Tri-iodothyronine inhibits multilayer formation of the osteoblastic cell line, MC3T3-E1, by promoting apoptosis

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Abstract

Cell death through apoptosis is a well-known mechanism for maintaining homoeostasis in many developmental and pathological processes. We have recently presented evidence for the occurrence of apoptosis during the formation of bone-like tissue in vitro. MC3T3-E1 osteoblast-like cells in culture develop features of the osteoblastic phenotype and form many cell layers embedded in extracellular matrix which can mineralise. Tri-iodothyronine (T₃), even though it enhances the expression of many osteoblastic features, attenuates the multilayer formation to about two layers. The aim of this study was to investigate how T₃ prevents multilayer formation. MC3T3-E1 cells were seeded at different densities and cultured for up to 2 weeks. Thereafter we analysed proliferation rate and the distribution of the phases of the cell cycle and studied apoptosis. We found that T₃ did not inhibit DNA synthesis. Analysis of the cell cycle phases showed an increase in the number of cells in G0/G1 with increasing cell density, but no significant effect of T₃ treatment was found. Morphological investigations showed apoptotic features in both cell layers and culture supernatants. The cells exhibited typical plasma membrane blebbings, chromatin condensation, DNA fragmentation and phagocytosed apoptotic bodies. T₃ treatment significantly increased the number of apoptotic cells. We conclude from our data that T₃ inhibits multilayer formation of MC3T3-E1 cells by increasing the rate of apoptosis and not by inhibition of proliferation. Because apoptosis is a fundamental regulatory event during bone tissue differentiation, our findings emphasise the importance of thyroid hormones in bone maintenance and development.


Introduction

Programmed cell death through apoptosis is a well-known mechanism for maintaining homoeostasis in many developmental and pathological processes (Thompson 1994, Jacobson et al. 1997, Su et al. 1997). Apoptosis was originally defined morphologically: cells undergo shrinkage and separation from their neighbours; plasma membrane blebbings form; a characteristic form of chromatin condensation occurs; there is nuclear membrane breakdown and cytolysis into condensed apoptotic bodies which are phagocytosed by surrounding cells and macrophages (Bursch et al. 1990, Thompson 1994).

Phosphatidylserine is located at the cytoplasmic face of the plasma membrane in healthy cells. Fadok et al. (1992) found that the early apoptotic phase is characterised by localisation of phosphatidylserine at the cell surface and can easily be detected by staining with annexin V, a protein that has strong natural affinity for phosphatidylserine (Martin et al. 1995). The TUNEL assay allows detection of DNA fragmentation in apoptotic alterations of the nuclei (Gavrieli et al. 1992). Among other factors, steroid hormones are important regulators of apoptosis via receptor-mediated processes (Thompson 1994). Thyroid hormone, the causative agent of amphibian metamorphosis, induces apoptosis in several tissues during organ transformation (Su et al. 1997). It has recently been shown that, in a myoblastic cell line derived from Xenopus laevis tadpole tail, tri-iodothyronine (T₃) induces the expression of CPP32, a putative cysteine protease, and apoptosis, which can be prevented by an inhibitor of this protein (Yaoita & Nakajima 1997). In semisolid cultures of chicken embryonic bone marrow cells, retinoic acid and thyroid hormones regulate cell differentiation at different levels (Gandrillon et al. 1994). On the one hand, they induce the early commitment of the erythroid cell progenitor cells, which can be blocked by v-ErbA, the viral counterpart of the thyroid hormone receptor α, and on the other, they induce differentiation in the presence of insulin and erythropoietin or apoptosis when these factors are absent. Similarly, in the promyeloleukaemic cell line, HL-60, retinoic acid and T₃ act synergistically to induce apoptosis.
during the differentiation process (Suzuki et al. 1997). All these data emphasise the importance of T₃ in differentiation processes that are often coupled to apoptosis.

Thyroid hormones are important regulators of bone development and metabolism (Mosekilde et al. 1990, Allain & McGregor 1993) and influence osteoblastic growth and differentiation (Kasahara et al. 1988, Klaushofer et al. 1985, Glantschnig et al. 1996, Fratzl-Zelman et al. 1997, Varga et al. 1997). Treatment of the osteoblastic cell line, MC3T3-E1, with T₃ induces the expression of osteoblastic markers such as alkaline phosphatase activity and osteocalcin (Kasahara et al. 1988, Fratzl-Zelman et al. 1997, Varga et al. 1997). In contrast with control cultures, MC3T3-E1 cells treated with T₃ formed only two cell layers at the most (Luegmayr et al. 1996). Inhibition of cell multiplication can be effected by either inhibiting proliferation or stimulating apoptosis. We recently presented evidence that, in long-term cultures of MC3T3-E1 cells, T₃ increased the frequency of cells containing apoptotic transformed nuclei (Fratzl-Zelman et al. 1997). In this paper we show, by a combination of biochemical and morphological techniques, that T₃ inhibits multilayer formation in confluent MC3T3-E1 cultures, not by inhibiting proliferation, but by increasing the rate of apoptosis.

Materials and Methods

Cell culture

MC3T3-E1 cells (kindly donated by Dr Kumegawa, Meikai University, Department of Oral Anatomy, Sakado, Saitama 35002, Japan) were cultured in αMEM (Sebak, Suben, Austria) supplemented with 4·5 g/l glucose, 5% fetal calf serum (Sebak) and 30 µg/ml gentamicin (Sebak) at 37 °C under 5% CO₂ in humidified air. They were subcultured twice a week using 0·001% Pronase E (Boehringer) and 0·02% EDTA in PBS; the washings were added to the medium. Thereafter the cells were detached using 0·003% Pronase E (Boehringer) and 0·02% EDTA in PBS and added to the medium again. The cells were pelleted at 1400 r.p.m. and washed once with PBS. After dispersion of the cells in 1 ml 2% fetal calf serum in PBS, 100 µl 1% Triton X-100 in 0·02 M HCl were added slowly. After the addition of 0·02 M HCl the nuclei were stained with 10 µg/ml propidium iodide. The flow cytometric analyses were performed with a FACScalibur Flow Cytometry System (Becton & Dickinson, San Jose, CA, USA).

5-Bromo-2′-deoxyuridine (BrdU) incorporation

A BrdU labelling and detection kit (Boehringer) was used according to the manufacturer’s instructions. Briefly, cells were seeded on coverslips at low or high density and grown for 4 or 8 days without or with 10⁻⁷ M T₃. For the assay, BrdU to a final concentration of 10 µM was added to the culture medium and cells were incubated for an additional hour to allow BrdU incorporation into cellular DNA. Thereafter the cells were immediately fixed with ethanol at −20 °C and incubated with a mixture containing an anti-BrdU monoclonal antibody and specific nucleases. Cells were then incubated with anti-mouse-IgG–fluorescein and finally embedded in anti-fade solution (Molecular Probes, Leiden, The Netherlands) containing propidium iodide (1 µg/ml) for nuclear staining.

TUNEL assay

The Boehringer in situ cell death detection kit with fluorescein-labelled dCTP was used according to the manufacturer’s instructions. Cells, seeded on coverslips at low or high density, were grown for 4 or 8 days without or with 10⁻⁷ M T₃ and fixed with 4% paraformaldehyde for 30 min at room temperature. To improve the sensitivity of the TUNEL assay, cells were pretreated with 0·1% Triton 33258 solution (10 µg/ml in 1 mM Tris–HCl, pH 8·0, 0·1 mM EDTA and 0·1% Triton X-100) was added to each well. DNA was quantified by measuring the fluorescence in a multiwell fluorimeter (excitation 355 nm; emission 460 nm; Tecan, Salzburg, Austria). The calibration curve was prepared using calf thymus DNA (Boehringer). Thereafter the cell layers were solubilised by adding 250 µl 0·2 M NaOH and after, the addition of 1·0 ml Optifluor 40 (Canberra-Packard Company, Meriden, CT, USA), the incorporated radioactivity was counted in a Topcounter for 30 min (Canberra-Packard Company).

Flow cytometry for analysis of the cell cycle phases

Cells were seeded in 6 cm Petri dishes and cultured as described above. After the indicated culture time, the medium was removed and the cell layers were washed with PBS; the washings were added to the medium. Thereafter the cells were detached using 0·003% Pronase E (Boehringer) and 0·02% EDTA in PBS and added to the medium again. The cells were pelleted at 1400 r.p.m. and washed once with PBS. After dispersion of the cells in 1 ml 2% fetal calf serum in PBS, 100 µl 1% Triton X-100 in 0·02 M HCl were added slowly. After the addition of 100 µl 10 mM EDTA, the nuclei were stained with 10 µg/ml propidium iodide. The flow cytometric analyses were performed with a FACScalibur Flow Cytometry System (Becton & Dickinson, San Jose, CA, USA).

Estimation of the amount of DNA and [³H]deoxythymidine ([³HdT]) incorporation

Cells were seeded in 24-well micro plates as described above. After 2 days of culture, 0·5 µCi/ml [³HdT (NEN, Boston, MA, USA) was added and the cells were incubated for another 16 h. The medium was then removed and the cell layers were washed three times with PBS and frozen at −20 °C. During thawing, 250 µl Hoechst
X-100 (2 min on ice) and irradiated with microwaves (Negoescu et al. 1996). Coverslips were transferred to a glass Petri dish containing 20 ml 0.01 M citrate buffer, pH 6, and irradiated for 5 min (Panasonic NN-6557 microwave, 180W) resulting in elevation of the buffer temperature to 80°C. Rapid cooling was achieved by immersing the coverslips in distilled water and PBS at room temperature. After these pretreatments, cells were incubated with the TUNEL mix for 60 min at 38°C in a moist chamber and, after three washing steps with PBS, mounted on to glass slides in Vectashield (Vector Laboratories, San Diego, CA, USA). A negative control for TUNEL was provided by omitting TdT from the labelling mix. The specificity was controlled by comparing TUNEL labelling with cellular morphology.

Semi-quantitative analysis of BrdU incorporation and TUNEL assay by confocal laser scanning microscopy (CLSM)

A total of 25 representative images were investigated per time point and treatment by using a CLSM (63 x objective, Leica TCS 4D). BrdU incorporation and the number of TUNEL-positive nuclei were determined by counting the total number of nuclei per image area and the number of BrdU- or TUNEL-positive nuclei respectively. In parallel, cellular morphology was investigated in the phase-contrast modus and the number of cells with a blebbing plasma membrane was determined.

Annexin V/propidium iodide staining for fluorescence microscopy

The ApoAlert annexin V apoptosis kit (Clontech, Palo Alto, CA, USA) was used according to the manufacturer’s instructions. Cells were washed with PBS and incubated with a mixture containing binding buffer, annexin V (1 μg/ml) and propidium iodide (2.5 μg/ml) for 15 min at room temperature. After being washed in PBS, cells were fixed with 4% paraformaldehyde for 30 min and mounted on to glass slides in Vectashield.

Annexin V/propidium iodide staining for flow cytometry

Cells were prepared for flow cytometry as described above. After they had been pelleted at 1400 r.p.m. and washed once with PBS, they were resuspended in a mixture containing binding buffer, annexin V (1 μg/ml) and propidium iodide (2.5 μg/ml) for 15 min at room temperature. This cell suspension was then analysed with a FACScalibur Flow Cytometry System.

Light and electron microscopy of embedded samples

For light and electron microscopic analysis, the samples were treated by routine procedures. After a short rinse in buffer, the cultures were fixed with 2% glutaraldehyde (in 0.1 M cacodylate buffer, pH 7.4) for 1 h, postfixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol and embedded in Epon 812. For light microscopy, 1 μm thick sections were stained with toluidine blue. Ultrathin sections stained with uranyl acetate/lead citrate were investigated with a Philips EM400 electron microscope.

Statistical analysis

Statistical significance was tested by ANOVA (posthoc test: Scheffe) using Statview 4.5 (Abacus Concepts Inc., Berkeley, CA, USA).

Results

Effects of T3 on growth and proliferation

In previous studies we have shown by morphological and biochemical methods that T3 prevents the multilayer formation of MC3T3-E1 cells in long-term cultures (Klaushofer et al. 1995, Glantschnig et al. 1996, Luegmayr et al. 1996, Fratzl-Zelman et al. 1997). Analysis of these data suggested that confluence rather than culture time determines the effects of T3. To confirm this, we seeded cells at different densities and treated them for 3 days without (Co) or with 10^-7 M T3 (T3). The amount of DNA was measured after staining with Hoechst 33258. The bars represent means ± S.E. (n=4). *P<0.05, ***P<0.001 (Co vs T3 treatment).

Figure 1

Effect of T3 on DNA synthesis in cultures seeded at different densities. MC3T3-E1 cells were seeded at 2800, 4200, 7000, 14 000, 28 000 or 70 000 cells/cm² and cultured for 3 days without (Co) or with 10^-7 M T3 (T3). The amount of DNA was measured after staining with Hoechst 33258. The bars represent means ± S.E. (n=4). *P<0.05, ***P<0.001 (Co vs T3 treatment).
nuclei after T3 treatment in all experimental groups. At significant density after 4 or 8 days (Fig. 4A). There were no poration was studied in cultures seeded at high or low densities after day 4 and about 7% on day 8. At high seeding density the percentage of BrdU-positive cells was about 7 at both time points investigated. All these data indicate that T3 treatment had no significant effect on DNA synthesis.

TUNEL assay and phase-contrast imaging
Apoptosis-induced DNA fragmentation was evaluated by the TUNEL assay. Figure 5 presents the semiquantitative data of the TUNEL assay experiments. In confluent cultures, either at day 4 seeded at high density or at day 8 seeded at low and high density, the number of TUNEL-positive apoptotic cells and apoptotic bodies was significantly increased after T3 treatment (Figs 4B, C and 5). Some of the apoptotic bodies, containing a TUNEL-positive fragment of the nucleus, were found to be incorporated by neighbouring cells (Fig. 4D).

The number of cells with a blebbing plasma membrane (Fig. 6A and B) was found to be increased tenfold after T3 treatment (Table 1), but again this was only the case in confluent cultures. Interestingly, these ‘blebbing’ cells...
Figure 4

*T₃* promotes apoptosis in osteoblasts

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failed to show any signs of DNA fragmentation as assayed by TUNEL staining (Fig. 4E).

Staining with annexin V and propidium iodide in flow cytometry and CLSM

To strengthen these data further, we performed annexin V–fluorescein isothiocyanate (FITC)/propidium iodide labelling and analysed the cultures by both flow cytometry and fluorescence microscopy. The quantitative flow cytometry analyses of confluent cultures are shown in Fig. 4J and K. T₃-treated cultures contained 2·5 times more annexin V–FITC-stained cells (1·04±0·12 vs 2·5±0·57, P<0·061, n=3). Fluorescence microscopy showed two different annexin V-staining patterns. Early apoptotic cells with more or less conspicuous plasma membrane blebbing showed punctate-granular annexin V staining of the plasma membrane, but nuclei were propidium iodide–negative, indicating the integrity of the plasma membranes (Fig. 4F and G). However, nuclei of these blebbing cells often showed typical apoptotic features such as chromatin condensation and the beginning of nuclear fragmentation. Such changes in nuclear morphology can be observed in Fig. 4G (compare n* with n) and Fig. 6A and B. On the other hand, in late apoptotic cells and apoptotic bodies, annexin V staining resulted in a diffuse fluorescent signal throughout the entire cytoplasm, with only a few positive granules (Fig. 4H and I). In these cases, the cell nucleus, being more or less fragmented, was always propidium iodide–positive, indicating rupture of the plasma membrane.

Demonstration of morphological features of apoptosis in numerous MC3T3-E1 cells after T₃ treatment

Blebbing cells, apoptotic bodies and apoptotic nuclei, frequently found in T₃–treated cultures, were investigated by light and electron microscopy. Figure 6A and B shows typically shrunken apoptotic cells with intense surface blebbing. These surface blebs, which often contain organelles and nuclear fragments, finally bud to form apoptotic bodies. In semithin sections, we found several cells that contained prominent intensely toluidine-blue-stained inclusions (Fig. 6C). Electron microscopy revealed that these inclusions were phagocytosed apoptotic bodies at different stages of degeneration (Fig. 6D and E). Apoptotic cells were found not only within the adherent cell layers, but also in the cell culture supernatant. Figure 6F shows two floating apoptotic cells containing nuclei with typical condensed chromatin. These cells are rounded, and the endoplasmic reticulum and Golgi cisterns are partially swollen.

Discussion

In long-term cultures, MC3T3-E1 cells proliferate continuously and form multilayers (Kasono et al. 1988, Matsumoto et al. 1991, Luegmayr et al. 1996). T₃ treatment attenuates this increase in DNA and limits the formation of cell layers to a maximum of two or three (Luegmayr et al. 1996). Two mechanisms could contribute to this effect of T₃: inhibition of cellular proliferation and stimulation of the rate of apoptosis. Evidence for the latter was found in a morphological investigation of long-term cultured MC3T3-E1 cells (Fratzl-Zelman et al. 1997). The present investigation was initiated to investigate these findings further and confirm the effect of T₃ on apoptosis in osteoblasts.
Figure 6

T₃ promotes apoptosis in osteoblasts

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MC3T3–E1 cells seeded at low density are subconfluent on culture day 4 and start to form a second cell layer on day 8 (Luegmayr et al. 1996, Fratzl-Zelman et al. 1997). Whereas confluent non-transformed cell lines stop proliferating, MC3T3–E1 cells in long-term cultures form up to 20 cell layers. Our cell cycle analysis showed that more cells are in the G1/G0 phase and fewer in the S or G2/M phases with prolonged culture time, indicating that the proliferation rate decreases. This is consistent with the finding that the amount of DNA per dish reaches a plateau (Matsumoto et al. 1991, Fratzl-Zelman et al. 1997). Such cultures develop many features of the differentiated osteoblastic phenotype.

Control of proliferation could depend on the state of differentiation, the culture time (Stein & Lian 1993, Ohishi et al. 1994) or the development of cell/cell contacts. In this study, we seeded cells at different densities, cultured them for 3 days and studied DNA synthesis by measuring $[^3]$H]thymidine incorporation. Depending on cell density, DNA synthesis was maximum when cells were subconfluent and decreased after they reached confluence. This indicates that the formation of cell/cell contacts determines the rate of proliferation. Our experiments with thyroid hormones had shown that $T_3$ in subconfluent cultures had no effect on cell number, whereas in confluent cells it attenuated the increase in DNA content. Surprisingly, $[^3]$H]thymidine incorporation in confluent cells was increased, indicating stimulation of DNA synthesis. This obvious contradiction could only be explained by an increased rate of apoptosis.

It is well known that $T_3$ is an important regulator of apoptosis. Besides their impressive effects on the tadpole tail during metamorphosis (Tata 1994), thyroid hormones also enhance the rate of apoptosis in promyeloleukaemic cells (Suzuki et al. 1997) and early erythrocytic progenitor cells (Gandrillon et al. 1994). We here demonstrate, using a combination of different techniques, namely TUNEL assay, annexin V/propidium iodide staining as well as light and electron microscopy, that $T_3$ significantly induces apoptotic processes in confluent osteoblast-like cells. For example, the number of cells with intense cell surface blebbings, a typical early apoptotic feature, was increased tenfold after treatment with $T_3$. These blebbing cells showed a punctate-granular annexin V-staining pattern of the plasma membrane, but were propidium iodide-negative, indicating the integrity of the plasma membrane. Although these blebbing cells showed apoptotic nuclear morphology including nuclear segmentation, they failed to show signs of DNA fragmentation as determined by the TUNEL assay. This is in accordance with recent findings of a time lag between nuclear morphological changes and major DNA fragmentation (Collins et al. 1997). Whereas nuclear condensation occurred early, usually at the time of initial surface blebbing, DNA strand breaks were demonstrated much later, only after the formation of separated apoptotic bodies. This is also true for our MC3T3–E1 cells, in which TUNEL-positive nuclei were found almost exclusively in apoptotic bodies. Light and electron microscopic investigations revealed that these apoptotic bodies were finally phagocytosed by neighbouring MC3T3–E1 cells. Again, the $T_3$ effect was dependent on confluence.

Apoptosis plays a fundamental role in the cellular organisation of bone tissue. It has been shown to occur during bone-like tissue development in vitro (Fratzl-Zelman et al. 1997, Lynch et al. 1998) and is coordinately regulated with osteoblast formation during bone healing (Landry et al. 1997). Our finding that $T_3$ increases the expression of the osteoblastic phenotype (Varga et al. 1997) and on the other hand regulates apoptosis further emphasises the importance of thyroid hormones in the development and maintenance of bone.

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