4-Tert-Octylphenol Regulates the Differentiation of C3H10T1/2 Cells into Osteoblast and Adipocyte Lineages

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The aim of this study was to investigate whether 4-tert-octylphenol (OP) affects the differentiation of multipotent C3H10T1/2 cells, a cell line established from mouse embryonic connective tissue, into osteoblast and adipocyte lineages. Confluent C3H10T1/2 cells were incubated for 7 days with (OP-treated cultures) or without (control cultures) 15 μg/ml of OP. The 7-day treatment of confluent cells with OP decreased alkaline phosphatase activity by 81%, inhibited the expression of transforming growth factor β2, and inhibited the morphological changes in cells to an osteoblastic appearance. These results indicate that the 7-day treatment of confluent C3H10T1/2 cells with OP inhibited their differentiation into osteoblasts. Since this treatment strongly induced the expression of peroxisome proliferator-activated receptor r (PPARr) but did not stimulate triacylglycerol (TG) accumulation in cells, C3H10T1/2 cells in the control and OP-treated cultures were incubated for 2 days with a hormone mixture (insulin [INS], dexamethasone, and 1-methyl-3-isobutyl-xanthine) and incubated for an additional 5 days with INS alone. The TG and adiponectin contents of the OP-treated cultures were 4.2 and 4.1 times higher, respectively, than those of the control cultures. There were many more Oil Red O-staining cells in the OP-treated cultures than in the control cultures. The expression of PPARr in the OP-treated cultures was higher than that in the control cultures. These results indicate that the OP-treated cultures contained a larger number of adipocytes than the control cultures. In conclusion, treatment of C3H10T1/2 cells with OP inhibited osteoblast differentiation, causing a lineage shift toward adipocytes.

Key Words: 4-tert-octylphenol; C3H10T1/2 cells; osteoblast differentiation; adipocyte differentiation.

Alkylphenol ethoxylates are a class of nonionic surfactants widely used in the manufacture of plastics, detergents, paints, and pesticides (Nimrod and Benson, 1996). The primary alkyl groups are branched octyl or nonyl chains positioned opposite the para-substituted ethoxylate chain. 4-tert-Octylphenol (OP) and 4-nonylphenol (NP) are major degradation products of alkylphenol ethoxylates. These chemicals are found in the wastewater and sludge of sewage treatment works and river sediments (Giger et al., 1984; Hernando et al., 2004). Alkylphenols are also found in drinking water (Kuch and Ballschmiter, 2001). Thus, alkylphenols are widely dispersed in the environment.

Alkylphenols mimic the actions of estrogens (Kwack et al., 2002; Laws et al., 2000; White et al., 1994). One of the critical roles of estrogens is to maintain bone volume (Riggs et al., 1998). The effect of environmental estrogens, including alkylphenols and diphenylalkanes, on bone metabolism has been reported. Beard et al. (2000) reported that chronic exposure to 1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane was associated with reduced bone mineral density in women. Dodge et al. (1996) reported that methoxychlor and zeranol prevented ovariectomy-induced bone loss in rats; the effect is similar to that of 17β-estradiol. OP and bisphenol A (BPA), however, did not exhibit similar estrogen-like effects on bone metabolism. In cultures of MC3T3-E1 cells, an osteoblast-like cell line established from newborn mouse calvaria, BPA enhanced bone mineralization, whereas NP did not have the ability to do so (Kanno et al., 2004).

To date, there has been no report of whether alkylphenols affect the early differentiation of mesenchymal stem cells into osteoblasts. C3H10T1/2 cells, a cell line established from mouse embryonic connective tissue, can differentiate into osteoblasts, adipocytes, chondrocytes, and myocytes, and they offer an excellent model system for the study of osteoblast differentiation (Reznikoff et al., 1973). In this study, we examined whether OP affects the differentiation of multipotent C3H10T1/2 cells into osteoblasts. We used two criteria for osteoblast differentiation: (1) the expression of alkaline phosphatase (ALP), an essential enzyme for mineralization (Bellows et al., 1991; Genge et al., 1988), and (2) the expression of transforming growth factor β2 (TGFβ2), a member of the TGFβ superfamily, which is induced dramatically in C3H10T1/2 cells by all-trans-retinoic acid (Gazit et al., 1993). In addition, we analyzed the expression of peroxisome proliferator-activated receptor r (PPARr), which regulates bone mass (Akune et al., 2004; Ali et al., 2005; Rzonca et al., 2004) and plays a critical role in the induction of osteoblast differentiation.
and maintenance of the fully differentiated adipocyte phenotype (Chawla et al., 1994; Cornelius et al., 1994; Gregoire et al., 1998).

Bone volume is maintained by the balance between bone formation by osteoblasts and bone resorption by osteoclasts. An imbalance between these two phases of bone remodeling causes bone diseases, such as osteoporosis and osteopenia. The decrease in bone volume associated with osteoporosis and age-related osteopenia is accompanied by an increase in bone marrow adipose tissue (Martin et al., 1990; Rozman et al., 1989). Therefore, whether treatment of C3H10T1/2 cells with OP causes a lineage shift toward adipocytes was also examined.

**MATERIALS AND METHODS**

**Materials.** C3H10T1/2 cells, a cell line (clone 8) established from C3H mouse embryo, were obtained from the Riken BRC Cell Bank (Ibaraki, Japan). OP, NP (a mixture of compounds with branched side chains), and bisphenol A-diglycidyl ether (BPA-DGE) were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). A rabbit polyclonal antibody to TGFβ2 (sc-90) and a rabbit polyclonal antibody to PPARγ (sc-7196) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An ECL Advance Western Blotting Detection Kit, Hyperfilm ECL, and Hybond-P membrane were obtained from GE Healthcare Bio-Sciences K.K. (Tokyo, Japan). Kits for ALP and triacylglycerol (TG) were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan). A mouse adiponectin ELISA kit was obtained from CycLex Co., Ltd (Nagano, Japan). All-trans-retinoic acid and β-glycerophosphate were obtained from Sigma (St Louis, MO). An AP-red substrate kit was obtained from Zymed Laboratories Inc. (South San Francisco, CA). Protease Inhibitor Cocktail Set III was obtained from Calbiochem-Novabiochem Co. (La Jolla, CA).

**Cell culture and measurements of ALP activity and TG.** C3H10T1/2 cells were grown to confluence in standard medium on 35-mm plates. One day after confluence, the medium was replaced with a differentiation medium containing OP at the indicated concentrations. The medium was changed every 2 days. After 7 days, the cells were harvested into 0.3 mL of lysis solution A (10mM Tris, 0.1% [vol/vol] Triton X-100, pH 7.5), sonicated briefly at 0°C, and centrifuged at 12,000 rpm and 4°C. The ALP activity in the supernatant was measured using a kit for ALP. The DNA in the supernatant was measured fluorometrically by the method of Hinegardner (1971), using calf thymus DNA as the standard.

For histological staining for ALP, the cells were washed three times with physiological saline, fixed for 1 min with 10% (vol/vol) formalin in methanol, and stained using an AP-red substrate kit. The cells were considered as ALP positive when they were stained red.

In another series of experiments, C3H10T1/2 cells that had been incubated for 7 days with or without 15 µg/mL OP in a differentiation medium were incubated for 2 days in standard medium supplemented with a combination of INS, DEX, and MIX, as described above. These cells were incubated for 3 days in standard medium containing 5 µg/mL INS alone. The medium was replaced with FBS-free DMEM containing 5 µg/mL INS alone, and the cultures were incubated for 24 h. The plates were then replenished with the same culture medium. After 24 h, the cells were harvested into 0.3 mL of lysis solution B (15mM Tris, 1% [vol/vol] Nonidet P-40, 150mM NaCl, 1mM EDTA, Protease Inhibitor Cocktail Set III [1:1000], pH 7.5), sonicated briefly at 0°C, and centrifuged at 12,000 rpm and 4°C. Aliquots of the infranatant were used to measure DNA and adiponectin. The amount of adiponectin secreted into the culture medium during the last 24-h incubation period was measured in aliquots of medium filtered through a 0.2-µm filter unit. Adiponectin was measured using an ELISA kit for adiponectin.

**Western blotting for TGFβ2 and PPARγ.** To analyze TGFβ2 expression, confluent C3H10T1/2 cells were incubated with or without 15 µg/mL OP in a differentiation medium and harvested at the indicated intervals, as described previously (Masuno et al., 2005). To analyze PPARγ expression, C3H10T1/2 cells that had been incubated for 7 days with or without 15 µg/mL OP in a differentiation medium were incubated for 2 days in standard medium supplemented with a combination of INS, DEX, and MIX and then incubated for an additional 5 days in standard medium containing INS alone, as described above. The cells were harvested at the indicated intervals.

The proteins (5 µg of protein per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 15% (wt/vol) acrylamide resolving gel for TGFβ2 and a 10% (wt/vol) acrylamide resolving gel for PPARγ. Proteins were transferred electrotheretically to Hybond-P membranes. The blotted membranes were incubated with either a rabbit polyclonal antibody to TGFβ2 (1:500) or a rabbit polyclonal antibody to PPARγ (1:1000) and then incubated with a horseradish peroxidase–conjugated secondary antibody (1:25,000). The blots were visualized with the ECL Advance Western Blotting Detection Kit. The membrane was exposed to Hyperfilm ECL with an intensifying screen for 1–5 min.

**Statistical analyses.** Statistical analysis of the group means was performed with ANOVA, followed by post hoc comparisons using Fisher protected least significant difference test. Significant differences between two independent groups were analyzed with the Student t-test. For all statistical analyses, the criterion for significance was p < 0.05. All values are expressed as means ± SE.

**RESULTS**

**Does OP Affect the Differentiation of C3H10T1/2 Cells into Osteoblasts?**

Confluent C3H10T1/2 cells were treated for 7 days with OP at the indicated concentrations. The control cultures, in which OP was absent during the treatment period, contained 7.66 µg per plate of DNA and 2.74 nmol/min/µg DNA of ALP activity. OP at concentrations of < 10 µg/ml did not cause any changes in the DNA content, but 15 µg/ml OP decreased the DNA content by 17% (Fig. 1A). ALP activity decreased with increasing OP concentrations (Fig. 1B). The presence of 5 µg/ml of OP caused a significant decrease in ALP activity. OP at concentrations of 10 and 15 µg/ml decreased ALP activity by 38 and 81%, respectively. In cultures treated with 20 µg/ml OP for more than 16 h, cells began to detach from the bottom of the

Eagle’s medium (DMEM). The differentiation medium contained 1µM all-trans-retinoic acid and 5mM β-glycerophosphate in standard medium.

**Measurement of adiponectin in cells and media.** C3H10T1/2 cells that had been incubated for 7 days with or without 15 µg/mL OP in the differentiation medium were incubated for 2 days in standard medium supplemented with a combination of INS, DEX, and MIX, as described above. These cells were incubated for 3 days in standard medium containing 5 µg/mL INS alone. The medium was replaced with FBS-free DMEM containing 5 µg/mL INS alone, and the cultures were incubated for 24 h. The plates were then replenished with the same culture medium. After 24 h, the cells were harvested into 0.3 mL of lysis solution B (15mM Tris, 1% [vol/vol] Nonidet P-40, 150mM NaCl, 1mM EDTA, Protease Inhibitor Cocktail Set III [1:1000], pH 7.5), sonicated briefly at 0°C, and centrifuged at 12,000 rpm and 4°C. Aliquots of the infranatant were used to measure DNA and adiponectin. The amount of adiponectin secreted into the culture medium during the last 24-h incubation period was measured in aliquots of medium filtered through a 0.2-µm filter unit. Adiponectin was measured using an ELISA kit for adiponectin.

**OCTYLPHENOL INHIBITS OSTEOGENESIS**

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plates. Therefore, the following experiments were performed using cultures treated with 15 μg/ml OP, termed the OP-treated cultures.

OP altered the appearance of the cells, resulting in a rounder morphology, unlike the elongated, fibroblastic, or osteoblastic appearance of the cells in the control cultures (Fig. 1C). These cells were fixed with formalin and histochemically stained for ALP. In the control cultures, the majority of cells were stained intensely (Fig. 1C, left panel). In the OP-treated cultures, there were fewer ALP-positive cells than in the control cultures, and the intensity of their staining was low (Fig. 1C, right panel).

The time courses of the OP-induced changes in DNA content, ALP activity, and expression of TGFβ2 and PPARr are shown in Figure 2. In both the control and OP-treated cultures, the DNA contents decreased during the first 4 days of the treatment period and then remained unchanged up to day 11 (Fig. 2A). The DNA contents of the OP-treated cultures were lower than those of the control cultures on the corresponding days. The ALP activity of the control cultures increased linearly up to 7 days and then increased slightly (Fig. 2B). The ALP activity of the OP-treated cultures also increased in a similar manner but was very low throughout the 11 days of treatment.

Confluent cells slightly expressed TGFβ2. In the control cultures, TGFβ2 was strongly induced during the 7-day treatment period; the TGFβ2 level on day 7 was higher than that on day 3 (Fig. 2C). Treatment of cells with OP inhibited the expression of TGFβ2; the TGFβ2 level on day 3 was similar to that in the confluent controls, while the level on day 7 was very low.

Confluent cells did not express PPARr. In the control cultures, PPARr was induced during the 7-day treatment period; the PPARr level on day 3 was detectable but very low, and the level on day 7 was higher than that on day 3 (Fig. 2C). Treatment of cells with OP markedly increased the expression of PPARr; the levels of PPARr on days 3 and 7 were higher than those of the control cultures on the corresponding days.

Effect of Another Alkylphenol, NP, on ALP Activity

The potency of NP to reduce ALP activity was compared with that of OP. When confluent cells were treated with 15 μg/ml NP, the cells detached from the bottom of the plates within 24 h. Therefore, this experiment was performed using alkylphenols at a concentration of 10 μg/ml. The DNA content and ALP activity decreased by 18 and 16%, respectively, in the cultures treated with NP, and by 9 and 39%, respectively, in the cultures treated with OP, relative to those in the control cultures (Fig. 3). Thus, the potency of NP to reduce ALP activity was lower than that of OP.

Effect of BPA-DGE on OP-Induced Decrease in ALP Activity

Confluent C3H10T1/2 cells were treated for 7 days with 15 μg/ml OP in the absence or presence of 50μM BPA-DGE,
Does C3H10T1/2 Cells in the OP-treated Cultures Differentiate into Adipocytes?

C3H10T1/2 cells in the control and OP-treated cultures were treated for 2 days with a combination of INS, DEX, and MIX and then for an additional 5 days with INS alone. The DNA content of the control cultures increased during the first 2 days of the 7-day treatment period and then remained unchanged, whereas that of the OP-treated cultures increased linearly during the first 4 days and then increased gradually (Fig. 4A).

The DNA content of the cultures on day 0 was lower in the OP-treated cultures than in the control cultures, but on days 4 and 7, it was higher in the former cultures than in the latter cultures.

The TG content of the cultures on day 0 was very low in both the control and OP-treated cultures (Fig. 4B). The TG content of the control cultures increased from 0.71 to 6.78 μg/g DNA during the 7-day treatment period, while that of the OP-treated cultures increased from 0.71 to 6.78 μg/g DNA over the same period. On day 7, there were many more Oil Red O-stained cells in the OP-treated cultures than in the control cultures (Fig. 4C).

Figure 4D shows the expression of PPARγ. In both the control and OP-treated cultures, the level of PPARγ increased after hormonal treatment. The level of PPARγ in the OP-treated cultures was higher throughout the 7 days of hormonal treatment than in the control cultures.

The amounts of adiponectin, one of the adipocytokines produced by adipocytes and a marker of adipocyte differentiation

**TABLE 1**

Effect of BPA-DGE on OP-Induced Decrease in ALP Activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>OP</th>
<th>BPA-DGE</th>
<th>ALP activity (nmol/min/μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.48 ± 0.02*</td>
</tr>
<tr>
<td>–</td>
<td>+</td>
<td>–</td>
<td>1.47 ± 0.03</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>–</td>
<td>0.34 ± 0.03*</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>–</td>
<td>0.15 ± 0.01*</td>
</tr>
</tbody>
</table>

*Note. C3H10T1/2 cells were treated for 7 days with 50 μM BPA-DGE alone or 15 μg/ml OP alone or a combination of 15 μg/ml OP and 50 μM BPA-DGE in a differentiation medium. The cells were harvested, sonicated briefly at 0°C, and centrifuged. The ALP activity in the supernatant was measured. The addition of BPA-DGE did not cause any changes in the DNA content of the corresponding cultures (data not shown).

*p < 0.01 (compared with the value obtained for the control cultures); **p < 0.01 (compared with the value obtained for the cultures treated with OP alone).
Control cultures 4.6 ± 1.2
OP-treated cultures 18.8 ± 6.4

†Mean ± SE of four plates.
* < 0.01 (compared with the value obtained for the control cultures).

(Combs et al., 2002; Ouchi et al., 2003), in cells and culture media were measured (Table 2). The amount of cellular adiponectin was 4.1 times higher in the OP-treated cultures than in the control cultures. The amount of adiponectin secreted into the culture medium over the last 24 h of the 7-day hormonal treatment period also was 4.7 times higher in the OP-treated cultures than in the control cultures.

**DISCUSSION**

Osteoblasts are the principal bone-forming cells, and adipocytes are the main fat-storing cells. These two different types of cells originate from the same multipotent mesenchymal stem cells. The aim of this study was to determine whether alkylphenols affect the differentiation of multipotent C3H10T1/2 cells into osteoblast and adipocyte lineages. In the first set of experiments, whether treatment of confluent C3H10T1/2 cells with alkylphenols affected the expression of osteoblast phenotypic markers was examined. The results of ALP activity measurements showed that the expression of ALP was lower in the OP- than OP. A Western blot analysis of TGF showed that the level of TGF was higher in the OP-treated cultures than in the control cultures. NP also had a similar decreasing effect on ALP activity but was less potent than OP. A Western blot analysis of TGFβ2 showed that OP inhibited the expression of TGFβ2. In addition, OP inhibited the morphological changes in cells to an osteoblastic appearance. These findings indicate that alkylphenols inhibited the differentiation of multipotent C3H10T1/2 cells into osteoblasts.

It has been reported that PPARr is associated with bone metabolism (Akune et al., 2004; Ali et al., 2005; Rzonca et al., 2004). For example, rosiglitazone, a ligand for PPARr, causes bone loss in mice by suppressing osteoblast differentiation and bone formation (Ali et al., 2005; Rzonca et al., 2004). We found that treatment of confluent C3H10T1/2 cells with OP strongly induced the expression of PPARr. These findings suggest the possibility that OP acts as a PPARr ligand, resulting in inhibition of osteoblast differentiation. To explore this possibility, we examined whether a PPARr antagonist, BPA-DGE, affected the OP-induced decrease in ALP activity. Since BPA-DGE was not able to reverse the OP-induced decrease in ALP activity, this indicates that the inhibitory action of OP on osteoblast differentiation was mediated by a mechanism that does not involve PPARr. Moreover, we found that an estrogen receptor antagonist, tamoxifen, did not block the decreasing effect of OP on ALP activity (data not shown), indicating that OP did not act through the estrogen receptor. Thus, the mechanism by which OP inhibited the differentiation of C3H10T1/2 cells into osteoblasts remains unclear.

Beresford et al. (1992) reported that there was a reciprocal relationship between osteogenesis and adipogenesis in cultures of rat marrow stromal cells. This raises the question of whether treatment of C3H10T1/2 cells with OP caused a lineage shift toward adipocytes. The 7-day treatment of confluent C3H10T1/2 cells with OP did not increase the TG content of the cultures (the control cultures [n = 4], 0.70 ± 0.08 μg/μg DNA; the OP-treated cultures [n = 4], 0.71 ± 0.11 μg/μg DNA). This result is consistent with our previous finding that OP by itself did not have the ability to stimulate TG accumulation in adipocytes (Masuno et al., 2005). Therefore, to explore the above mentioned question, C3H10T1/2 cells that had been treated for 7 days with or without OP were treated with lipogenic hormones. In this set of experiments, we used three criteria for adipocyte differentiation: (1) TG accumulation in cells, (2) the expression of PPARr, and (3) the level of adiponectin. The results of TG measurements and Oil Red staining showed that there were more lipid-containing cells in the OP-treated cultures than in the control cultures. A Western blot analysis of PPARr showed that the level of PPARr was higher in the OP-treated cultures than in the control cultures. The results of adiponectin measurements showed that the OP-treated cultures produced and secreted larger amounts of adiponectin than the control cultures. These results indicate that the OP-treated cultures contained a larger number of adipocytes than the control cultures. Based on these findings, we conclude that OP inhibits the differentiation of multipotent C3H10T1/2 cells into osteoblasts, causing a lineage shift toward adipocytes.

Several members of the nuclear receptor superfamily play an important role in the induction of adipocyte differentiation and regulate alterations in gene expression during adipocyte differentiation (Chawla et al., 1994; Cornelius et al., 1994; Gregoire et al., 1998). One of these members is PPARr, which is expressed in an adipocyte-specific manner and serves as a transcriptional activator that induces the expression of adipocyte genes. PPAR response elements (PPREs) have been identified in a number of adipocyte genes including the adiponectin (Iwaki et al., 2003), 422/aP2 (Tontonoz et al., 1994), and lipoprotein lipase (Schoonjans et al., 1996) genes. Iwaki et al. (2003) showed that PPARr plays a significant role in the transcriptional activation of adiponectin gene via the PPRE in its promoter. Taken together, our present results indicate that OP enhanced the expression of PPARr, resulting in an increase in the production and secretion of adiponectin.
In the present study, we used high concentrations (2.42 × 10^{-5} to 7.27 × 10^{-5}M) of alkylphenols, which were similar to those used by others. Our previous study showed that both OP (4.85 × 10^{-5}M) and NP (4.54 × 10^{-5}M) increased cell proliferation in cultures of fully differentiated 3T3-L1 cells (Masuno et al., 2003). Abraham and Frawley (1997) reported that OP at 10^{-6} to 10^{-4}M stimulated prolactin gene expression in pituitary cells. Kwack et al. (2002) reported that OP at 10^{-5}M stimulated cell proliferation in cultures of human estrogen-sensitive MCF-7 breast tumor cells, but OP at > 10^{-6}M induced slight cytotoxicity. Kanno et al. (2004) reported that NP at 10^{-3} to 10^{-4}M was cytotoxic in cultures of MC3T3-E1 cells. Thus, cytotoxicity of alkylphenols differs depending on the types of cells. We found that OP at < 4.85 × 10^{-5}M did not inhibit DNA replication but significantly decreased ALP activity. Moreover, the cells in the cultures treated with OP at the highest concentration (7.27 × 10^{-5}M) excluded trypan blue (data not shown). These results indicate that a decrease in ALP activity in the OP-treated cultures was not due to the cytotoxic effect of OP in C3H10T1/2 cells.

Hernando et al. (2004) reported that the concentrations of OP found in wastewater ranged from 16.7 to 48.4 ng/l. Kuch and Ballschmieder (2001) reported that the concentrations of OP ranged from 0.8 to 54 ng/l in river water and from 0.2 to 4.9 ng/l in drinking water. These concentrations were very low compared with those used in this study. Shiraishi et al. (1989) reported that the fat tissue of carp in the Trenton Channel of the Detroit River in the United States contained very high concentration (140 ppm) of 2,4-di-tert-pentylphenol. This concentration was greater than that in the water or sediment, suggesting that bioaccumulation of alkylphenols occurs in adipose tissue. These findings suggest that chronic exposure to low levels of alkylphenols may cause deleterious effects to wildlife and humans. Therefore, it will be important to examine the relationship between chronic exposure to alkylphenols and bone metabolism in further studies.

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