute estrogen depletion owing to ovariectomy [6]. IL-6 was also shown to be involved in other processes associated with accelerated bone turnover such as Paget disease, multiple myeloma, rheumatoid arthritis and renal osteodystrophy [7-10]. Moreover selective inhibitor of IL-6 activity has been documented to suppress bone mass reduction [11]. However, recently published results of the study on IL6KO mice have demonstrated that in hyperparathyroidism IL-6 is not required for the osteoclast formation and bone loss [12]. Lately we have observed highly elevated osteocalcin levels in IL6KO mice in thyrotoxicosis, suggesting crucial role of IL-6 in bone formation in hyperthyroidism [13]. Data on role of IL-6 in bone turnover in hypothyroidism are lacking.

Thus, the aim of the present study was to estimate serum markers of bone turnover: osteoclast-derived tartrate-resistant acid phosphatase form 5a (TRACP 5b) reflecting resorption, and osteocalcin as a marker

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of bone formation in IL-6-deficient mice to assess the role of IL-6 in the bone turnover in hypothyroidism.

Materials and Methods

Experimental animals. The study was performed on forty, 14-15 weeks old, female mice: C57BL/6J (wild-type; WT) and C57BL/6J$^{IL6-/-Kopf}$ (IL6KO) weighing 18-22 g at the beginning of the experiment. The IL6KO mice used in this study were obtained from the Department of Cardiology and Angiology of Medical School in Hannover. All animals had free access to standard granulated diet and drinking water. The animals were housed in plastic cages at 22.1°C and constant humidity, with a 12/12 light/dark cycle, beginning at 7 am.

The mice were randomly divided into 4 groups with 10 mice in each one: 1/ WT mice in hypothyroidism (WT-ht), 2/ WT controls (WT-ctrl), 3/ IL6KO mice in hypothyroidism (IL6KO-ht) and 4/ IL6KO controls. Experimental model of hypothyroidism was induced by intraperitoneal injection of propylthiouracyl at the dose of 60 μg/kg daily over 21 days. Control mice were injected vehicle under the same experimental conditions.

Genotyping. Genomic DNA for IL-6 genotyping was isolated from mouse tails using "Genomic mini" kit (A&A Biotechnology, Gdansk, Poland) according to the enclosed protocol. PCR was performed using DNA polymerase Taq "Marathon" (A&A Biotechnology, Gdansk, Poland) and custom made primers (F 5'-AAGTGCATC ATCGTTGTTCATAC-3'; R 5'-CCATCCAGTTGCCTTCTTG-3'). Twenty nine PCR cycles were performed under following conditions: 94°C 5 seconds, 94°C 20 seconds, 55°C 30 seconds, 72°C 2 min 50 seconds, 72°C 7 seconds, 10°C at the end of the procedure. Then DNA was separated by electrophoresis on the 1% agarose gel with ethidium bromide.

Thyroid tissues. To verify an animal model of hypothyroidism mice were thyreoidectomized. Both thyroid lobes were fixed in Bouin's fluid for 24 hours in temperature of +4°C and embedded in paraffin in a routine procedure. The specimens were cut into 5 μm slices and stained by haematoxylin-eosin. The histological preparations were subjected to analysis, using Olympus Bx50 microscope.

Osteodensitometry. Bone mineral density (BMD) was examined in a-p position by the dual X-ray absorptiometry using Lunar DPX densitometer (Lunar Corporation, Madison, WI, USA) with small animal software. BMD measurements were performed by the same experienced operator. The densitometer was calibrated everyday with a standard phantom specimen. The following sites were examined: the total body, tibia, trochanter and vertebrae L2-L4. BMD results were obtained in absolute values (mg/cm^2).

Blood specimens. At the end of the experiment, the animals were anaesthetized with ketamine (100 mg/kg) and xylazine-HCL (10 mg/kg), their abdomen was opened by midline incision and the blood was taken from the abdominal aorta of each mouse for measurement of serum concentrations of osteoclast-derived tartrate-resistant acid phosphatase form 5a (TRACP 5b), osteocalcin and creatinine. The blood was collected to polypropylene tubes without anticoagulant and was incubated in room temperature until the clot was formed and then centrifuged (2500 × g, 15 minutes). The supernatant (serum) was removed and stored at -70°C until a consequtive analysis. The serum levels of TRACP 5b and osteocalcin were determined by enzyme-linked immunosorbent assay commercial kits: TRACP 5b (MouseTRAP Assay, SBA Sciences, Turku, Finland; sensitivity 0.1 U/l; intra-assay precision 6.5%; inter-assay variation 8%) and osteocalcin (Mouse Osteocalcin EIA kit, Biomedical Technologies Inc., Stoughton, USA; sensitivity 1 ng/ml; intra-assay precision 6%; inter-assay variation 8%). Serum creatinine concentration was measured by autoanalyzer using standard laboratory methods.

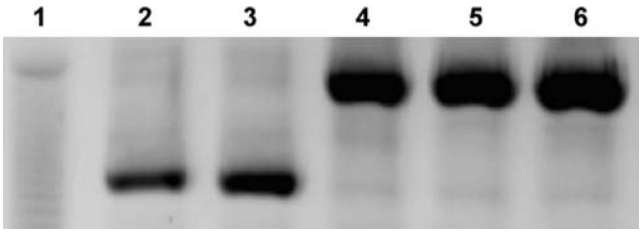

Fig. 1. IL-6 Genotyping of wild-type and IL6KO animals. DNA electrophoresis on agarose gel. Lane 1- DNA ladder, lanes 2 and 3- wild-type (C57BL/6J) animals, lane 4 to 6- C57BL/6J$^{IL6-/-Kopf}$ mice.

Statistical analysis. The statistical significance was estimated by Mann-Whitney U-test. To evaluate relationships between variables Spearman's test was performed using Statistica 6.0 for Windows XP (StaSoft, Tulsa, USA).

Ethical issues. All procedures were performed in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Local Ethics Committee in Bialystok.

Results

IL-6 genotyping

Material from wild-type animals yielded DNA fragments with size ca. 900 bp, whereas DNA fragments from IL6KO animals contained also a fragment of neomycin cassette and were size about 1400 bp (Fig. 1).

Thyroid specimens

The efficacy of the animal model of hypothyroidism has been confirmed by the histological picture of thyroid glands. The thyroids had a follicular, encapsulated structure in the control and experimental mice. The differences between the central and peripheral follicles in thyroids obtained from both IL6KO and WT control mice were observed. The central follicles had a smaller diameter, the colloid was less dense, and the follicular epithelium was higher, whereas the peripheral follicles were larger, delimited by flat cuboid epithelium and homogenous, intensely stained colloid (Fig. 2A and 2B). Examination of thyroid sections of IL6KO and WT animals treated with propylthiouracyl pointed to thyroid function blockade with predominance of microfollicular hyperplasia with poor in colloid hyperplastic follicles of irregular shape. (Fig. 2C and 2D).

Osteodensitometry

BMD values (in mg/cm^2) are shown in Table 1. We have not found any significant differences between hypothyroid an control WT and IL6KO mice.

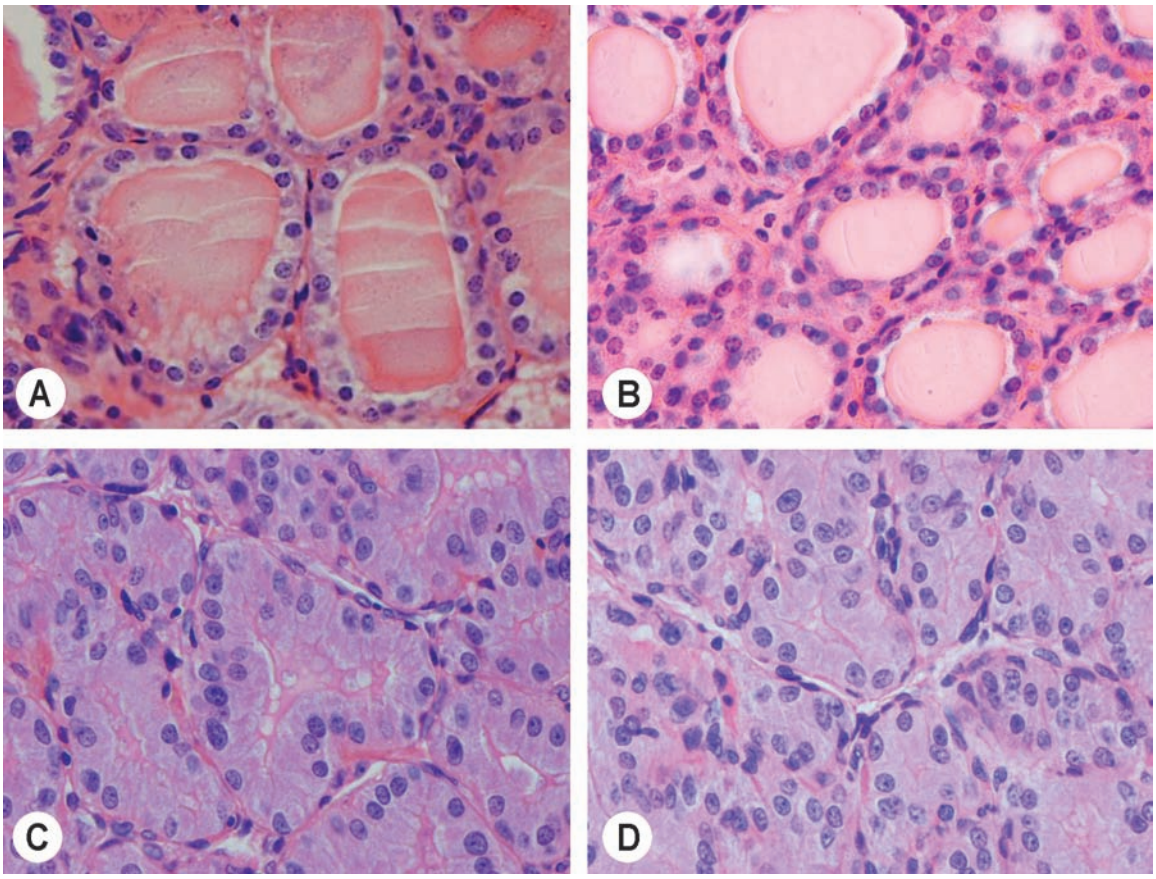

Fig. 2. Light micrograph of thyroid gland (magnitude × 400.000): (**A**) C57BL/6J, (**B**) C57BL/6J$^{IL6-/-Kopf}$, (**C**) C57BL/6J treated with propylthiouracyl, (**D**) C57BL/6J$^{IL6-/-Kopf}$ treated with propylthiouracyl.

Bone turnover serum markers

Moreover, significantly diminished values of TRACP 5b and osteocalcin as markers of bone turnover in propylthiouracyl injected mice as compared to relative controls may also serve as the confirmation of efficacy of the animal model of hypothyroidism. Serum concentrations of TRACP 5b (median and interquartile ranges) were significantly decreased in both groups of mice with hypothyrosis (Fig. 3): WT (3.2 (2.5-4.7) U/l) and IL6KO (2.6 (1.8-3.5) U/l) as compared to the respective controls: WT (8.4 (6.4-10.5) U/l) and IL6KO (8.0 (5.0-9.8) U/l).

Also, as can be seen in Fig.4, serum osteocalcin concentrations (median and interquartile ranges) were significantly diminished in both groups of mice in experimental hypothyroidism: WT (25.8 (23.0-28.2) ng/ml) and IL6KO (21.5(19.0-24.6) ng/ml) as compared to the respective controls: WT (32.5 (27.6-37.3) ng/ml) and IL6KO (30.5 (26.3-42.5) ng/ml).

There were no significant differences between creatinine serum concentrations (median and interquartile ranges) in studied groups of mice with hypothyroidism: WT (0.17 (0.12-0.22) mg/dl) and IL6KO (0.18 (0.12-0.25) mg/dl) as compared to the respective controls: WT (0.17 (0.12-0.23) mg/dl) and IL6KO (0.20 (0.13-0.28) mg/dl).

Discussion

The exact mechanism by which thyroid hormones regulate osteoblasts and osteoclasts function at a cellular level remains unclear. Osteoblasts were documented to synthesize thyroid receptors [14]. The presence of osteoblasts seems to be a necessary condition for osteoclasts to resorp bone in response to triiodotyronine [15]. Triiodotyronine was reported to stimulate

Table 1. Bone Mineral Density measures of total body, tibia, trochanter and lumbar vertebrae 2-4 of studied mice (in mg/cm^2). No significant differences between groups were found ($p>0.05$).

	WT-ht	WT-ctrl	IL6KO-ht	IL6KO-ctrl
Total body	220±6	220±5	222±8	219±6
Tibia	151±7	154±8	150±8	153±7
Trochanter	153±9	156±10	154±10	154±8
Vertebrae L2-L4	151±8	154±8	153±8	153±6

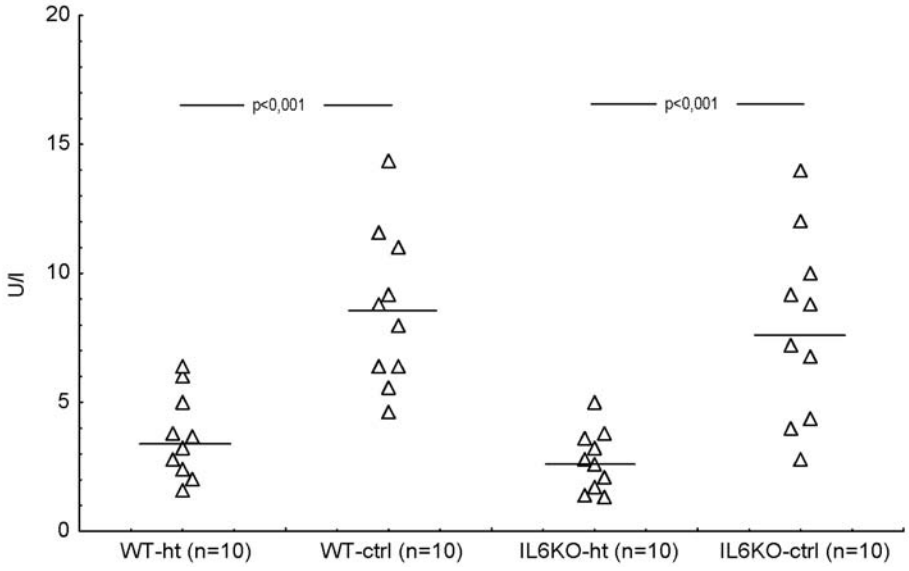

Fig. 3. The individual and median (–) TRACP 5b serum levels of hypothyroid and control WT mice as well as hypothyroid and control IL6KO mice.

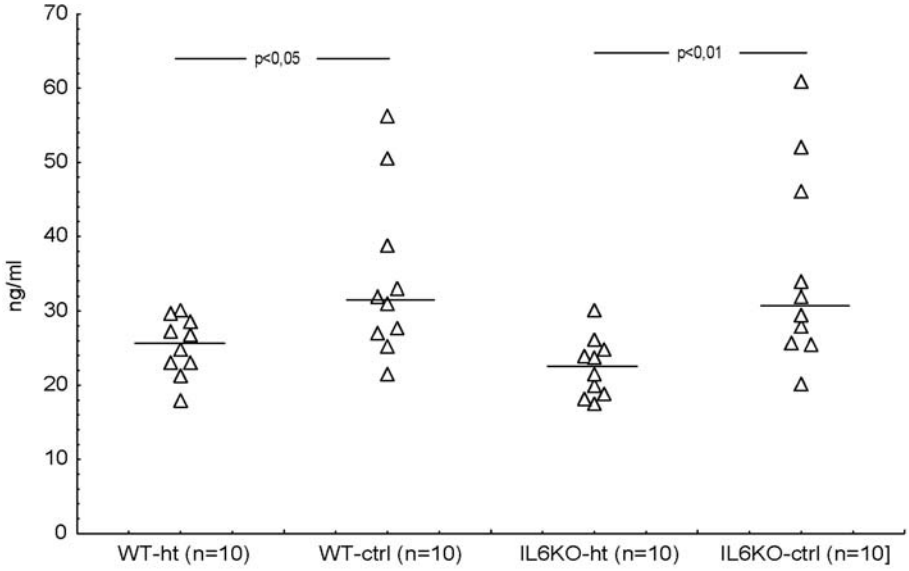

Fig. 4. The individual and median (–) osteocalcin serum levels of hypothyroid and control WT mice as well as hypothyroid and control IL6KO mice.

osteoblast activity both directly and indirectly via growth factors and cytokines [16,17]. The action of triiodotyronine on osteoclastic bone resorption has been suggested to be mediated predominantly by osteoblast-derived osteotrophic cytokines such as IL-1, IL-6, IL-8 and Tumour Necrosis Factor α [16,18]. Data from the studies on osteoblastic bone marrow stromal cells and osteoblast cell lines have reported that triiodotyronine stimulates IL-6 expression and augments IL-1-induced stimulation of IL-6 production [19,20].

IL-6 has been shown to be involved in the pathogenesis of several bone diseases characterized by a negative balance between bone resorption and formation. We have found lately unexpectedly increased osteocalcin serum concentration in response to elevated bone resorption in IL6KO mice in experimental thyrotoxicosis [13]. This finding suggest that IL-6 inhibits osteocalcin production and probably generally bone formation in hyperthyroidism in mice. There are no data in literature on the role of IL-6 in bone turnover in hyperthyrosis. Our present study has shown that both bone formation and resorption markers are decreased in hypothyroidism both in WT and IL6KO. There were no differences between WT and IL6KO hypothyroid mice as to BMD and TRACP 5b, reflecting resorption rate as well as to osteocalcin, as bone formation marker. Diminished indicators of bone turnover in both IL6KO and WT mice may serve as an indirect confirmation of efficacy of hypothyroidism. Decreased bone metabolism markers were found in hypothyroid patients [21]. Comparable levels of TRACP 5b in IL6KO and WT mice may suggest that

IL-6 does not play an important role in the induction of bone resorption in normal and hypothyroid mice, as far as TRACP 5b molecules has been considered a useful marker of bone resorption rate [22,23]. IL-6 was suggested to be not a powerful bone-resorping factor in its own right, but a molecule capable to enhance the effects of other factors on bone resorption, such as NF-kappa B ligand (RANK-L) [24]. Moreover, in culture IL-6 was found to stimulate osteoclast formation and bone resorption of mouse calvaria only slightly in comparison to IL-1 [25]. Also in hyperthyroid patients the increase in the levels of serum IL-6 was not related directly with bone resorption seen in hyperthyroidism [26]. Experimentally induced increase in bone resorption leading to bone loss is in general accompanied with elevated bone formation, probably as an attempt of the organism to maintain the bone balance [27]. In the previous study in thyrotoxic IL-6 lacking mice we observed greatly higher elevation of osteocalcin, suggesting a crucial role of this cytokine in inhibition of bone formation. Osteocalcin is an osteoblast product that constitutes the most abundant noncollagenous protein present in bone and its serum levels closely correlate with histomorphometric parameters of bone formation [28]. In the present study lack of difference in serum osteocalcin as well as TRACP 5b in hypothyroid WT and IL6KO mice suggest that IL-6 is not crucial for the maintenance of the balance between bone resorption and formation in hypothyroidism in mice. It seems that IL-6 is in general an important osteotropic factor in the state of increased bone turnover and has minor role in physiological bone remodeling and during processes associated with inhibited bone turnover.

To sum up, the results of the present study suggest that IL-6 does not play an important role in bone turnover in both euthyroid and hypothyroid mice.

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