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Thyroid hormones increase insulin-like growth factor mRNA levels in the clonal osteoblastic cell line MC3T3-E1

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Abstract

Thyroid hormones are known to affect skeletal growth and maturation by influencing both bone resorption and bone formation. Their exact mechanism of action, however, is still unknown. Local factors such as prostaglandins, TGF-β or IGF-I were suggested to mediate their effects. Thyroid hormones were reported to stimulate expression of IGF-I mRNA in liver and kidney and to increase IGF-I release from bone organ cultures and osteoblast-like cells. Therefore we studied the effect of thyroid hormones on IGF-I mRNA expression in MC3T3-E1 cells. The cells were grown in culture for 5 to 7 days and treated with triiodothyronine (10^{-11}–10^{-6} M) and thyroxin (10^{-6} M) for 1–24 h. Cellular mRNA was isolated and subjected to Northern hybridization. The amount of IGF-I mRNA, which is already expressed in this cell line under control conditions, was markedly enhanced by T3 and T4. This effect was found to be dose-dependent with a maximum at 10^{-7}M and could already be seen after 3 h increasing up to 24 h. Our findings indicate that IGF-I expression in osteoblasts is directly regulated by thyroid hormones. We conclude that IGF-I expression belongs to the phenotypic characteristics of mature osteoblasts, and that thyroid hormones play an important role in differentiation of MC3T3-E1 cells along the osteoblastic lineage.

Key words: Bone; Osteoblast-like cell; Thyroid hormone; IGF-I

1. Introduction

Thyroid hormones are essential for normal growth and development in children and affect bone remodelling in adults. In vivo and in vitro data indicate that they influence both bone formation as well as bone resorption. Hyperthyroidism and hypothyroidism are accompanied by disturbances of bone and calcium metabolism [1]. Thyroidectomy in experimental animals resulted in delayed growth and skeletal maturation which effect was reversed by thyroxin (T4) treatment [2].

Based on in vitro data a direct effect of T4 and triiodo-L-thyronine (T3) on bone cells was postulated in previous investigations. Direct stimulation of bone resorption was observed in cultures of fetal rat limb bones [3,4] and of neonatal mouse calvaria [5–7]. This is supported by the finding of specific thyroid hormone receptors in isolated nuclei from neonatal mouse calvaria [8], MC3T3-E1 cells [9] and in rat osteosarcoma cells [10,11].

Although direct influence of thyroid hormones on bone cell functions was clearly established, their exists some evidence that their effects on bone remodelling are mediated by local factors produced by the bone cells. Thus prostaglandins [7] and TGF-β [12] seem to mediate partially the effect on bone resorption. On the other hand some investigators suggested IGF-I as a likely candidate to mediate their effect on bone formation [13,14]. Indeed, IGF-I, one of the most prevalent growth factors synthesized by bone cells and present in bone, is known to be regulated by systemic hormones such as parathyroid hormone [15–17] and estrogens [18] as well as local factors such as prostaglandin E_2 [19].

More recently it was shown that thyroid hormones and growth hormone interact to regulate IGF-I mRNA and circulating levels in the rat and that thyroid hormones induce IGF-I expression in liver and kidney cells at the mRNA and protein level [20]. Based on these observations we were interested whether thyroid hormones could influence IGF-I synthesis in bone cells at the mRNA level. We used MC3T3-E1 cells since previous investigations showed that this osteoblastic cell line has the capacity to synthesize IGF-I under basal and hormone stimulated culture conditions [21,22]. We now show that both T4 and T3 increase IGF-I mRNA levels in MC3T3-E1 cells. Since this work had been initiated it was reported that T3 stimulates IGF-I and IGF-I binding protein-2 production by rat osteoblasts in vitro [23] as well as IGF-I content in the medium of rat bone tissue [24].

2. Materials and methods

2.1. Cell culture

MC3T3-E1 cells were kindly donated by Dr. Kumegawa (Meikai University, Department of Oral Anatomy, Sakado, Saitama 35002, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Laboratories, Grand Island, NY) with 4.5 g/l glucose, supplemented with 5% or 10% fetal calf serum (FCS, Boehringer Mannheim, Germany) 30 μg/ml Gentamycin (Sigma, Germany) at 37°C under 5% CO_2 in humidified air. They were subcultured twice a week using 0.001% pronase E and 0.02% EDTA in Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS). For experiments MC3T3-E1 cells were plated at a density of 2×10^5 cells/cm^2. The stimulation experiments were performed 5 to 7 days after seeding at about 80% confluency. For treatment the medium was removed and the cell layer was

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washed with PBS. After preincubation in DMEM supplemented with 2% horse serum (cut-off MW 1000, heat-inactivated at 56°C, Sigma, Germany) for 1 h, the cells were incubated for 24 h in the same medium with or without T4 (10^{-6} M) and T3 (10^{-11}–10^{-6} M) for the induction experiments. The dialysed horse serum was used to be sure to have no unspecific effects caused by low molecular weight factors present in the serum supplement. For the time course experiments culture was stopped at the respective time points.

2.2. RNA-isolation and Northern hybridization

Cytoplasmic RNA was isolated using the mini-prep method of M. Wilkinson [25] and the total amount of RNA was estimated by measuring the absorption at 260 nm with a Hitachi spectrophotometer. Northern hybridization was performed by fractionating 10μg total RNA on a 1% agarose gel containing 2.2 M formaldehyde according to Maniatis et al. [26]. After electrophoresis the gel was soaked in 50 mM NaOH for about 20 min and transferred to a nylon filter (NEN, Brussels) with 20 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M Na-citrate). After baking the filter for 2 h at 80°C, hybridization was done for about 10–16 h in 10% Dextransulfate, 1 M NaCl and 1% sodium-dodecylsulfate after 10 min pre-hybridization in the same solution. For estimation of the amount of mRNA, the filters were exposed for 6 to 10 days and the autoradiograms were digitised and loaded into the NIH-Image program (Wayne Rasband, NIH). The density was estimated using the gel blotting macro. The hybridization probe was generated by amplification of reverse transcribed RNA with both primers TCTTCACATCTCTTCTACCTGG (5′ primer) and GGTCTTGTTTCCTGCACTTCC (3′ primer). The amplified fragment was cloned into the *SmaI* site of the SK⁻ bluescript Phagemid (Stratagene, La Jolla, CA). The cloned IGF-I cDNA fragment was verified by sequencing. As a control we hybridized the same Northern blots using the *XhoII* to *BglI* fragment of rat GAPDH kindly provided by Dr. Busslinger (Research Institute of Molecular Pathology, Vienna). Probe labelling was performed either by transcription with [^{32}P]UTP using the phage promoter T3 or T7 according to the suppliers suggestions or with PCR [27] using the described PCR-primer.

3. Results

Fig. 1a shows the Northern blot of RNA isolated from untreated and thyroid hormone-treated (10^{-7} M T3 or 10^{-6} M T4) cells. Both mRNAs for IGF-I [28] are detectable in our cell line but are expressed in different amounts. Compared to controls an about 3-fold increase of IGF-I mRNA was found after treatment with 10^{-7} M T3 or 10^{-6} M T4 for 24 h. Concerning the two types of IGF-I mRNAs no difference in enhancement was detectable. Fig. 1b shows the hybridization of the same filter with the GAPDH probe for control of loaded RNA. The experiment was repeated four times with similar results concerning the stimulatory effect of T3 and T4 although the control levels of IGF-I mRNA showed interexperimental variance. We assume that the IGF-I mRNA levels in untreated MC3T3-E1 cells depend on the differentiation state of the cell line as we observed a constant rise of IGF-I mRNA during prolonged incubation time of the cells (see also time course experiments in Fig. 3).

As we could not find any significant differences between the effects of T4 and T3 we used only T3 for further experiments. Fig. 2a shows the dose–response curve of T3 (10^{-6}–10^{-11} M) on IGF-I mRNA expression. In these experiments the increase of IGF-I mRNA levels at the highest concentration (10^{-6} M T3) was about

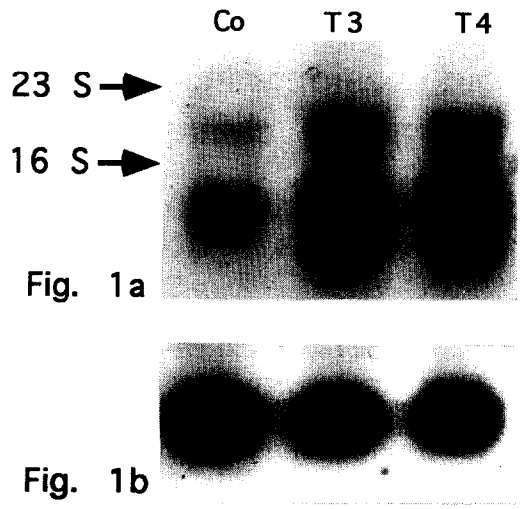

Fig. 1. (a) Expression of IGF-I mRNA in untreated (Co), T3 (10^{-7} M) and T4 (10^{-6} M) treated MC3T3-E1 cells (treatment time: 24 h). Total RNA (10 μg) was analyzed by Northern blotting using the IGF-I probe. Both IGF-I mRNAs are detectable. Arrows indicate the position of the markers (16 S/23 S rRNA, *E. coli*). (b) Control hybridization of the blot shown in a with the GAPDH probe to show the amount of loaded mRNA.

4-fold. The dose–response relationship found in our experiments is comparable to results from the literature concerning the effects of T3 on alkaline phosphatase activity in these cells [10].

To get a further insight into the mechanism of the stimulatory effect of T3 on IGF-I expression, time course experiments (1, 3, 6 and 24 h) were performed. As shown in Fig. 3a there was no increase of IGF-I mRNA levels found after 1 h. Already 3 h after start of the incubation a stimulatory effect of T3 could be detected with an increase at 24 h. It also can be seen that the control levels of IGF-I mRNA in untreated cells shows a constant rise with prolonged incubation time which might reflect growth and differentiation of these cells during culture. Nevertheless at 3, 6 and 24 h a distinct stimulatory effect of T3 was measured which reached an extent of about twice to threefold at 24 h.

4. Discussion

The result of our study show for the first time that thyroid hormones have the capacity to enhance IGF-I mRNA levels in the osteoblastic cell line MC3T3-E1. This stimulatory effect was dose-dependent with a maximum between 10^{-7} M and 10^{-6} M T3 which is in good agreement with data concerning other biological effects of thyroid hormones in bone organ cultures or osteoblastic cells such as alkaline phosphatase activity [9]. Our finding is also consistent with recent studies which report an increase in medium IGF-I in response to thyroid hormones in cultures of fetal rat tibiae [29], UMR-106

cells and in fetal rat limb bones [24]. Significantly increased IGF-I levels were reported in the media of UMR-106 cells by the second day of culture with no further increase up to five days [23]. However, the increase of the message in MC3T3-E1 cells occurs already during the first 24 h.

Interestingly, T3 has not only the capacity to stimulate IGF-I expression, synthesis and secretion in osteoblastic cells but could also stimulate the expression of IGF-I receptor mRNA as reported for chondrocytes [30]. This could indicate that at the level of the basic multicellular units (BMUs) under the influence of thyroid hormones certain cell types produce local growth factors while others express the respective receptors.

In our earlier study we found that locally produced prostaglandins could act as mediators of the effects on bone formation and resorption [7]. Prostaglandin E2 is known to be a strong stimulator of IGF-I synthesis in osteoblastic cells [19] therefore we also tested whether the effect of T3 on IGF-I expression could be influenced by indomethacin. The lack of inhibitory influence of the cyclooxygenase inhibitor (data not shown) indicates that the effect of T3 on IGF-I expression is not mediated by prostaglandins. Thus prostaglandins and IGF-I independently could cooperate in mediating the anabolic effects of thyroid hormones on bone as it is also discussed for parathyroid hormone [31,32].

Studies with isolated osteoblasts and cocultures of osteoblasts and osteoclasts indicated that osteoblasts are the primary site of action for thyroid hormones [33,34]. Indeed specific receptors are present on osteoblastic cells [8–11] and thyroid hormones are believed to exert their effects on bone cells through these nuclear receptors. In addition, putative plasma membrane binding sites for thyroid hormones in bone tissue mediating direct non genomic stimulation of the inositol phosphate second messenger system were postulated [35]. From our experiments we believe that neither a direct action of the thyroid hormone/receptor complex on the IGF-I gene nor the stimulation of second messenger pathways can explain the observed increase in IGF-I message. We conclude this from the time course experiments which show a delayed effect (after 3 h) and from experiments with cycloheximid which itself stimulates IGF-I expression

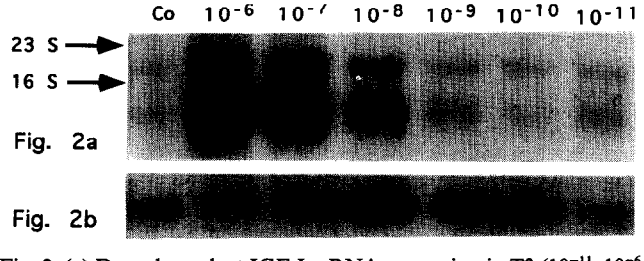

Fig. 2. (a) Dose-dependent IGF-I mRNA expression in T3 (10^{-11}–10^{-6} M) treated MC3T3-E1 cells (Co, no treatment; treatment time, 24 h). 10 μg RNA were analyzed by Northern blotting. (b) Control hybridization of the blot shown in a with the GAPDH probe.

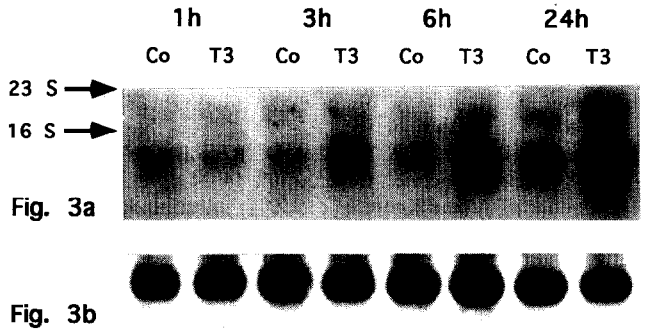

Fig. 3. (a) Time course of the IGF-I mRNA expression in untreated (Co) and T3 (10^{-7} M) treated MC3T3-E1 cells. 10 μg RNA were analyzed by Northern blotting. (b) Control hybridization of the blot shown in a with the GAPDH probe.

and does not alter the effect of T3 (data not shown). The latter result could be explained by the existence of a regulated repressor protein [36].

MC3T3-E1 cells in prolonged cultures are known to differentiate from a preosteoblastic phenotype to mature osteoblasts [37]. T3 treatment accelerates this differentiation, shown by stop of proliferation and increase of differentiated functions such as alkaline phosphatase activity [9] and prostaglandin synthesis [38]. We speculate that the observed increase in IGF-I expression is also a consequence of accelerated osteoblastic differentiation of MC3T3-E1 cells after T3 treatment. The primary effect of thyroid hormones on bone cells therefore could be stimulation of osteoblastic differentiation. IGF-I expression and synthesis, consequently, belong to the important phenotypic characteristics of mature osteoblasts which lay down and calcify the bone matrix.

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