Thyroid hormone stimulates basal and interleukin (IL)-1-induced IL-6 production in human bone marrow stromal cells: a possible mediator of thyroid hormone-induced bone loss

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Abstract

It is well known that excessive thyroid hormone in the body is associated with bone loss. However, the mechanism by which thyroid hormone affects bone turnover remains unclear. It has been shown that it stimulates osteoclastic bone resorption indirectly via unknown mediators secreted by osteoblasts. To determine if interleukin-6 (IL-6) or interleukin-11 (IL-11) could be the mediator(s) of thyroid hormone-induced bone loss, we studied the effects of 3,5,3'-tri-iodothyronine (T_3) on basal and interleukin-1 (IL-1)-stimulated IL-6/IL-11 production in primary cultured human bone marrow stromal cells. T_3 at 10^{-12} – 10^{-8} M concentration significantly

increased basal IL-6 production in a dose-dependent manner. It also had an additive effect on IL-1-stimulated IL-6 production, but failed to elicit a detectable effect on basal or IL-1-stimulated IL-11 production. Treatment with 17 β -estradiol (10^{-8} M) did not affect the action of T_3 on IL-6/IL-11 production. These results suggest that thyroid hormone may stimulate bone resorption by increasing basal and IL-1-induced IL-6 production from osteoblast-lineage cells, and these effects are independent of estrogen status.

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Introduction

It has been established that excessive thyroid hormone in the body is associated with bone loss. Bone mass is reduced in patients with hyperthyroidism or receiving long-term thyroid hormone suppression therapy (Toh *et al.* 1985, Diamond *et al.* 1991, Allain & McGregor 1992), and individuals with a history of thyrotoxicosis have an increased risk of fracture (Cummings *et al.* 1995). Studies on biochemical markers (Lee *et al.* 1990, Harvey *et al.* 1991) and bone histomorphometry (Bordier *et al.* 1967, Mundy *et al.* 1976, Mosekilde & Melsen 1978) showed increased bone turnover in thyrotoxic patients, suggesting that predominantly osteoclastic activity is responsible for the bone loss in thyrotoxicosis. However, the exact mechanism by which thyroid hormone stimulates bone resorption remains unclear.

It has been shown that thyroid hormone directly stimulates bone resorption in organ culture of neonatal mouse calvaria (Klaushofer *et al.* 1989) and fetal rat limb bones (Mundy *et al.* 1976, Hoffmann *et al.* 1986). Thyroid hormone receptors have been demonstrated in osteoblastic cell lines such as ROS 17/2.8 (Rizzoli *et al.* 1986), UMR-106 (LeBron *et al.* 1989) and MC3T3-E1 (Kasano *et al.* 1988), and direct effects of tri-iodothyronine (T_3) on

proliferation and differentiation of human osteoblast-like cells have also been documented (Kassem *et al.* 1993). In contrast, there has been a paucity of data on the direct functional effects of thyroid hormone on osteoclasts, despite a report that human osteoclastoma cells express T_3 receptors (Allain *et al.* 1996). Previous studies suggested that the action of T_3 on bone is mediated by osteoblasts. Allain *et al.* (1992) and Britto *et al.* (1994) showed that T_3 stimulated osteoclastic bone resorption in the presence of osteoblasts, but not in their absence. Furthermore, stimulation of resorption also occurred if osteoblasts were pretreated with T_3 and then osteoblast-osteoclast coculture was carried out in the absence of T_3 . These findings imply that thyroid hormone indirectly stimulates osteoclasts via a mediator produced by osteoblasts. However, the mediator responsible for the activation of osteoclasts by thyroid hormone has yet to be elucidated.

Osteoblasts and bone marrow stromal cells produce bone-active cytokines such as interleukin-6 (IL-6) and interleukin-11 (IL-11) which are critical for osteoclast formation and bone resorption (Manolagas 1995, Girasole *et al.* 1992, 1994). These cytokines have been suggested to mediate the effects of many stimulators of bone resorption such as parathyroid hormone, interleukin-1, and tumor necrosis factor- α . On the basis of these studies, it can be

postulated that thyroid hormone stimulates IL-6 and/or IL-11 production by osteoblastic cells, resulting in increased bone resorption. To test this hypothesis, we examined the effects of thyroid hormone on basal and IL-1-stimulated IL-6 and IL-11 production in primary cultured human bone marrow stromal cells (hBMSCs). In addition, we investigated the possible influence of estrogen on thyroid hormone action.

Materials and Methods

Materials

3,5,3'-Tri-iodothyronine (T_3), 3,3',5'-tri-iodothyronine (reverse T_3 , rT_3), recombinant human IL-1 α and α -modified minimum essential medium (α -MEM) were purchased from Sigma Chemical Co. (St Louis, MO, USA). IL-6 ELISA kits were obtained from Genzyme (Cambridge, MA, USA), and IL-11 ELISA kits from R&D Systems (Minneapolis, MN, USA). The detection limits of IL-6 and IL-11 assays were 18 and 8 pg/ml, and coefficients of variation were 9.5 and 8.9% respectively.

hBMSC culture

hBMSCs were isolated from ribs discarded at the time of open thoracotomy as described previously (Kim *et al.* 1996, 1997b). None of the patients from whom the ribs were obtained had metabolic bone disease, thyroid disease, or were taking thyroid hormone. Briefly, the ribs were excised aseptically, cleaned of soft tissues, and opened longitudinally. The exposed bone marrow was flushed out using several washes of serum-free α -MEM. The medium with flushed bone marrow was centrifuged at 1400 r.p.m. for 10 min. Cell pellets were resuspended in culture medium, and enriched bone marrow stromal cells were obtained by Ficoll/Hypaque (specific gravity 1.077) gradient centrifugation. The cells were seeded into a 75 cm² plastic culture flask at a density of 4×10^5 cells/cm² and cultured in α -MEM containing 10% fetal bovine serum, penicillin and streptomycin (100 U/ml and 100 μ g/ml respectively). The medium was changed twice weekly from the second week, and when the cells were grown to 80% confluence, they were then subcultured using 0.01% trypsin and 0.05% EDTA. The second-passage cells were used for the experiments.

Effect of T_3 on basal IL-6 and IL-11 production by hBMSCs

T_3 and rT_3 were dissolved in ethanol and the initial 10^{-5} M stock solution was serially diluted to concentrations of between 10^{-8} M and 10^{-12} M. The vehicle was added to control cultures, and the final concentration of ethanol in the culture medium did not exceed 0.1%.

The cells (3×10^4 /well) were subcultured in a 48-well plate containing α -MEM and 5% charcoal-stripped serum for 2 days. Subsequently, the medium was replaced with fresh medium containing various doses of T_3 (10^{-12} – 10^{-8} M) or rT_3 (10^{-8} M), and then cultured for an additional 72 h. After 72 h, the conditioned medium was collected, centrifuged free of cell debris, and stored at -20°C until IL-6 and IL-11 assay. The concentration of IL-6 and IL-11 in the medium was measured by ELISA using commercial kits. The number of cells in each well was determined by quadruplicate hemocytometer counts of trypsin-EDTA-released cells at the end of the culture. Data are expressed as the amount of IL-6 or IL-11 produced per 10^5 cells.

Effect of T_3 on IL-1-stimulated IL-6 and IL-11 production by hBMSCs

Cells were seeded into a 48-well plate, cultured as described above, and treated with various doses of IL-1 (10, 100, 1000 U/ml) with or without the addition of T_3 (10^{-8} M). Then the amounts of IL-6 and IL-11 produced in the subsequent 72 h of culture were measured in the conditioned medium.

Influence of 17 β -estradiol on the effect of thyroid hormone

To examine the possible influence of estrogen on the effect of thyroid hormone on basal and IL-1-stimulated IL-6/IL-11 production, the cells were treated with 17 β -estradiol (10^{-8} M) for 24 h before the addition of T_3 and/or IL-1. The results were compared with those from groups not treated with estrogen in the same set of experiments.

Statistics

All experiments were repeated at least three times using different hBMSC preparations, and representative data are shown in the figures. The significance of the differences between treatment groups was assessed using the Mann-Whitney U-test or ANOVA and post-hoc analysis with Duncan's multiple range test as appropriate. Dose-response relationships were examined by Spearman's rank correlation analysis.

Results

Effect of T_3 on basal IL-6 and IL-11 production by hBMSCs

Treatment with T_3 , over the concentration range 10^{-12} – 10^{-8} M, significantly increased hBMSC IL-6 production in a dose-dependent manner (Fig. 1A). rT_3 , an inactive analog of T_3 , did not elicit a response at a dose of 10^{-8} M (data not shown).

Figure 1 Effects of T_3 on basal IL-6 (A) and IL-11 (B) production in hBMSCs. Cells were treated with various concentrations of T_3 for 72 h, and the amount of IL-6/IL-11 produced in the medium was expressed relative to the number of cells in each well. Values are means $\pm$ S.D. ($n=6$). * $P<0.05$, ** $P<0.01$ vs control.

In contrast, T_3 had no detectable effect on IL-11 production (Fig. 1B).

Effect of T_3 on IL-1-stimulated IL-6 and IL-11 production by hBMSCs

As previously reported (Kim *et al.* 1997b), IL-1 (10–1000 U/ml) dose-dependently stimulated both IL-6 and IL-11 production in hBMSCs. T_3 (10^{-8} M) additively increased the IL-1-induced IL-6 production at various concentrations of IL-1 (Fig. 2A). However, IL-1-induced IL-11 production was not affected by T_3 treatment (Fig. 2B).

Influence of 17β -estradiol on the effect of thyroid hormone

Treatment with 17β -estradiol (10^{-8} M) inhibited IL-1-induced IL-6 production, but not T_3 -stimulated IL-6 production (Fig. 3A). IL-6 production co-stimulated by T_3 and IL-1 was partially inhibited by 17β -estradiol, but it was still greater than that stimulated by T_3 or IL-1 alone.

Figure 2 Effects of T_3 on IL-1-induced IL-6 (A) and IL-11 (B) production in hBMSCs. Cells were stimulated with IL-1 (10–1000 U/ml) with or without T_3 (10^{-8} M), and the amounts of IL-6/IL-11 produced during 72 h were measured in the medium. Values are means $\pm$ S.D. ($n=6$). ** $P<0.01$ vs group without T_3 .

There was no significant difference in IL-11 production in the presence or absence of 17β -estradiol (Fig. 3B).

Discussion

In the present study, T_3 significantly increased basal and IL-1-stimulated IL-6 production in hBMSCs. These results support the possibility that increased IL-6 production by osteoblast-lineage cells plays an important role in thyroid hormone-induced bone loss. In line with this possibility, Lakatos *et al.* (1997) reported that serum IL-6 concentrations are elevated in patients with hyperthyroidism, and blood mononuclear cells derived from hyperthyroid patients secrete more IL-6 than those taken from healthy subjects. They also showed that bone turnover is increased and radius bone mineral content is reduced in patients with hyperthyroidism. Our results are also consistent with the report of Siddiqi *et al.* (1998) demonstrating that T_3 increased both secretion and mRNA expression of IL-6 and IL-8 by hBMSCs and MG63 osteoblast-like cell lines.

Figure 3 Effects of 17 β -estradiol (E₂) on T₃ and/or IL-1-stimulated IL-6 (A) and IL-11 (B) production in hBMSCs. The cells were treated with 17 β -estradiol (10⁻⁸ M) for 24 h before the addition of T₃ (10⁻⁸ M) and/or IL-1 (1000 U/ml). The amounts of IL-6/IL-11 produced during 72 h were compared with those in groups not treated with estrogen. Values are means $\pm$ S.D. (n=6). *P<0.05, **P<0.01 vs group without E₂.

Tarjan & Stern (1994, 1995) reported that T₃ potentiated the stimulatory effect of IL-1 on IL-6 production in neonatal mouse calvarial osteoblasts and fetal rat limb bone cultures, which is in agreement with our results with hBMSCs. However, our results show that T₃ alone significantly increased basal IL-6 production. Tarjan & Stern (1995) failed to observe the stimulatory effect in fetal rat limb bone culture of T₃ on basal IL-6 production over a 10⁻¹¹–10⁻⁶ M concentration range. They observed that T₃ alone increased ⁴⁵Ca release significantly only at a fairly high concentration (10⁻⁶ M), but in the presence of IL-1, 10⁻⁸ M T₃ significantly potentiated the IL-1 effect on ⁴⁵Ca release. Treatment with IL-1 receptor antagonist blocked the potentiating effects of T₃. They therefore speculated that the enhancement of IL-1-induced IL-6 production might be a biologically relevant mechanism of thyroid hormone-induced bone loss. On the other hand, our data showed that T₃, in the absence of IL-1, also directly stimulated IL-6 production. The reason for this discrepancy is not clear, but may be related to the difference in species of cell origin or stage of osteoblast phenotype expression (Williams *et al.* 1994). It has also

been suggested that the presence/absence or relative levels of various forms of thyroid hormone receptor (α , β_1 , β_2) in osteoblastic cells may explain the variability of the response to thyroid hormone (Suwanwalaikorn *et al.* 1997). Alternatively, we cannot exclude the possibility that endogenous IL-1 production is higher in human than rat bone cell cultures. However, it was reported that human bone marrow stromal cells did not constitutively produce significant amounts of IL-1 β , although low levels of expression of IL-1 β mRNA were detected (Aman *et al.* 1994).

Our results show that the effect of thyroid hormone on IL-6 production was not affected by estrogen. Consistent with this, Lakatos *et al.* (1997) observed that there was no difference in pre- and post-menopausal IL-6 levels in hyperthyroid patients. Taken together, the results suggest that thyroid hormone and estrogen act via different mechanisms in regulating IL-6 production. A recent meta-analysis report showed that suppressive doses of thyroid hormone therapy had a detrimental effect on all skeletal sites in postmenopausal women, whereas it had little effect in premenopausal women (Uzzan *et al.* 1996). Our experimental data and those of others (Tarjan & Stern 1994, 1995) showing the synergistic effect of thyroid hormone and IL-1, which is increased in the estrogen-deficient state, on IL-6 secretion may explain this phenomenon. However, further studies are needed to clarify this controversial issue.

IL-11 is a fairly recently discovered cytokine which has a role in osteoclast development (Girasole *et al.* 1994). The effects of thyroid hormone on IL-11 secretion have not yet been studied. We observed in this study that neither basal nor IL-1-induced IL-11 production is affected by thyroid hormone. However, we cannot exclude the possibility that thyroid hormone affects IL-11 production synergistically with other systemic or local cofactors (Manolagas 1995, Kim *et al.* 1997b) that exist *in vivo*.

In this study, T₃ had a detectable effect on IL-6 secretion at concentrations much lower than those found in human plasma (about 10⁻⁹ M). Under the experimental conditions used, free hormone concentrations in the medium may be higher than those found *in vivo*, as culture medium containing 5% serum would contain less T₃-binding protein than *in vivo*. At present, however, the physiological implication of this finding is difficult to clarify even if we measure the free T₃ concentrations in the culture medium, because the local thyroid hormone concentration to which bone cells are exposed *in vivo* may be different from the circulating concentration. Further studies are needed.

It should be pointed out that the hBMSCs used in this study are not mature osteoblasts. However, previous studies have shown that, when cultured to confluence in the presence of serum, these cells possess many of the phenotypic characteristics of differentiated osteoblasts, including deposition of mineralized matrix (Kassem *et al.* 1991, Cheng *et al.* 1994). They produce type I procollagen

and osteocalcin in response to 1,25-dihydroxyvitamin D₃ (Kassem *et al.* 1991) and also increase cAMP in response to parathyroid hormone (Cheng *et al.* 1994). We also confirmed that these cells deposit calcium in the extracellular matrix, and express mRNA characteristic of osteoblastic cells, such as alkaline phosphatase, α 1(I)collagen, osteopontin, and decorin (Kim *et al.* 1997a). Another possible limitation of using these cells is that they may exhibit heterogeneity between samples because of different proportions of preosteogenic, preadipogenic and prechondrogenic cell precursors. However, this should not be a significant problem because we cultured the cells under the same standardized conditions and we repeated the experiments at least three times using different hBMS cells preparations to confirm the consistency of the results.

In conclusion, these results suggest that thyroid hormone may increase bone resorption by stimulating osteoclast formation and activity by increasing basal and IL-1-induced IL-6 production by osteoblastic cells.

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