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Triiodothyronine, a Regulator of Osteoblastic Differentiation: Depression of Histone H4, Attenuation of c-fos/c-jun, and Induction of Osteocalcin Expression

F. Varga, M. Rumpler, E. Luegmayr, N. Fratzl-Zelman, H. Glantschnig, K. Klaushofer

Ludwig Boltzmann-Institute of Osteology, 4th Medical Department, Hanusch Hospital, Heinrich Collinstrasse 30, A-1140 Vienna, Austria

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Abstract. Thyroid hormones influence growth and differentiation of bone cells. *In vivo* and *in vitro* data indicate their importance for development and maintenance of the skeleton. Triiodothyronine (T3) inhibits proliferation and accelerates differentiation of osteoblasts. We studied the regulatory effect of T3 on markers of proliferation as well as on specific markers of the osteoblastic phenotype in cultured MC3T3-E1 cells at different time points. In parallel to the inhibitory effect on proliferation, T3 down-regulated histone H4 mRNA expression. Early genes (c-fos/c-jun) are highly expressed in proliferating cells and are down-regulated when the cells switch to differentiation. When MC3T3-E1 cells are cultured under serum-free conditions, basal c-fos/c-jun expressions are nearly undetectable. Under these conditions, c-fos/c-jun mRNAs can be stimulated by EGF, the effect of which is attenuated to about 46% by T3. In addition, T3 stimulated the expression at the mRNA and protein level of osteocalcin, a marker of mature osteoblasts and alkaline phosphatase activity. All these effects were more pronounced when cells were cultured for more than 6 days. These data indicate that T3 acts as a differentiation factor in osteoblasts by influencing the expression of cell cycle–regulated, of cell growth–regulated, and of phenotypic genes.

Key words: Triiodothyronine — MC3T3-E1 cells — c-fos — Osteocalcin — Histone H4.

The osteoblastic cell line MC3T3-E1 is used as a model system to study the differentiation of osteoblasts *in vitro*. This immortalized cell line derived from mouse calvaria [1] shows a culture time dependent pattern of gene expression reflecting different stages of osteoblastic differentiation [2] similar to primary osteoblasts isolated from mouse [3] or rat calvaria [4].

The ordered expression of genes during osteoblastic differentiation can be divided into three distinct periods [5]. The first period is characterized by proliferation and expression of genes of the mitotic activities like the early genes of the activator protein-1 complex (AP-1), c-jun and c-fos, but also of c-myc and the core histones. During this period also proteins involved in the formation of the extracellular matrix (ECM) are expressed. At the end of this period, proliferation ceases and a transition from proliferation to differentiation can be observed. At this transition point a down-regulation of the cell cycle regulated histone was described [6, 7, 8]. Proteins of the mature bone cell phenotype like alkaline phosphatase (ALP) are now detected. After the transition point c-myc and c-fos are nearly undetectable [7, 8, 9]. The third period is characterized by expression of more bone-related proteins like bone sialoprotein, osteopontin, and osteocalcin (OC) and by mineralization of the extracellular matrix. The expression of OC, a bone-specific protein with unknown function, not only depends on the differentiation state of the bone cells but also is regulated by hormones and local factors. The complex regulation of OC is reflected by its promoter with numerous regulatory elements [10]. Several glucocorticoid responsive elements [11] and at least three AP-1 binding places were found within the rat and mouse OC promoter [12]. One is located directly within a vitamin D responsive element. At this region a ligand-activated vitamin D receptor (VDR) binds to induce OC gene expression [9]. Several reports exist that the thyroid hormone receptor (TR) also binds to this regulatory area [13] and that T3 is able to induce OC gene expression in osteoblasts [14]. Besides regulation of OC, thyroid hormones induce several other bone-related genes [15, 16, 17], but, more important, they stop proliferation of MC3T3-E1 cells [16, 17, 18, 19] and of primary rat osteoblasts [20].

The regulatory protein c-fos is considered to be important for the regulation of skeletal development [21]. Deregulated c-fos expression interferes with normal bone development [22], leading to excessive new bone formation and/or tumors [23]. The heterodimer with c-jun (AP-1) is generally accepted to play a key role in the transition between proliferating and differentiating osteoblasts. Expression of AP-1 can prevent differentiation by inhibition of the expression of tissue-specific genes. These findings together with the observations that T3 influences both proliferation and expression of tissue-specific genes in osteoblasts opens the possibility that T3 regulates OC expression directly by binding to the gene promotor but also indirectly by influencing c-fos/c-jun expression during down-regulation of proliferation.

Correspondence to: K. Klaushofer

In this article we show that T3, which is known to inhibit proliferation of osteoblasts, also down-regulates the histone H4 gene expression, followed by an up-regulation of the OC gene expression. In addition, T3 attenuates epidermal growth factor (EGF) induced c-fos mRNA expression in nonproliferating MC3T3-E1 cells. These results indicate that T3 plays an important role in the osteoblastic differentiation process.

Materials and Methods

Cell Culture

MC3T3-E1 cells (kindly donated by Dr. Kumegawa, Meikai University, Department of Oral Anatomy, Sakado, Japan) were cultured in αMEM (Sebak, Suben, Austria) supplemented with 4.5 g/liter glucose, 5% FCS (Sebak, Suben, Austria), and 30 μg/ml Gentamycin (Sebak, Suben, Austria) at 37°C under 5% CO_2 in humidified air. They were subcultured twice a week using 0.001% pronase E (Boehringer Mannheim, Germany) and 0.02% EDTA in Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS). To prevent a potential phenotypic drift during repeated subcultures, the cells were not used for more than 4 weeks after thawing.

Alkaline Phosphatase Activity and Cell Growth

Cells were seeded in 96 multiwell microplates and cultured with or without 10^{-7} M T3 for 4 and 8 days. Then the media were removed and the cells were washed twice with PBS and frozen at −20°C. During subsequent thawing, 50 μl Hoechst 33258 solution (5 μg/ml, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1 mM EDTA) were added to each well. The amount of DNA was quantitated by measuring the fluorescence in a multiwell fluorometer (excitation 355/emission 460; SLT, Salzburg, Austria). DNA was estimated using a standard curve prepared from calf thymus DNA (Boehringer Mannheim, Germany). Before measuring ALP activity in the same multiwells, the plates were frozen again. After thawing, the cells were incubated for 20 minutes with 50 μl 20 mM p-nitrophenylphosphate in 100 mM diethanolamine and 10 mM $MgCl_2$ at pH 9.5. The reaction was stopped with 0.1 M EDTA in 1M NaOH. The absorption was measured at 405/490 nm in a microplate reader. ALP activity was estimated using a standard curve prepared from calf intestinal alkaline phosphatase (Boehringer Mannheim, Germany). Statistical evaluation was done with Statview-4.0 (Abacus Concepts, Inc., Berkeley, CA) using ANOVA.

Expression of Osteocalcin, c-fos, c-jun, and Histone H4 mRNA

For long-term T3 treatment cells were seeded at a density of 50,000 cells/cm^2 in Petri dishes cultured overnight in αMEM, supplemented with 4.5 g/liter glucose, 5% FCS, and 30 μg/ml Gentamycin at 37°C under 5% CO_2 in humidified air. Thereafter cultures were continued to grow with or without 10^{-7} M T3 for 4 days or 8 days. On day 4 untreated cells reached a density of about 2.1×10^5/cm^2 and T3-treated cells about 1.7×10^5/cm^2. On day 8 untreated cells reached a density of about 3.6×10^5/cm^2 and T3-treated cells about 2.0×10^5/cm^2. Medium change was performed every two days. OC mRNA expression was studied on days 4 and 8 without any further treatment. For study of c-fos and c-jun expression on days 4 and 8, medium was changed to αMEM containing 0.1% bovine serum albumin (BSA, Boehringer Mannheim, Germany) with or without 10^{-7} M T3. After 2 hours, some cells were treated with 10 ng/ml EGF (Boehringer, Mannheim, Germany) for 30 min.

Short-term T3 treatment was performed in cells seeded at 50,000 cells/cm^2 in 100 mm Petri dishes and cultivated for 4 days or 8 days in αMEM supplemented with 5% FCS. For the time course experiments, 10^{-7} M T3 was added to the culture medium and cultivated further for 1, 3, 6, and 24 hours. Dose response was studied after 5 hours of T3 treatment with 10^{-11} M, 10^{-9} M, 10^{-7} M, 10^{-6} M T3 or without T3 in culture medium without a medium change. Calculation of the influence of cycloheximide (CHX) on T3-induced OC expression was done both for cultures treated with 10^{-7} M T3 and 10 μg/ml CHX for 5 hours and for those not so treated.

Expression studies of histone H4 were done in cultures 4 days or 6 days after seeding, and some cultures were treated with 10^{-7} M T3 for 24 hours.

RNA-Isolation and Northern Hybridization

Cytoplasmic RNA was isolated from about 3.6×10^7 cells using a mini-prep method [24] at the time points given above. The total amount of RNA was estimated by measuring the absorption at 260 nm with a Hitachi spectrophotometer. Northern hybridization was performed by fractionating 10 μg total RNA on a 1% agarose gel containing 2.2 M formaldehyde [25]. After electrophoresis the gel was soaked in 0.1 M Tris-HCl and 0.15 M NaCl for 5 min and transferred to a nylon filter (NEN, Brussels) with 20 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate). After baking the filter for 2 hours at 80°C, hybridization was done overnight in 10% dextransulfate, 10 μg/ml shared salmon sperm DNA, 1 M NaCl, and 1% sodium dodecylsulfate after 1 hour prehybridization in the same solution. For estimation of the amount of mRNA, the filters were evaluated in an Instant Imager (Packard Instrument Company, Meriden, CT). As a hybridization probe we used the c-fos cDNA [26], c-jun cDNA [27], the histone H4 cDNA, and the mouse OC gene [28]. As a control we hybridized the same northern blots using the Pst I fragment of rat GAPDH. All probes were kindly provided by Dr. Busslinger (Research Institute of Molecular Pathology, Vienna, Austria). Probe labeling was performed by multiprime labeling according to the supplier's suggestions (Boehringer Mannheim, Germany).

Radioimmunoassay of OC

To estimate the concentration of OC protein in culture media and in the cell layer/matrix fraction we used a commercial RIA kit (Biomedical Technologies Inc. [BTI], Stoughton, MA). According to the supplier's description of the RIA, there is no cross-reaction with bovine OC. The RIA did not detect any measurable OC in our culture media. Addition of 0.2% Triton X-100 in PBS had no influence on the measurements.

Cells were seeded at a density of 50,000 cells/cm^2 in 96 multiwell culture plates in αMEM supplemented with 5% FCS and cultured overnight. On the next day cultures were treated with 10^{-7} M T3. For short-time treatment T3 was added 24 hours before analysis of the culture media. At days 4, 7, 10, and 13 the culture media were removed and frozen. We performed single measurements of 10 μl culture media from each well. The results are given as μg OC/ml culture media. In addition, OC levels were measured in the cell layer/matrix fraction on days 4 and 8.

For this purpose the whole fraction was solubilized by a short sonification with 0.2% Triton X-100 in PBS and the extracts clarified by a brief centrifugation step. 40 μl of the supernatants were used for the RIA and 20 μl for the protein BCA assay with BSA as standard (Sigma, Germany). The results are given as ng OC/μg protein. Statistical analysis was performed with ANOVA.

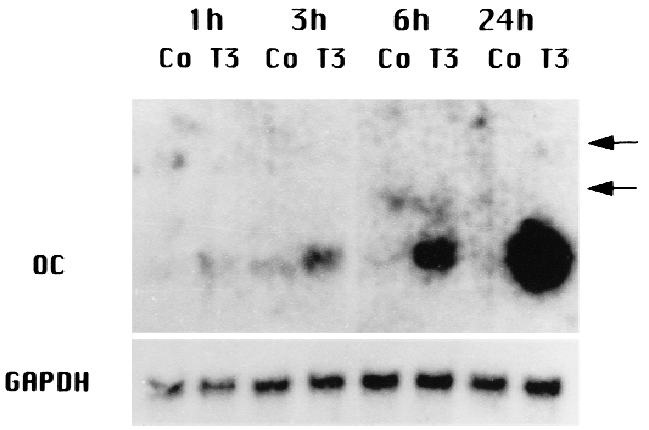

Fig. 1. Time course of T3-stimulated OC expression. Northern blots of RNA isolated from MC3T3-E1 cells treated for 1, 3, 6, and 24 hours with 10^{-7} M T3 (T3) or without (Co) after 8 days of culture are shown. Control hybridizations were performed using a rat GAPDH probe. Arrows indicate the 23S/16S ribosomal RNA markers.

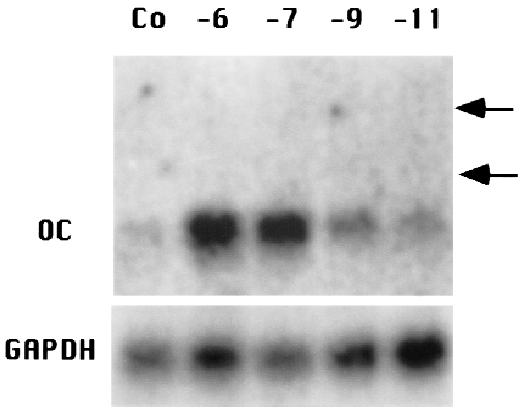

Fig. 2. Dose-response of T3-stimulated OC expression. Northern blots of RNA isolated from MC3T3-E1 cells treated for 5 hours without (Co) or with 10^{-11} M, 10^{-9} M, 10^{-7} M, or 10^{-6} M T3 after 8 days of culture are shown. Control hybridizations were performed using a rat GAPDH probe. Arrows indicate the 23S/16S ribosomal RNA markers.

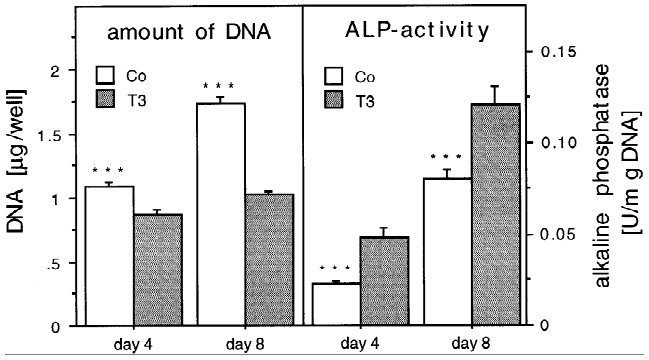

Fig. 3. T3 inhibits proliferation of MC3T3-E1 cells and stimulates ALP activity on days 4 and 8. The amount of DNA and ALP activity of cells treated for 4 or 8 days with (T3) or without (Co) 10^{-7} M T3 were measured. Bars represent means ± SEM from 8 cultures. ***, $P < 0.001$ (treated vs. control).

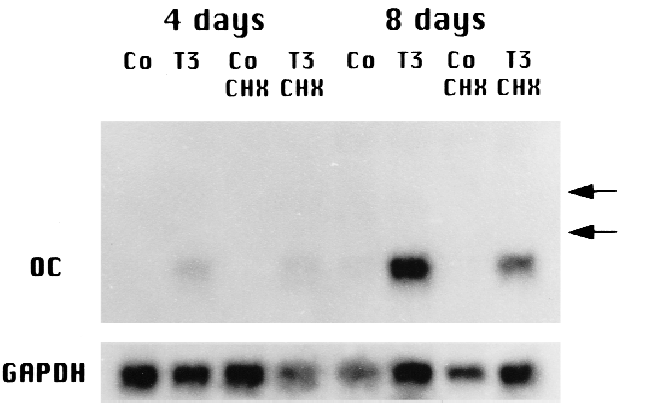

Fig. 4. T3-stimulated OC mRNA expression depends on culture time. Northern blots of RNA isolated from MC3T3-E1 cells treated for 5 hours with (T3) or without (Co) 10^{-7} M T3 after 4 and 8 days of culture are shown. Cycloheximide (CHX) was added to inhibit *de novo* protein synthesis. Control hybridizations were performed using a rat GAPDH probe. Arrows indicate the 23S/16S ribosomal RNA markers.

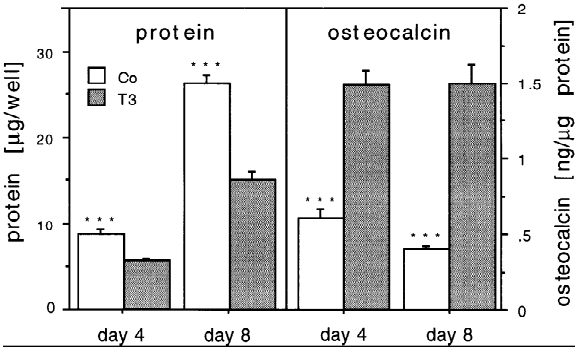

Fig. 5. T3 stimulates OC protein synthesis in the cell layer/matrix fraction on days 4 and 8. The amount of Triton-X100 soluble proteins and the OC concentration were measured. MC3T3-E1 cells were treated for 4 or 8 days with (T3) or without (Co) 10^{-7} M T3. Bars represent means ± SEM from 8 cultures. ***, $P < 0.001$ (treated vs. control).

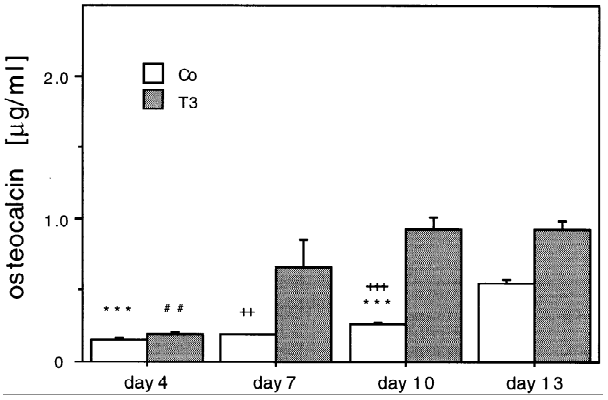

Fig. 6. T3-stimulated OC protein synthesis depends on culture time. Estimation of the OC protein concentration measured with RIA in the culture media. MC3T3-E1 cells were treated for 24 hours with (T3) or without (Co) 10^{-7} M T3 after 4, 7, 10, and 13 days of culture. Bars represent means ± SEM from 4 cultures each determined twice (8 values). ***, $P < 0.001$ (treated vs. control). ††, $P < 0.01$; †††, $P < 0.001$ (control, compared to the consecutive time point.). ##, $P < 0.01$ (T3-treated, compared to the consecutive time point.).

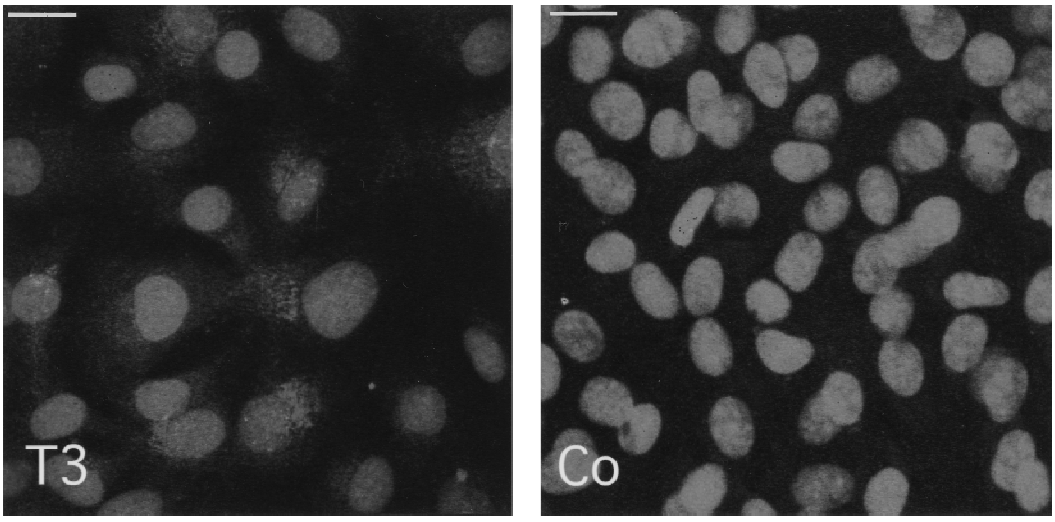

Fig. 7. T3-induced OC accumulation in the golgi of MC3T3-E1 cells. Immunohistological staining of OC of MC3T3-E1 cells treated continuously for 8 days with (T3) or without (Co) 10^{-7} M T3 are shown. (Scale bars = 10 μm.)

Immunohistological Staining

The immunolocalization of OC in MC3T3-E1 cells was performed with an anti-mouse osteocalcin antibody from goat (RIA-grade; Biomedical Technologies Inc., Stoughton, MA). MC3T3-E1 cells were cultured for 3 days or 8 days with or without 10^{-7} M T3 in chamber slides (Nunc, Naperville, IL) as described above. Thereafter, cells were fixed in methanol/acetone (3 + 1) for 10 min at −20°C. After fixation, cells were washed 3 times with PBS. Incubations were performed at room temperature for 60 min with goat anti-mouse OC antibody in a dilution of 1:10 in PBS. Immunostaining was performed with a FITC labeled mouse monoclonal antibody against goat IgG (1:30, 60 min; Sigma, Germany). Negative controls (to detect unspecific staining) were performed without the OC antibody. After staining of the nuclei with 1 μg/ml propidiumiodide in PBS the cells were viewed for immunofluorescence in a confocal laser scanning microscope (LSM TCS^{4D}, Leica, Heidelberg, Germany). For histomophometrical determination of OC-positive cells a total of 150 cells in about 14 images was counted using a 63x objective. Statistical analysis was performed with ANOVA.

Results

Treatment of 8-day-old MC3T3-E1 cells with 10^{-7} M T3 resulted in an increase of OC mRNA levels (Fig. 1). This stimulatory effect was already seen after 3 hours and increased constantly during longer treatment. The effect was dose dependent (10^{-11} to 10^{-6} M T3) and reached a plateau at 10^{-7} M (Fig. 2). The concentration 10^{-7} M T3, although clearly pharmacologic, was used for the further experiments because this concentration is widely used in the literature. In agreement with the OC results, T3 treatment also caused a significant stimulation of alkaline phosphatase activity, another osteoblastic marker (Fig. 3). Experiments with cycloheximide resulted in about a 43% inhibition of the stimulatory effect of T3 on OC mRNA levels (Fig. 4).

The stimulatory effect was also found at the protein level. When OC/μg protein was measured in the detergent extract of the cell layer/matrix fraction a slight but significant decrease was found in untreated cultures between days 4 and 8 (Fig. 5). T3 treatment resulted in significant increase of the amount of OC at both time points investigated, although the values (ng OC/μg protein) were equal on days 4 and 8 (Fig. 5). As shown in Figure 6, the OC concentration in the culture media measured by RIA was significantly increased when cells were treated for 24 hours with T3. Immunofluorescence studies using a polyclonal antibody were performed to show that T3 stimulates OC protein synthesis on the single-cell level. These experiments revealed a strong perinuclear staining indicating localization of OC in the Golgi regions (Fig. 7). By histomorphometry the percentage of OC-positive cells was determined on days 3 and 8. For this purpose a total of 150 cells for each treatment and time point was counted (Fig. 8). At both time points T3 treatment resulted in a significant increase of OC-positive cells. In contrast to MC3T3-E1 cells cultivated for 8 days, in 4-day-old cultures treated with T3 a weaker stimulatory effect on OC mRNA and protein in the culture media was found (Figs. 4 and 6).

MC3T3-E1 cells are known to differentiate along the osteoblastic phenotype during prolonged culture. This was indicated in our experiments by a small but significant increase of OC in the culture media with time as shown in Figure 9. When 10^{-7} M T3 was present during the whole culture period the concentrations of OC in the culture media were significantly higher compared to controls at all time points investigated (Fig. 9). However, the stimulation of OC production by T3 becomes very strong at day 7 after cells have reached confluence. This is also reflected at the mRNA level. The northern blot of Figure 10 shows only a weak stimulation of OC mRNA by T3 on day 4 but a strong increase on days 8 and 10.

To investigate further the relationship between OC expression and growth behavior of MC3T3-E1 cells, we studied the expression of histone H4, a marker gene of proliferation, and of c-fos/c-jun, which are important regulatory proteins. In 4-day-old cultures treated with T3 for 24 hours the expression of histone H4 mRNA was equal to that in controls. However, in 6-day-old cultures the expression of this gene was significantly reduced by 24 hours of T3 treatment (Fig. 11), supporting our published findings that T3 inhibits proliferation of confluent MC3T3-E1 cells [19]. In

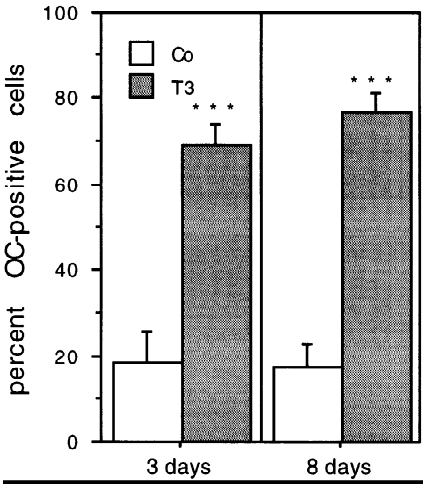

Fig. 8. Histomorphometrical determination of OC-positive cells. MC3T3-E1 cells were continuously treated with (T3) or without (Co) 10^{-7} M T3 for 3 and 8 days. Bars represent means ± SEM. ***, $P < 0.001$ (treated vs. control).

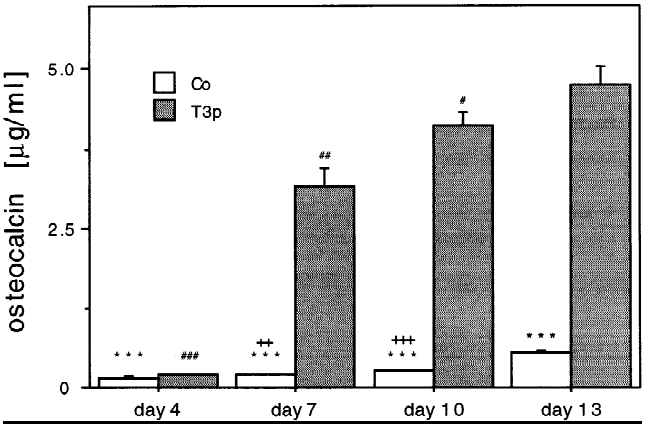

Fig. 9. Accumulation of OC in culture media permanently treated with T3. Estimation of the OC protein concentration measured by RIA in the culture media. MC3T3-E1 cells were continuously treated with (T3) or without (Co) 10^{-7} M T3 for 4, 7, 10, and 13 days. Bars represent means ± SEM from 4 cultures each determined twice (8 values). ***, $P < 0.001$ (treated vs. control). ††, $P < 0.01$; †††, $P < 0.001$ (control, compared to the following time point). #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ (T3-treated, compared to the following time point).

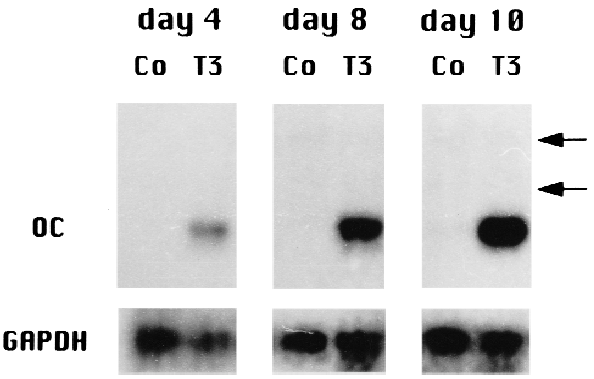

Fig. 10. Long-term treatment with T3 stimulated continuously OC expression. Northern blots of RNA isolated from MC3T3-E1 cells treated continuously for 4, 8, and 10 days with (T3) or without (Co) 10^{-7} M T3 are shown. Control hybridizations were performed using a rat GAPDH probe. Arrows indicate the 23S/16S ribosomal RNA markers.

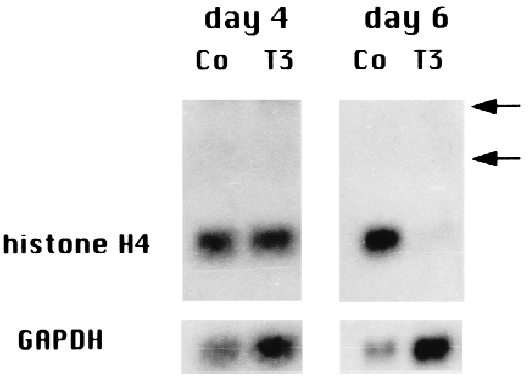

Fig. 11. T3 down-regulated histone H4 mRNA on day 6 but not on day 4 of culture. Northern blots of RNA isolated from MC3T3-E1 cells treated for 24 hours with (T3) or without (Co) 10^{-7} M T3 after 4 and 6 days of culture are shown. Control hybridizations were performed using a rat GAPDH probe. Arrows indicate the 23S/16S ribosomal RNA markers.

untreated MC3T3-E1 cells, c-fos/c-jun mRNA expression was found to be too low to be able to study down-regulating factors quantitatively. Therefore we used EGF-stimulated c-fos/c-jun expression. In cultures continuously treated with T3 for 4 days, no significant difference in c-fos/c-jun expression between controls (unstimulated or EGF-stimulated) and T3-treated cells was found (Fig. 12). Stimulation of confluent cells (after 8 days of culture) with 10 ng/ml EGF resulted in a strong induction of c-fos expression. This stimulatory effect was attenuated to 46% by T3 treatment (Fig. 12A and 12B). The effect on c-jun expression as studied by northern analysis was found to be comparable and therefore not densitometrically quantified (Fig. 12A).

Discussion

In vivo and *in vitro* data indicate that thyroid hormones influence development growth and maintenance of the skeleton and belong to the important regulators of bone formation and bone resorption [16, 29–32]. Thyroid dysfunction states result in severe disturbances of bone and calcium metabolism. Although the importance of the euthyroid state for the skeleton is well accepted from clinical observations, the exact mechanism of thyroid hormone action on bone metabolism remains unresolved. In this manuscript we present new evidence for a regulatory role of thyroid hormones in osteoblastic proliferation and differentiation. In several studies it was shown that T3 inhibits proliferation of MC3T3-E1 cells (16–19). In this article, we present evidence for a T3-induced down-regulation of the mRNA of the proliferation marker histone H4, which reflects the antiproliferative effect of T3. In parallel, T3 induces OC expression and stimulates ALP activity, both marker proteins of mature osteoblasts. Furthermore, we found that T3 attenuates EGF-induced c-fos expression. Together with c-jun, which is also attenuated by T3 at the mRNA level, c-fos

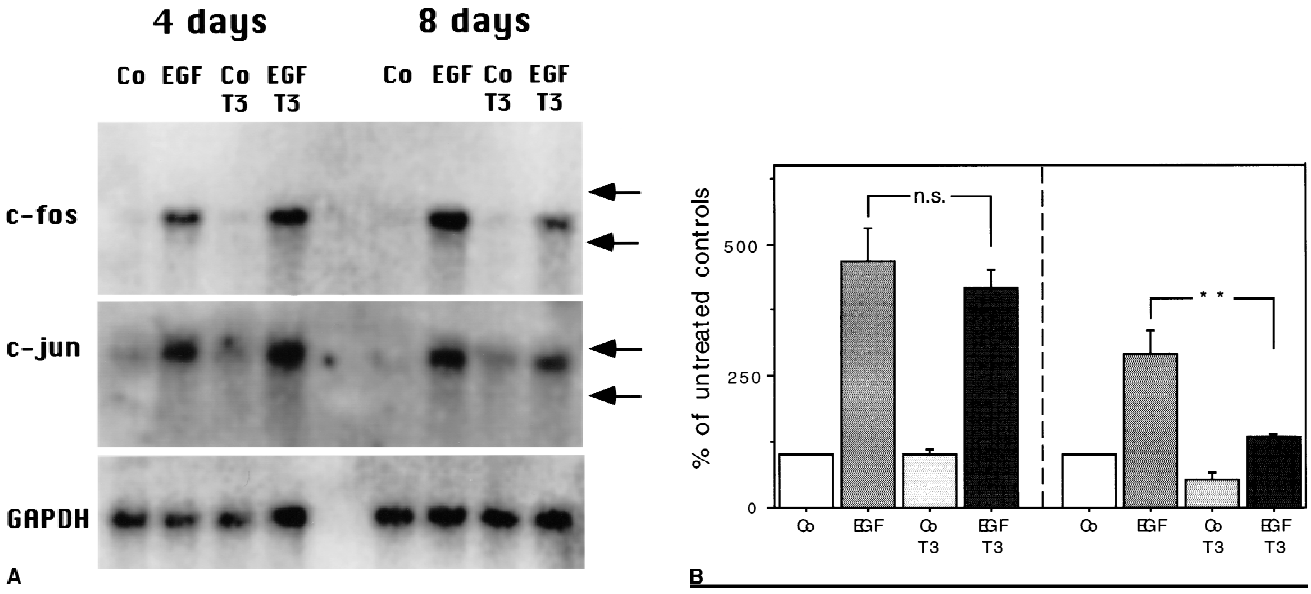

Fig. 12. T3 attenuates EGF-stimulated c-fos expression on day 8 but not on day 4 of culture. **(A)** Northern blots of RNA isolated from MC3T3-E1 cells are shown. Cells have been continuously treated with (T3) or without (Co) 10^{-7} M T3 for 4 and 8 days. Thereafter c-fos/c-jun mRNA expression was stimulated with 10 ng/ml EGF in T3 treated (T3/EGF) as well as untreated cells (Co/EGF) for 30 minutes. Control hybridizations were performed using a rat GAPDH probe. Arrows indicate the 23S/16S ribosomal RNA markers. **(B)** Densitometric analysis of the northern blots of c-fos shown in **(A)** after normalization to GAPDH. Data are given as percent of the control. Mean ± SE, $n = 3$, ** $P < 0.01$ (treated vs. control).

is a putative negative regulator of OC expression [33]. We observed all these effects in confluent MC3T3-E1 cells cultivated for more than 6 days only.

There exist several reports that thyroid hormones stimulate OC synthesis in osteoblastic cells. In primary rat calvarial osteoblasts cultured for more than 9 days T3 induces OC synthesis dose dependently during a 48-hour treatment [20]. In a recent paper, cells representing different differentiation states were studied concerning T3-induced OC expression [14]. In the fibroblastic cell line ROS 25/1 and in the undifferentiated osteosarcoma cell line UMR 106, there was no basal OC expression and T3 was unable to induce OC synthesis. However, the more differentiated osteosarcoma cell line ROS 17/2.8 displays both basal and also T3-stimulated OC synthesis [14]. Younger MC3T3-E1 cells show no basal OC expression. When cultivated for longer periods (6–8 days) they start to express OC at the mRNA and protein level in parallel to their ability to synthesize organic matrix [34]. Under our culture conditions MC3T3-E1 cells produced increasing amounts of OC mRNA and protein with culture time.

Concerning the extent of the effect of T3 on OC synthesis, proliferating MC3T3-E1 cells showed no strong increase of OC mRNA. This finding is comparable with the results found in undifferentiated UMR 106 cells [14]. However, in older MC3T3-E1 cells T3 displayed a strong induction of OC mRNA and protein which is comparable with the stimulatory effect of T3 on OC synthesis in the more differentiated ROS 17/2.8 cell line. These findings support the opinion that T3 enhances functional activities of mature osteoblasts [20].

In addition, T3 stops proliferation of MC3T3-E1 cells [16–19]. The inhibition of proliferation is accompanied by an attenuation of the signal transduction from EGF to c-fos expression. In cultured primary osteoblasts proliferation and expression of c-fos and histone genes are inversely related to the expression of the osteoblastic phenotype [5–10]. Therefore, we interpret the negative effect on proliferation and the attenuation of c-fos expression as a result of T3-induced differentiation of osteoblastic cells. Our finding could also explain that T3 stimulates OC synthesis after 8 days but not after 4 days. It was described that T3 attenuates c-fos mRNA expression in pituitary cells resulting in reduced AP-1 activity [35]. One AP-1 binding place is located in the area of the vitamin D responsive element in the OC promoter which also binds the TR [13] and was found to be sufficient for T3-regulated expression in transfection experiments. When the concentration of AP-1 is attenuated after stop of proliferation, this regulatory element can become accessible to activated TR and VDR, resulting in expression of phenotypic proteins like OC. Our data could indicate that T3 regulates OC synthesis by down-regulation of AP-1 followed by induction of OC expression, which effect could be additionally amplified by a direct activation of the OC promoter by the activated TR.

Several reports using different experimental systems postulate that matrix formation and collagen type I synthesis are obligatory prerequisites for OC expression [34, 36–38]. By use of a three-dimensional MC3T3-E1 cell culture system it was shown that in particular two factors, namely cell-to-cell contacts and cell-matrix contacts, promote the expression of the osteogenic potential [36]. We have reported that T3-treated MC3T3-E1 cells stop to proliferate forming two cell layers at the maximum [19]. However, the morphology shows that extra cellular matrix is present in T3-treated cultures. Both prerequisites, cell-matrix and cell-to-cell contacts, are fulfilled in particular on day 8 when all cells are confluent. This could reveal a possible explanation for the differences between days 4 and 8.

We report strong evidence that T3, which is known to stop proliferation of MC3T3-E1 cells, in parallel depresses histone H4 expression, attenuates EGF-stimulated c-fos/c-

jun expression, and stimulates OC synthesis. Increased bone formation without impaired bone resorption was found in OC-deficient mice, indicating a role of OC as a determinant of bone formation without affecting mineralization [39]. Modulation of OC synthesis in osteoblasts could be one mechanism by which T3, at physiologic as well as pathologic concentrations, influences bone turnover. We conclude that thyroid hormones by their regulatory influence on proliferation and expression of phenotypic genes in osteoblasts control crucial steps in osteoblastic differentiation and bone turnover.

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