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Inhibition of ossification in vivo and differentiation of osteoblasts in vitro by tributyltin

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Abstract

Tributyltin is ubiquitous in the environment and an endocrine disruptor for many wildlife species. However, minimal information is available regarding the effect of this chemical on bone formation. When tributyltin chloride (TBT) (1 mg/kg body weight) was administered subcutaneously to pregnant mice at 10, 12, and 14 days post coitus (dpc), fetuses at 17.5 days post coitus revealed the inhibition of calcification of supraoccipital bone. In contrast, 1 mg/kg body weight monobutyltin trichloride (MBT) did not affect the fetal skeleton. Therefore, we examined the effects of TBT and its metabolites (dibutyltin dichloride, DBT, and MBT) on bone metabolism using rat calvarial osteoblast-like cells (ROB cells). The viability of ROB cells was not affected by the exposure of the cells to 10^{-10} to 10^{-7} M TBT. However, TBT reduced the activity of alkaline phosphatase (ALPase) and the rate of deposition of calcium of ROB cells. In addition, the expression levels of mRNA for ALPase and osteocalcin, which are markers of osteoblastic differentiation, were depressed by the treatment with TBT. TBT inhibited ALPase activity and the deposition of calcium to a greater extent than did DBT. MBT had no effect on the osteoblast differentiation of ROB cells. Tributyltin is known to inhibit the activity of aromatase. However, the aromatase inhibitor aminoglutethimide did not reproduce the inhibitory effects of TBT on osteoblast differentiation. Our findings indicate that TBT might have critical effects on the formation of bone both in vivo and in vitro although its action mechanism is not clarified.

Keywords: Tributyltin chloride; Differentiation; Mineralization; Bone formation; Osteoblast

1. Introduction

Butyltin compounds are used mainly as antifoulants on ships and fishnets, as wood preservatives, and as biocides for cooling systems, and have been shown to be ubiquitous in the aquatic environment. In animal experiments, exposure to tributyltin compounds caused thymus atrophy with peripheral T cell depletion and suppression of T cell-mediated immune responses [1,2]. Tributyltin chloride (TBT) also causes the imposition of male sex characteristics on females in shellfish [3,4]. In vitro, TBT has been reported to cause apoptosis in various lines of cells [5–7]. Butyltins also inhibit the activity of human aromatase from transfected cells [8] or a granulose cell-like tumor cell line [9].

Bone is a highly specialized form of connective tissue that is nature's provision for an internal support system in all higher vertebrates. The formation of bone involves a complex series of events that include the proliferation and differentiation of osteoprogenitor cells and eventually result in the formation of a mineralized extracellular matrix. Several model systems have been developed for studies of the proliferation and differentiation of bone-forming cells in vitro and the molecular biology of the mineralization process [10–14]. The sequential expression of type I collagen, alkaline phosphatase (ALPase) and osteocalcin, and the deposition of calcium are known as markers of osteoblastic differentiation. The formation of

Abbreviations: ALPase, alkaline phosphatase; dpc, days post coitus; DBT, dibutyltin dichloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MBT, monobutyltin trichloride; ROB cells, rat calvarial osteoblast-like cells; RT-PCR, reverse transcriptase-polymerase chain reaction; TBT, tributyltin chloride

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bone is easily influenced by the exposure of osteoblasts and osteoclasts to chemical compounds [15–18]. However, information on the effects and action mechanisms of environmental contaminants in the modeling and remodeling of mammalian bone is not fully available. We screened various endocrine disruptors for the ability to regulate the proliferation, differentiation, and function of cultured osteoblasts and osteoclasts. Recently, we reported that 3-methylcholanthrene (3MC), a dioxin receptor ligand, inhibited the proliferation and differentiation of cultured osteoblastic cells and caused a delay of ossification in vivo [19]. 3MC also inhibited the formation of osteoclast-like cells by inhibiting the expression of the osteoclast differentiating factor of osteogenic/stromal cells [20]. In the present study, we examined the effects of TBT on the mineralization by osteoblastic cells of fetuses in pregnant mice and on the proliferation and differentiation of osteoblasts in vitro.

2. Materials and methods

2.1. Materials

Tributyltin chloride, dibutyltin dichloride (DBT), and monobutyltin trichloride (MBT) were purchased from Wako Pure Chemical Industries. α-Modified minimum essential medium (α-MEM), RPMI 1640, the penicillin/streptomycin antibiotic mixture, fetal bovine serum, and aminoglutethimide were obtained from Sigma.

2.2. Preparation of animals

BALB/c female mice (10-week-old) from Charles River Japan Breeding Laboratories were examined the morning after mating for the presence of vaginal plugs. Noon on the day of evidence of a vaginal plug was considered to be 0.5 days post coitus (dpc). Pregnant mice were injected subcutaneously with 1 mg of TBT or MBT/kg body weight, dissolved in mineral oil, at 10, 12, and 14 dpc (i.e. three injections per mouse). Control dams received the solvent only. Fetuses at 17.5 dpc were obtained by Cesarean section under anesthesia with diethyl ether. Fetuses were kept in sterile distilled water. Animals were treated and maintained in accordance with ethical guidelines and approved by the Tokyo Women's Medical University animal protocol.

2.3. Analysis of fetal skeletons

For whole-mount skeletal analysis, fetuses at 17.5 dpc were placed in water for 2 days, skinned, eviscerated, fixed in 99% ethanol for 6–7 days, and finally stained with alcian blue solution (30 mg alcian blue: 80 mL 99% ethanol: 20 mL glacial acetic acid) for 2 days. Skeletons were rinsed and dehydrated in 99% ethanol for 2 days. Samples were stained in alizarin red solution (50 mg alizarin red in 2% potassium hydroxide) for 4 h and then rinsed in 2% KOH. The samples were then cleared in 0.8% KOH plus 20% glycerol, in 0.5% KOH plus 50% glycerol, and in 0.2% KOH plus 80% glycerol, and stored in 100% glycerol [21].

2.4. Culture of osteoblastic cells

ROB cells were isolated enzymatically from the calvariae of newborn Wistar rats as described previously [10]. Cells were maintained in 75 cm^2 flasks in α-MEM, supplemented with 10% fetal bovine serum, 50 units/mL penicillin and 50 μg/mL streptomycin, in a humidified atmosphere of 5% CO_2 in air at 37 °C. After cells had reached 70% confluence, they were detached by treatment with 0.05% trypsin, replated in either 55 cm^2 dishes or 12-well plates (area of each well, 3.8 cm^2) at a density of 1×10^4 cells/cm^2 and grown in α-MEM supplemented with 10% fetal bovine serum, 50 units/mL penicillin, 50 μg/mL streptomycin, 5 mM β-glycerophosphate, and 50 μg/mL ascorbic acid. Fresh medium was supplied to cells at 3-day intervals. TBT was dissolved in ethanol (final concentration of 0.1%) and added to the culture medium after confluency had been reached (day 3). Ethanol at 0.1% did not affect the proliferation or differentiation of ROB cells.

2.5. Viability of ROB cells following treatment with TBT

We used 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT; Dojindo) to determine the viability of ROB cells. ROB cells (1×10^3 cells/well; 96-well plates), exposed to TBT at various concentrations, were subcultured for the indicated periods. Fresh medium and TBT were supplied to cells at 3-day intervals. After the cells were washed twice with serum-free RPMI 1640 medium, they were treated with MTT (50 μg/well) for 4 h and then the absorbance at 570 nm was measured.

2.6. Measurement of alkaline phosphatase activity

ROB cells were subcultured in 12-well plates (3.8 cm^2/well) in α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/mL ascorbic acid. After cells had reached confluence (day 3), TBT and its metabolites were added at various concentrations to the culture medium. Fresh medium containing chemical compounds was supplied at 3-day intervals. At the indicated times, cells were washed with 10 mM Tris–HCl, pH 7.2, and sonicated for 15 s in 1 mL of 50 mM Tris–HCl, pH 7.2, that contained 0.1% Triton X-100, in a sonicator (Ultrasonic Disruptor UD-201; Tomy Co.). The alkaline phosphatase activity of each sonicate was determined by an established technique with *p*-nitrophenyl phosphate as the substrate. Concentrations of protein were determined with

the BCA protein assay reagent (Pierce Chemical Co.) with bovine serum albumin as the standard.

2.7. Quantitation of the deposition of calcium

ROB cells were subcultured in α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/mL ascorbic acid. After cells had reached confluence (day 3), TBT and its metabolites were added at various concentrations to the culture medium. The amount of calcium, deposited in the cell layer, was measured as follows. The layers of cells in 12-well plates (3.8 cm^2/well) were washed with PBS and incubated overnight with 1 mL of 2N HCl with gentle shaking. The Ca^{2+} ions in the samples were quantitated by the *o*-cresolphthalein complexone method with a Calcium C kit (Wako Pure Chemical Industries). This kit is specific for Ca^{2+} ions and has a limit of detection of 1 μg/mL. We used the solution of Ca^{2+} ions (20 mg/dL) in the kit as the standard solution.

2.8. von Kossa staining

ROB cells that had been cultured with or without 10^{-7} and 10^{-8} M TBT for 15 days in 12-well plates (3.8 cm^2/well) were fixed in 10% formaldehyde for 30 min at room temperature and then washed three times with 10 mM Tris–HCl, pH 7.2. The fixed cells were incubated with 5% silver nitrate for 5 min in sunlight, washed twice with H_2O, and then treated with 5% sodium thiosulfate.

2.9. Reverse transcriptase-polymerase chain reaction (RT-PCR)

We detected mRNAs for rat ALPase and osteocalcin in osteoblasts using RT-PCR. We extracted RNA from ROB cells using ISOGEN™ (Wako Pure Chemical Industries) according to the manufacturer's protocol and then we reverse transcribed the total RNA (2 μg) using Moloney murine leukemia virus reverse transcriptase, Superscript™ (200 units; Promega, random primers) and a 25 μL reaction mixture. The cDNA was amplified in 20 μL of Taq DNA polymerase mixture (Takara) that contained 1 μM sense primer, 5′-AGGCAGGATTGACCACGG-3′, and 1 μM antisense primer, 5′-TGTAGTTCTGCTCATGGA-3′, for rat ALPase (accession number J03572; 1151–1590, 440 bp); 1 μM sense primer, 5′-CAGACCTAGCAGACAC-CATGAG-3′, and 1 μM antisense primer, 5′-CGTCCA-TACTTTCGAGGCAG-3′, for rat osteocalcin (accession number M11777; 13–428, 416 bp); or 1 μM sense primer, 5′-ATTGTTGCCATCAACGACC-3′, and 1 μM antisense primer, 5′-CATGGACTGTGGTCATGAGC-3′, for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (accession number AF106860; 931–1381, 451 bp). Each amplification cycle, repeated a total of 32, 33, and 30 times for ALPase, osteocalcin, and GAPDH, respectively, consisted of incubation at 94 °C for 30 s, at 60 °C for 30 s, and 72 °C for 45 s. PCR products were subjected to electrophoresis on a 2% agarose gel and visualized by staining with ethidium bromide. DNA marker fragments (100 bp DNA ladder; New England BioLabs Inc.) were used as size markers.

3. Results

We examined the effects of TBT and MBT on the formation of bone in the fetuses of pregnant mice. We collected 31 control (exposure to mineral oil only) fetuses, 16 fetuses that had been exposed to TBT (1 mg/kg of body weight), and 11 fetuses that had been exposed to MBT (1 mg/kg of body weight) from 11 untreated pregnant dams, 4 TBT-treated pregnant dams, and 5 MBT-treated pregnant dams, respectively. There were no differences in body weight between the fetuses from TBT-treated and control dams. Fetuses at 17.5 dpc were stained with Alcian Blue and Alizarin Red to visualize cartilage and bone, respectively, in the forming skeletal structures. Fig. 1 shows typical photographs of fetal bones. The supraoccipital bone (arrowheads in A and C) is seen in alizarin-stained material as two distinct centers of ossification, one located on either side of the dorsal midline in control fetuses at 17.5 dpc. In contrast, we noted a delay in the ossification of the supraoccipital bone (arrowheads in B and D) in eight fetuses in the TBT-treated group as compared with the control group. No variations in skeletal ossification or skeletal malformations were observed in other bones. MBT-treated fetuses showed ossification of the supraoccipital bone similar to that of the control group. Furthermore, we collected eight fetuses from 0.1 mg TBT-treated dams and two of the fetuses showed a delay of ossification of the supraoccipital bone (data not shown).

Because TBT inhibited the calcification of the supraoccipital bone in mouse fetuses, we examined the effects of TBT on the metabolism of cultured rat calvarial osteoblasts that had previously been well characterized. We first confirmed, using MTT, that 10^{-10} to 10^{-7} M TBT did not affect cell viability at the proliferative (A) and post-proliferative (B) stages of ROB cells (Fig. 2). ROB cells also exhibited no changes in morphology during exposure to TBT at concentrations from 10^{-10} to 10^{-7} M.

When ROB cells were cultured with TBT, the differentiation and mineralization of ROB cells were inhibited in a dose-dependent manner. As shown in Fig. 3A, TBT decreased the activity of ALPase. The ALPase activity of ROB cells that had been exposed to 10^{-7} M TBT was significantly lower than that of control cells (treated with ethanol) after 9 and 12 days. The deposition of calcium by ROB cells was also strongly suppressed, to 10% of control values, by 10^{-7} M TBT (Fig. 4A). In addition, von Kossa staining revealed that 10^{-7} M TBT decreased the formation of mineralized nodules of ROB cells (Fig. 4C). TBT

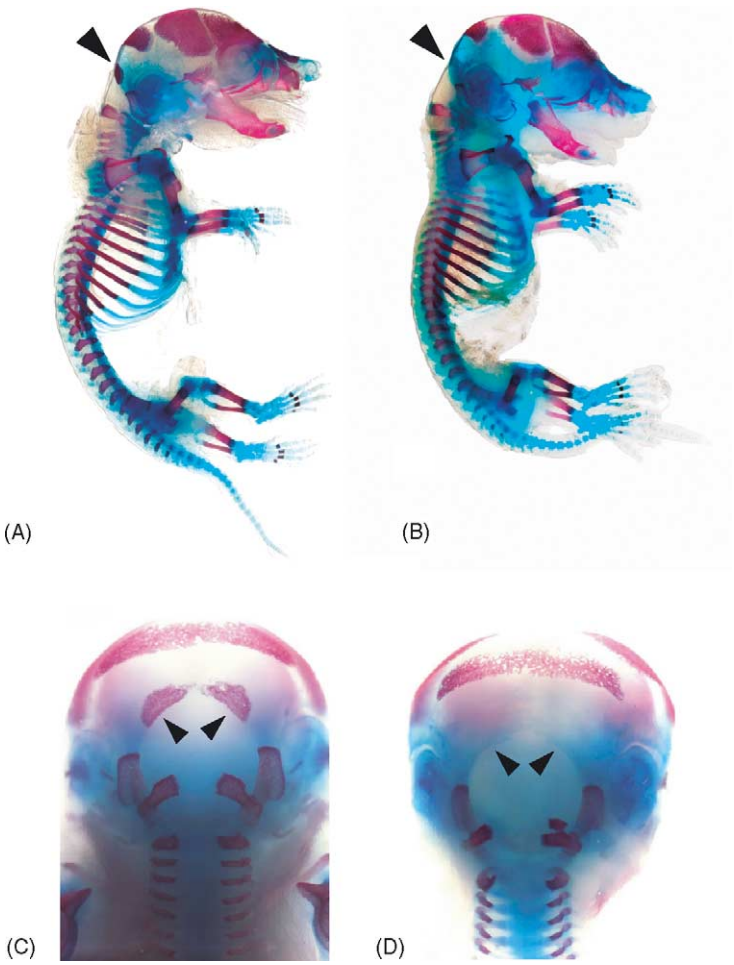

Fig. 1. Effects of TBT on mouse fetuses. Lateral views of control (A) and TBT-exposed (B) fetuses. Dorsal views of head skeletons of control (C) and TBT-exposed (D) fetuses. Pregnant mice were injected subcutaneously with 1 mg of TBT/kg body weight, dissolved in mineral oil, at 10, 12, and 14 dpc (i.e. three injections per mouse). E17.5 fetuses were stained with alcian blue and alizarin red to visualize cartilage and bone, respectively, in the forming skeletal structures. The supraoccipital bone (arrowheads in A and B) is seen in alizarin-stained material of E17.5 normal fetus. In TBT-treated fetuses, the supraoccipital bone (arrowheads in C and D) is not formed.

inhibited ALPase activity on day 12 and the deposition of calcium on day 15 to a greater extent than did DBT (Figs. 3B and 4B). MBT had no effect on the osteoblast differentiation of ROB cells. When TBT was added to the culture medium only during the proliferative phase of the culture (days 0–3), it inhibited osteoblast differentiation, as manifested by ALPase activity (30% inhibition of control value) and the deposition of calcium (40% inhibition of control value). RT-PCR analysis showed that TBT inhibited the expression of mRNAs for ALPase and osteocalcin, which are markers of osteoblastic differentiation, in a dose-dependent manner (Fig. 5). On day 12, osteocalcin mRNA began to be expressed in control ROB cells and its evel of expression continued to increase with time. Low expression of osteocalcin mRNA was detected in ROB cells that had been treated with 10^{-8} M TBT, and we were not able to detect this mRNA in 10^{-7} M TBT-treated cells.

TBT is known to inhibit the activity of aromatase. Therefore, we determined whether the inhibitory effects of TBT on osteoblast differentiation were caused by the inhibition of aromatase activity. When we added the aromatase inhibitor aminoglutethimide at concentrations from 2.5×10^{-5} to 2×10^{-4} M to the culture medium of ROB cells under the same conditions as those of TBT addition, aminoglutethimide had no effect on the ALPase activity on day 12 or the deposition of calcium on day 15 of ROB cells.

4. Discussion

The specific risk to bone metabolism in mammals exposed to environmental endocrine disruptors is not well understood. The aim of this study was to clarify the potential effects of butyltin compounds on bone metabolism. We found that injection of TBT into pregnant dams inhibited the calcification of the supraoccipital bone in mouse fetuses. Therefore, we examined the effects of TBT using rat calvarial primary osteoblast-like cells and we

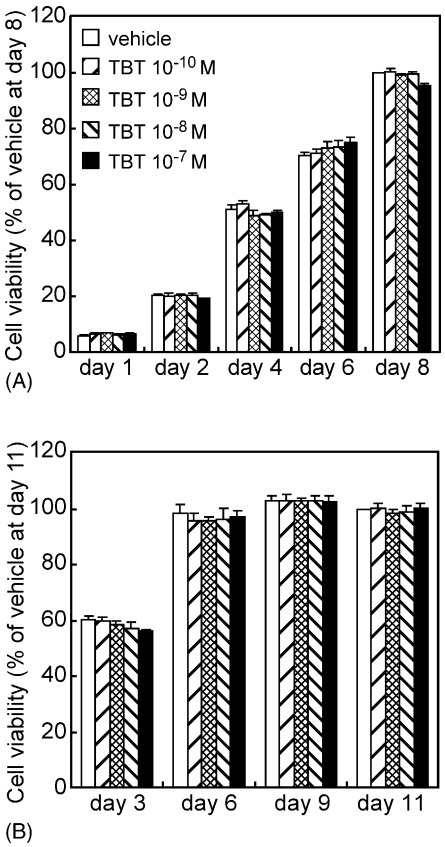

Fig. 2. Effects of TBT on the viability of osteoblasts. ROB cells (1×10^3 cells/well; 96-well plates) exposed to TBT at various concentrations were subcultured for the indicated periods. TBT did not affect cell viability at the proliferative (A) and post-proliferative (B) stages of ROB cells. Fresh medium with TBT was supplied at 3-day intervals. Cells were treated with MTT (50 μg/well) and then the absorbance at 570 nm was measured. Values represent the means ± S.D. of the results for three wells. Data are representative of results from three separate experiments.

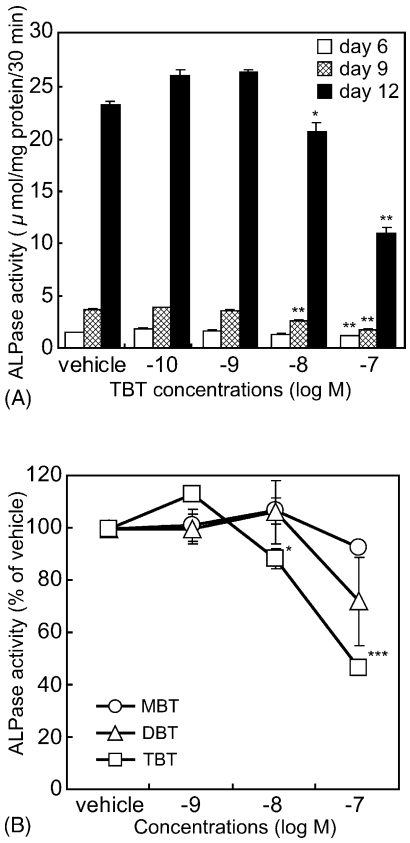

Fig. 3. Effects of TBT and its metabolites on the alkaline phosphatase activity of ROB cells. ROB cells were cultured in 12-well plates (3.8 cm^2/well) with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/mL ascorbic acid, and TBT at various concentrations for 6, 9, and 12 days (A), and TBT and its metabolites at various concentrations for 12 days (B). Fresh medium containing the test chemicals was supplied at 3-day intervals. At the times indicated, alkaline phosphatase activity was measured as described in the text. Values represent the means ± S.D. of the results for three wells. Data are representative of results from three separate experiments. $^{*}P < 0.05$ vs. controls; $^{**}P < 0.01$ vs. controls; $^{***}P < 0.001$ vs. controls.

showed that TBT inhibited the differentiation and mineralization of osteoblasts.

In our study, pregnant mice received injections of 1 mg of TBT or MBT/kg body weight at 10, 12, and 14 dpc during development. A delay of ossification was observed in the supraoccipital bone of the TBT-treated group compared with the control group. In contrast, no variations in skeletal ossification were seen in the MBT-treated group. The ossification of the supraoccipital bone of the skull usually commences at 17.5 dpc [22]. Delayed ossification was also seen in some metacarpals and metatarsals of TBT-treated limbs, although ossification of the metacarpals and metatarsals commences at 17.5 dpc [22]. Therefore, we considered that the delay of ossification of the supraoccipital bone caused by TBT is due to the timing of TBT administration and analysis. Very recently, Adeeko et al. [23] reported that in utero exposure of rats to a high concentration (20 mg/kg body weight) of TBT for 0–19 dpc delayed ossification of the fetal pelvic girdle, skull, and limbs.

To clarify the mechanism underlying the delayed ossification of the supraoccipital bone in TBT-treated fetuses, we determined the effects of TBT on the viability and differentiation of ROB cells. TBT inhibited the levels of expression of markers characteristic of the osteoblast phenotype, namely, ALPase and osteocalcin, in ROB cells. Moreover, the deposition of calcium by ROB cells was inhibited by TBT. These parameters are characteristic of the later stages of osteoblast differentiation and are essential for both the development and maintenance of bone. In contrast, TBT did not affect the viability of ROB cells. These results indicated that delayed ossification of the fetal skeleton might be due to the inhibition of the differentiation of osteoblasts by TBT. The mechanism of the TBT-induced alteration in the differentiation of osteoblasts is unknown. TBT has been shown to inhibit the activity of human aromatase [7,8] and this enzyme is expressed in human osteoblasts [24,25]. Therefore, we determined whether the inhibitory effects of TBT on osteoblast differentiation were caused by the inhibition of aromatase activity using the aromatase inhibitor aminoglutethimide.

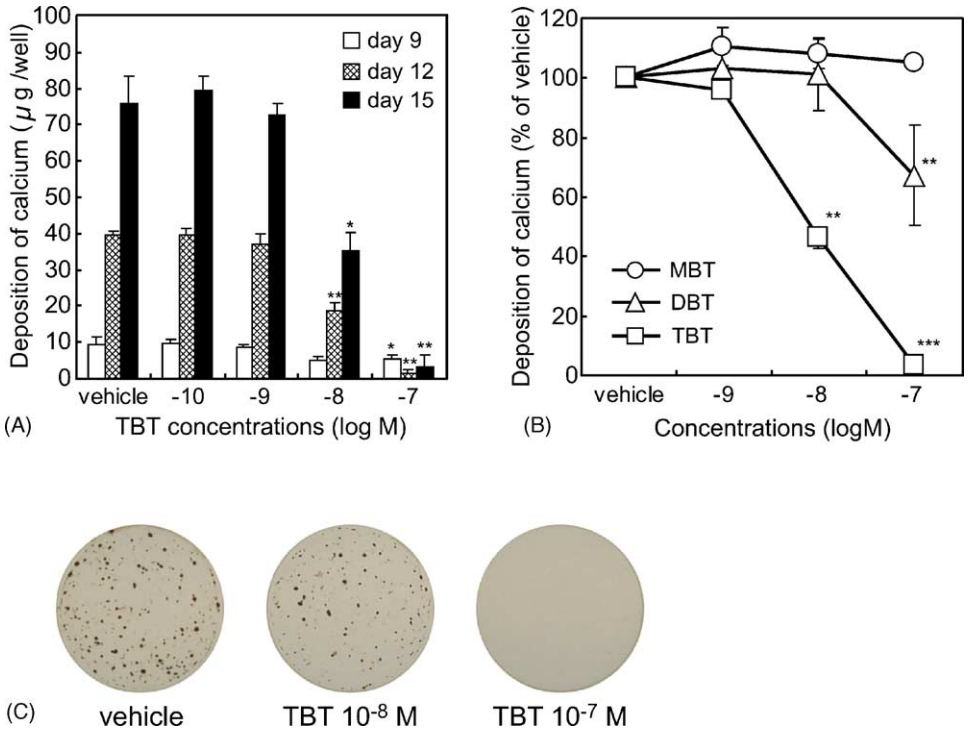

Fig. 4. Effects of TBT and its metabolites on the deposition of calcium by ROB cells. ROB cells were cultured in 12-well plates (3.8 cm^2/well) with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/mL ascorbic acid, and TBT at various concentrations for 9, 12, and 15 days (A), and TBT and its metabolites at various concentrations for 15 days (B). Fresh medium containing the test chemicals was supplied at 3-day intervals. At the times indicated, the deposition of calcium was measured as described in the text. Values represent the means ± S.D. of the results for three wells. Data are representative of results from three separate experiments. $^{*}P < 0.05$ vs. controls; $^{**}P < 0.01$ vs. controls; $^{***}P < 0.001$ vs. controls. (C) Phase-contrast photomicrographs. ROB cells were cultured with 10^{-7} and 10^{-8} M TBT for 15 days and subjected to von Kossa staining for visualization of mineralized nodules.

However, aminoglutethimide had no effect on ALPase activity and the deposition of calcium of ROB cells. On the other hand, TBT has been reported to increase the basal levels of intracellular calcium of rat hepatocytes [26], keratinocytes [27], rat pheochromocytoma cells [28], and human T lymphoblastoid cells [29]. Calcium and its signaling pathways play important roles in osteoblastic differentiation [30,31]. Therefore, TBT might influence osteoblastic differentiation via suppression of the signaling pathway(s) of calcium in ROB cells.

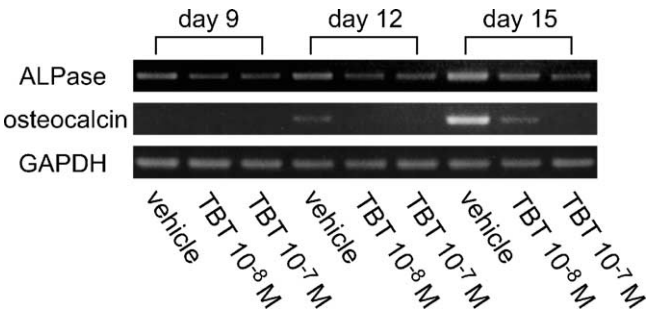

Fig. 5. Expression levels of mRNAs for osteocalcin and ALPase in ROB cells. Total RNA was isolated from cells after treatment with 10^{-7} and 10^{-8} M TBT for 9, 12, and 15 days. Levels of mRNAs for rat osteocalcin (416 bp) and rat ALPase (440 bp) in the cells were determined by RT-PCR with specific primers (see text for details). Amplification of rat GAPDH mRNA was used as an internal control. Data represent results typical of those from three separate experiments.

It has been reported that the inhibitory effects of the butyltin compounds on MAPK phosphorylation [29] and the function of human natural killer lymphocytes [32] followed the order of TBT > DBT > MBT. This order was similar to the order of the inhibition of osteoblast differentiation by TBT and its metabolites observed in the present study. It is unclear why the inhibition of osteoblast differentiation increases with the number of butyl groups in the side-chain.

In the present study, the exposure of ROB cells to 10^{-7} M TBT inhibited ALPase activity (40% of control value) and the deposition of calcium (5% of control value). TBT at 10^{-8} M also had significant effects on the metabolism of ROB cells. The concentrations (10^{-8} to 10^{-7} M) of TBT that we used in the in vitro study were lower than the effective range of those (10^{-6} to 10^{-4} M) used in studies on the inhibition of human aromatase activity [8] and the release of calcium ions from intracellular calcium stores [26,29]. TBT has been shown to be ubiquitous in the aquatic environment. The TBT concentrations in the livers of marine mammals from Asian coastal, North Pacific, and US Atlantic waters ranged between 5 and 1100 ng/g wet weight [33–35]. Furthermore, the mean concentration of TBT for 32 human blood samples from central Michigan, USA was 8.18 ng/mL (2.5×10^{-8} M) and the highest concentration of TBT was 85 ng/mL (2.6×10^{-7} M) [36].

These correspond closely to the concentrations that inhibited the differentiation of osteoblasts in this study. Thus, our study revealed that the formation of bone and the differentiation of osteoblasts might be more easily affected by exposure to TBT than the formation and differentiation of other tissues and cells.

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