PHENYTOIN STIMULATES CHONDROGENIC DIFFERENTIATION IN MOUSE CLONAL CHONDROGENIC EC CELLS, ATDC5

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ABSTRACT — Phenytoin (DPH) is known to affect bone formation. However, the mechanism of this effect has not been well understood. In this study, we evaluated the effects of DPH on cartilage formation in a model system using ATDC5 cells, a clonal murine chondrogenic cell line. Alcian blue staining for cartilage nodules and real-time reverse-transcription polymerase chain reaction for the expression of genes encoding type II collagen, aggrecan, transforming growth factor (TGF)-β1, bone morphogenetic protein (BMP)-4, parathyroid hormone-related peptide (PTHrP), indian hedgehog (Ihh), and patched (Ptc) were performed in ATDC5 cells cultured with DPH. The ATDC5 cells demonstrated enhanced cartilage formation in cultures with DPH. During promoted chondrogenic differentiation, it was observed that DPH increased the mRNA expression of TGF-β1, BMP-4, Ihh, and Ptc, in a dose-dependent manner on Days 5 to 15. In contrast, other antiepileptic drugs, phenobarbital and valproic acid had no effects on chondrogenesis in ATDC5 cells and osteogenesis in MC3T3-E1 cells. Our results provide fundamental evidence that DPH has a direct stimulatory effect on cartilage formation by regulating TGF-β and hedgehog signaling molecules, and that DPH effect on bone formation, including chondrogenesis and osteogenesis, is distinct from other antiepileptic drugs as suggested in clinical settings.

KEY WORDS: Antiepileptic drugs, ATDC5, Chondrogenesis, EC cells, Phenytoin

INTRODUCTION

Phenytoin (diphenylhydantoin, DPH) is a widely used antiepileptic drug, but has been found to affect bone metabolism during long-term use. In patients receiving chronic DPH therapy, increased thickness and density of maxillary and calvarial bones has been shown (Kattan, 1970; Lefebvre et al., 1972; Johnson, 1984). Sasaki et al. (1999) reported a clinical case of excessive exostosis that had formed in a patient chronically treated with DPH. In addition, there have been many in vivo and in vitro studies which suggest that DPH has direct positive effects on osteogenesis. In rabbits, the healing of experimentally produced fractures of mandibular bone was accelerated by DPH (Sklans et al., 1967). In human culture cells, DPH has osteoblastic effects at micromolar concentrations (Lau et al., 1995). Moreover, DPH increased the osteoblast number and osteoid thickness, as well as the volume of bone tissues in rats (Ohta et al., 1995). However, bone formation is a complex process including osteogenesis and chondrogenesis, and the effect of DPH on chondrogenesis has not yet been elucidated. The evaluation of DPH effects on chondrogenesis provides fundamental information for understanding the effect of DPH on bone formation in clinical settings.

Chondrocytes undergo coordinated proliferation, differentiation and apoptosis to produce a cartilage scaffold that is mineralized during new bone formation. The process of chondrocyte differentiation is regulated by glucocorticoids (GCs) and thyroid hormones (THs). In growing rats treated with corticosterone, the growth plate was reduced, which was attributed to impaired chondrocyte proliferation and increased hypertrophic chondrocyte apoptosis (Silvestrini et al., 2000). It was reported that hypothyroid growth arrest results from disorganization of a growth plate in which there is a relative failure of hypertrophic chondrocyte differentiation as well as production of an abnormal cartilage matrix (Stevens et al., 2000). These findings clearly
demonstrate that GCs and THs have opposite actions on chondrogenic proliferation and differentiation in vivo. In addition, DPH is known to have teratogenic potency inducing cleft palate in humans and laboratory animals (Dansky and Finnell, 1991; Finnell and Dansky, 1991), as GCs do. (Goldman et al., 1978). Katsumata et al. (1982) demonstrated that DPH and GCs share a common receptor which is responsible for the teratogenic effects of these chemicals. This DPH mechanism has been supported by later experiments (Goldman, 1984; Katsuyama et al., 1985; Kay et al., 1990). The significant interaction of DPH with TH receptor (TR) was also reported previously (Franklyn et al., 1985). These findings imply, in terms of the molecular mechanism, that DPH may act in two ways to inhibit or stimulate chondrocyte differentiation, through GC receptor (GR) or TR signaling, respectively. Thus it is of great interest to identify whether DPH has stimulatory or inhibitory effects on chondrogenesis.

In this study, we employed the clonal murine cell line ATDC5. This line of embryonal carcinoma (EC) cells is isolated from the feeder-independent teratocarcinoma stem cell line AT805 (Atsumi et al., 1990), and is well established as an in vitro model for the differentiation of chondrocytes (Shukunami et al., 1997). In the presence of insulin, there appeared to be areas of the condensation in the culture from which proliferating chondrocytes were generated to form cartilage nodules. The progressive expression of type II collagen and aggrecan mRNA was initiated along with condensation and the subsequent growth of the nodules (Shukunami et al., 1997). The differentiation of ATDC5 cells is coordinately regulated by the molecules fundamental for the normal chondrogenesis in vivo. Previous studies demonstrated that opposite inhibitory and stimulatory effects were exhibited in ATDC5 cells treated with GCs and THs, respectively, as in vivo (Miura et al., 2002; Siebler et al., 2002), suggesting that the evaluation for determining the chondrogenic effects of DPH in this cell line was accurate. Moreover, in these cells, the supplementation of Indian hedgehog (Ihh) stimulated chondrogenic differentiation (Akiyama et al., 1999; Enomoto-Iwamoto et al., 2000), and parathyroid hormone-related peptide (PTHrP) inhibited cellular condensation and the subsequent formation of cartilage nodules (Shukunami et al., 1996). The progression of chondrogenic differentiation of ATDC5 cells also involves autocrine signaling by transforming growth factor (TGF)-β and bone morphogenetic protein (BMP)-4 (Kawai et al., 1999; Shukunami et al., 2000).

Firstly, we evaluated the direct effects of DPH on chondrogenic differentiation in ATDC5 cells. Next, to understand the molecular mechanism of DPH-affected chondrogenesis, the expression of TGF-β1, BMP-4, Ihh, patched (Ptc, a Ihh receptor), and PTHrP were investigated by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). Additionally, effects of other widely used antiepileptic drugs, phenobarbital (PBT) and valproic acid (VPA), on chondrogenesis and osteogenesis were examined on ATDC5 and MC3T3-E1 cells, respectively. Since PBT and VPA have toxicological and teratological effects similar to DPH, the comparison of the effect on bone formation between these chemicals may be critical to understanding the effects of DPH and its mechanism in bone formation. The results obtained herein show that DPH directly promoted not only osteogenic differentiation, but also chondrogenic differentiation. PBT and VPA had no effect on either the osteogenic or the chondrogenic differentiation processes in vitro, confirming the distinctness of the effect of DPH on bone formation in clinical settings.

**MATERIALS AND METHODS**

**Reagents**

Alpha-modified minimum essential medium (α-MEM), a 1:1 mixture of Dulbecco’s modified Eagle’s and Ham’s F12 medium (DMEM/F12), and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA). β-Glycerophosphate, bovine insulin (I), human transferrin (T), and sodium selenite (S) were obtained from Sigma Chemical Co. (St. Louis, MO). 5,5-Diphenylhydantoin sodium salt (DPH) was from Sigma. Both phenobarbital sodium (PBT) and sodium valproate (VPA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Cell culture and treatment with antiepileptics**

ATDC5 and MC3T3-E1 cells were purchased from RIKEN cell bank (Ibaraki, Japan). Stock cultures were grown in growth media of DMEM/F12 containing 5% FBS or α-MEM containing 10% FBS with 50 µg/mL ascorbic acid for ATDC5 or MC3T3-E1 cells, respectively, at 37°C in a humidified atmosphere of 5% CO2 in air. After purchasing, cells were not used beyond passage 10, and were passed every 3 days. They were plated at an initial density of 1.0 × 10^4 cells/cm² in culture dishes. For ATDC5, after a 48 hr precul-
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The medium was changed to the growth medium which included ITS (10 µg/mL insulin, 10 µg/mL transferrin and 3 × 10⁻⁸ M sodium selenite) with or without each concentration of antiepileptics (culture day 0). While for MC3T3-E1, after a 48-hr preculture period, the medium was changed to the growth medium with 10 mM β-glycerophosphate and each concentration of antiepileptics (culture day 0). The medium was replaced every other day. Duplicate or triplicate cultures were used for each experiment. The selected concentrations for the antiepileptics were 1, 10, and 100 µM of DPH, 10 and 100 µM of PBT, and 100 and 1,000 µM of VPA, which showed low cytotoxicity in these cells (data not shown).

Alcian blue and Alizarin red S stainings
ATDC5 and MC3T3-E1 cells were plated in 6-multiwell plates (Corning Incorporated, Corning, NY) and cultured. At each time point, cells were rinsed with phosphate-buffered saline, and fixed on ice with 99.5% methanol for 2 min or 70% ethanol for 15 min for alcian blue or alizarin red stainings, respectively. Fixed ATDC5 and MC3T3-E1 cells were then stained with 0.1% alcian blue 8 GX (Sigma) for 2 hr and 1% alizarin red S (Kanto Kagaku Co., Inc., Tokyo, Japan) for 5 min, respectively, at room temperature. The plates were washed with tap water, air-dried, and photographed.

Assay for Alkaline Phosphatase (ALPase) activity
MC3T3-E1 cells were seeded in 24-multiwell plates (Asahi Techno Glass Corp., Tokyo, Japan) and cultured. At each time point, the activity of ALPase was measured using p-nitrophenyl phosphate (pNPP) as a substrate. Cell layers were homogenized on ice in 0.9% NaCl containing 0.2% Triton X-100, and then centrifuged. The supernatant was assayed in a reaction mixture of 0.5 mM pNPP and 0.5 mM MgCl₂. The reaction was carried out for 15 min at 37°C, and the concentration of p-nitrophenol generated was measured at 415 nm using a SPECTRAmax 250 Microplate Spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). The serial dilutions of p-nitrophenol (Sigma) were also measured as the standard. Protein content in the cell layer was determined by the Bradford method using a Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Values of ALPase activity were standardized by protein content, and expressed as nmol/µg protein/hour.

Total RNA preparation and qRT-PCR
ATDC5 cells were cultured in 6 cm dishes (Corning). Procedures for total RNA preparation and qRT-PCR were described previously (Okada et al., 2002). Primer sequences used in this study are shown in Table 1. Quantitative real-time PCR was carried out in an ABI Prism 7900 Sequence Detector (Applied Biosystems, Foster, CA). The number of template copies present at the start of the reaction was determined by comparison to a standard scale prepared from mouse genomic DNA. The expression level of each target gene was calculated by standardizing the target gene copy number with the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) copy number in a sample. The analysis of the results is based on triplicate samples from two independent experiments.

Statistical analysis
Statistical analysis was carried out using Duncan’s multiple comparison test for qRT-PCR as well as ALPase activity studies. Data are reported as the mean ± SD, and are considered significantly different at p<0.05.

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a: GenBank accession number.
RESULTS

Effects of DPH on cartilage formation in ATDC5 cells

To evaluate chondrogenic potency of DPH, ATDC5 cells were cultured both with and without DPH. Two days after inoculation, ATDC5 cells rapidly proliferated to form a confluent monolayer. At this point, they remained undifferentiated with a fibroblastic morphology. After reaching confluency, insulin and each concentration of DPH were added to the culture medium. As shown in Photo 1, without DPH, ATDC5 cells showed chondrogenic differentiation through the condensation stage to form cartilage nodules. The size and number of cartilage nodules increased with each day of culture. DPH stimulated cell condensation and increased the formation of cartilage nodules in a dose-dependent manner. Changes in cartilage nodule formation were verified by staining ATDC5 cells with alcian blue, as shown in Photo 2. No alcian blue staining was seen on Day 6 for any culture group. In the absence of DPH on Day 12, spotty staining of cartilage nodules appeared in the center area of the culture. The degree of staining then spread out through the dish over time and reached its highest intensity on Day 20 (Photo 2). On Day 12, DPH had no influence on staining compared to the control (not shown). However, on Day 20, an apparent increase in the number of stained spots was noted in ATDC5 cells cultured with 100 µM DPH (Photo 2). Thus, DPH at 100 µM stimulates chondrogenic differentiation to form cartilage nodules in ATDC5 cells.

Effects of DPH on chondrogenic marker genes in ATDC5 cells

A number of extracellular matrix (ECM) marker genes

Photo 1. Phase contrast micrographs of ATDC5 cells with and without DPH. In the absence of DPH (Control), ATDC5 cells are at the undifferentiated condensation stage on Day 5, and typical cartilage nodules are formed by Day 15. The bar represents 200 µm.
molecules, including type II collagen and aggrecan, are well characterized during chondrogenesis *in vivo* and in ATDC5 cells (Shukunami *et al.*, 1997). To support the stimulatory effect of DPH on the chondrogenic differentiation, the expressions of type II collagen and aggrecan mRNAs were measured using qRT-PCR in ATDC5 cells cultured with DPH at 1, 10, and 100 µM on Days 5, 10, and 15. Both mRNAs were expressed on Day 5, and expression increased with each culture day (Fig. 1). On Day 5, expression levels of type II collagen treated with DPH were comparable to the control at 1 and 10 µM, but significantly higher at 100 µM. In the absence of DPH, type II collagen was markedly increased at Day 10 and slightly decreased at Day 15. DPH induced the higher expression of type II collagen dose-dependently on Days 10 and 15 at 10 and 100 µM. While, in the control cells, aggrecan mRNA increased continuously from Days 5 to 15. At 10 and 100 µM, an increase in aggrecan was detected when compared to the control on Days 10 and 15 (Fig. 1). These results taken together indicate that DPH induces type II collagen and aggrecan expressions in ATDC5 cells in a dose-dependent manner. Thus, changes in the expression of ECM molecules strongly confirm the DPH effects in promoting the chondrogenic differentiation of ATDC5 cells.

**Effects of DPH on the expression of TGF-β1, BMP-4, PTHrP, Ihh, and Ptc mRNAs during the chondrogenic differentiation of ATDC5 cells**

In order to understand the mechanism of DPH effects on chondrogenesis, we next examined the expression of signaling molecules important for chondrogenesis in ATDC5 cells. TGF-β and hedgehog signaling cascades were selected to examine the effect of DPH in ATDC5 cells. These cascades are fundamental for normal chondrogenesis both *in vivo* and in ATDC5 cells, and, therefore, their alteration in ATDC5 cells may be clear evidence for demonstrating DPH effects in clinical settings. In control cells, TGF-β1 mRNA was detected on Day 5 and increased on Day 10, meanwhile, BMP-4 was expressed on Day 5 and maintained to Day 15. In the presence of DPH, significantly higher expressions of TGF-β1 were exhibited throughout Days 5 to 15 in ATDC5 cells exposed to 100 µM concentrations (Fig. 2). Similarly, the expression of BMP-
4 was induced by treatment with 10 and 100 µM of DPH on Day 10 (Fig. 2). According to hedgehog signaling, an elevation of Ihh and Ptc occurred on culture Days 5 to 15 in cells cultured without DPH. At 10 and/or 100 µM, increased expressions in both Ihh and Ptc were observed on Day 10 or 15 (Fig. 3). These findings indicate that DPH promotes chondrogenic differentiation of ATDC5 cells by stimulating TGF-β and hedgehog signaling molecules. There was no marked change in PTHrP expression in cells exposed to any concentration of DPH (Fig. 3).

**Effects of VPA and PBT on cartilage formation and mineralization**

PBT and VPA are other antiepileptic drugs widely used in clinical settings. In addition to their pharmacological potency, these chemicals are known to cause a variety of toxicological and teratological effects similar to DPH in humans and laboratory animals (Dansky and Finnell, 1991; Finnell and Dansky, 1991). Since the osteogenic and chondrogenic potencies of PBT and VPA have not yet been reported *in vivo* and *in vitro*, the evaluation of the effect of these chemicals may provide information important for understanding the similarities or differences in the effect mechanisms of antiepileptic drugs on adult and fetal bone formations. Thus the alcin blue staining and qRT-PCR for type II collagen and aggrecan were performed in ATDC5 cells cultured

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**Fig. 1.** Effects of DPH on the expression of type II collagen and aggrecan. The level of mRNA expression for type II collagen (upper) and aggrecan (lower) was determined for ATDC5 cells cultured with DPH on Days 5, 10, and 15. The data represents the mean ± SD from triplicate samples in 2 independent experiments. *a p<0.05 and b p<0.01 vs. control.*

**Fig. 2.** Effects of DPH on the expression of TGF-β1 and BMP-4. The level of mRNA expression for TGF-β1 (upper) and BMP-4 (lower) was determined for ATDC5 cells cultured with DPH on Days 5, 10, and 15. The data represents the mean ± SD from triplicate samples in 2 independent experiments. *a p<0.05 and b p<0.01 vs. control.*
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Moreover, to compare the effects on mineralization with that of DPH, alizarin red S staining for mineralized nodules and the measurement of ALPase activity were performed on MC3T3-E1 cells treated with and without DPH, PBT, or VPA. The clonal murine calvarial cell line MC3T3-E1 has been well used as an in vitro model for osteoblast differentiation and maturation in the presence of β-glycerophosphate (Kodama et al., 1981; Sudo et al., 1983).

In ATDC5 cells, no differences in the formation of cartilage nodules or their staining with alcian blue was observed in the presence or absence of PBT or VPA at any concentration (Fig. 4). Both type II collagen as well as aggrecan mRNA expression were comparable in ATDC5 cells cultured with PBT or VPA with 10 and 100 μM PBT, or 100 and 1,000 μM VPA.

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**Fig. 3.** Effects of DPH on the expression of PTHrP, Ihh, and Ptc. The level of mRNA expression for PTHrP (upper), Ihh (middle), and Ptc (lower) was determined for ATDC5 cells cultured with DPH on Days 5, 10, and 15. The data represents the mean ± SD from triplicate samples in 2 independent experiments. *p<0.05 and †p<0.01 vs. control.

**Fig. 4.** Effects of PBT and VPA on the chondrogenesis of ATDC5 cells. ATDC5 cells cultured with or without PBT (10 and 100 μM) or VPA (100 and 1,000 μM) were stained with 0.1% alcian blue on Day 20 (upper). The figure represents a typical well at each time point. The lower columns show the level of mRNA expression for type II collagen and aggrecan in ATDC5 cells cultured with PBT or VPA on Day 15. The data represent the mean ± SD from triplicate samples in 2 independent experiments. *p<0.01 vs. control.
as compared to the control on Day 15 (Fig. 4), confirming that PBT and VPA have no direct effect on the chondrogenic differentiation of ATDC5 cells. Mineralized nodules stained with alizarin red S were absent in all groups including the control on Day 10, and then appeared moderately on Day 20 in the control for the MC3T3-E1 cells (Photo 3). However, DPH at 100 µM markedly increased both the number and the area of the stained spots in the dish in which the MC3T3-E1 cells had been cultured for 20 days (Photo 3). Treatment with DPH at 10 and 100 µM also induced ALPase activity in a dose-dependent manner on Day 7, when

Photo 3. Effects of DPH, PBT, and VPA on the formation of mineralized nodules. MC3T3-E1 cells cultured with DPH, PBT, or VPA were stained with 1% alizarin red S on Days 10 (control) and 20 (control and others). The figure represents a typical well at each time point and concentration.
enzyme activity began to increase in the untreated-culture (Fig. 5, Kodama et al., 1981). In contrast, no effects of PBT and VPA were observed on the formation of mineralized nodules and ALPase activity in MC3T3-E1 cells (Photo 3 and Fig. 5). These findings clearly demonstrate that the stimulatory effects of DPH on chondrogenesis and osteogenesis are distinct from that of PBT and VPA in the systems used in this study.

**DISCUSSION**

Much *in vivo* and *vitro* evidence indicates that DPH has a promoting effect on bone formation, especially osteogenesis, in clinical and laboratory settings. However, the effect of DPH on chondrogenesis has not been well elucidated, and the molecules responsible for the DPH effect remain unknown. This study is an initial report to demonstrate that DPH has a stimulatory effect on chondrogenic differentiation in ATDC5 cells as well as osteogenic differentiation in MC3T3-E1 cells. This study also demonstrates that the effect of DPH on bone formation is distinct from that of PBT and VPA. Importantly, our findings show that the enhancement of the expression of signaling molecules such as TGF-β1, BMP-4, Ihh, and Ptc occurs during chondrogenesis promoted by DPH. Thus this study provides fundamental information for understanding the effects of DPH treatment on bone formation in a clinical setting.

ATDC5 and MC3T3-E1 cells are well characterized in *in vitro* models and have been used for investigating the molecular mechanisms of chondrogenic and osteogenic differentiation, respectively. In this study, we showed that DPH stimulated the osteogenic differentiation of MC3T3-E1 cells at concentrations of 10-100 µM. This effect is in agreement with previous studies in rats treated intraperitoneally at 1-5 mg/kg/day (serum DPH levels were not determined) (Ohta et al., 1995), fetal rat calvaria cells at 12.5-200 µM (Ikedo et al., 1999), and human osteoblasts at 5-10 µM (Lau et al., 1995). Thus it has been confirmed that DPH is an apparent osteogenic compound *in vivo* and *in vitro* at micromolar concentrations. It is of interest that the induction of chondrogenic differentiation has been shown here in ATDC5 cells cultured with DPH at concentrations of 10-100 µM ATDC5 showed an increased-formation of cartilage nodule in a dose-dependent manner with DPH on Day 20, as shown in Photos 1 and 2. However, the expression of chondrogenic markers was a more sensitive indicator than the alcian blue staining for cartilage nodules, as expression increased for both type II collagen and aggrecan mRNA, starting from Days 5 and 10, respectively. Later effects on aggrecan expression reflected a normal sequential expression of these markers, suggesting that DPH accelerates the differentiation process in ATDC5 cells. Following these stages, ATDC5 cells transited to the mineralized stage by changing the CO2 concentration to 3% on Day 21 (Shukunami et al., 1997). However, we focused on the effects of DPH on the early phases of the ATDC5 cells, and the later effect on mineralization was studied in MC3T3-E1 cells. Taken together, these findings clearly show that DPH at 10-100 µM directly stimulates both the osteogenic and chondrogenic processes during bone formation *in vitro*. The recommended therapeutic range of serum DPH concentration for treating epileptic patients is between 10 and 20 µg/ml (equivalent to 36.5-73 µM) (Levy, 1980), indicating that the *in vitro* effect observed in this study could be clinically relevant.

Concerning the chondrogenic signaling molecule, the stimulatory effect of DPH was detected in the expression of TGF-β1, BMP-4, Ihh, and Ptc during chondrogenic differentiation in ATDC5 cells. Previous studies demonstrated that autocrine signaling by TGF-β...
and BMP-4 are involved in the progression of early chondrogenic differentiation, including initiation and conversion of cell condensation, in ATDC5 cells (Kawai et al., 1999; Shukunami et al., 2000). Thus the induction of TGF-β1 and BMP-4 may be responsible for the early differentiation promoted by DPH treatment. Especially BMP-4 is expressed highly in early culture stages (Days 3 to 14) in ATDC5 cells and declines thereafter (Akiyama et al., 2000), and its signaling was critically required for the conversion of condensing undifferentiated cells into chondrocytes (Shukunami et al., 2000). Thus in this study, the transient increase in BMP-4 expression noted on Day 10 of culture with DPH may reflect the critical requirement of BMP-4 in this stage of ATDC5 cell differentiation. In addition, DPH stimulated the increase in Ihh expression as well as Ptc expression as shown in Fig. 3. That the supplementation of Ihh stimulated the late-phase chondrogenic differentiation of ATDC5 cells (Akiyama et al., 1999; Enomoto-Iwamoto et al., 2000) suggests that DPH-increased Ihh could accelerate the late-phase chondrogenesis even more. Moreover, the induction of Ptc observed in the culture with DPH was possibly due to the increased Ihh, since the recombinant of Ihh induced Ptc expression (Akiyama et al., 1999). In contrast,PTHrP is a potent inhibitor of chondrogenic differentiation of chondrocytes (Lee et al., 1996; Schipani and Provot, 2003), inhibited cellular condensation and the subsequent formation of cartilage nodules in ATDC5 cells (Shukunami et al., 1996), and is known to be induced by Ihh in pre-chondrocytes (Vortkamp et al., 1996). In this study, although changes in PTHrP were not evident in ATDC5 cells cultured with DPH during chondrogenic differentiation by Day 15, its expression may increase sequentially in the later termination stages of chondrogenesis. Taken together, these findings suggest that the expression of molecules involved in TGF-β and hedgehog signaling is coordinately induced by DPH treatment and highly responsible for DPH-accelerated chondrogenesis in ATDC5 cells.

DPH is known to have the teratogenic potency to induce cleft palate and axial skeletal malformations in humans and laboratory animals (Danksy and Finnell, 1991; Finnell and Danksy, 1991). The involvement of GR in DPH-induced cleft palate has been suggested since DPH and GCs share a common receptor (Katsumata et al., 1982; Goldman 1984; Katsuyama et al., 1985; Kay et al., 1990). Therefore, it is speculated that DPH inhibits chondrogenic differentiation of ATDC5 cells via GR. A previous study by Shibata et al. (1996) detected the inhibitory effects of the teratogenic compound YM9429, which induces cleft palate in rats, in the chondrogenic differentiation of ATDC5 cells. However, our ATDC5 results show an opposite effect, suggesting a lesser possibility of DPH action through GR. In contrast, the pattern of skeletal abnormalities closely resembles that observed in mice exposed to retinoic acid (RA), and in phenotypes of mice lacking the genes encoding homeobox (Hox) proteins (Ross et al., 2000). In MC3T3-E1 cells, retinoids induced their osteogenic differentiation (Park, 1997), and the expression of Hox genes were regulated by TGF-β superfamily members (Kloen et al., 1997). In addition, the expression of Hox genes was induced in the BMP-implanted rat tissue (Iimura et al., 1994). These findings demonstrate that there may be an association between RA, TGF-β, and Hox signaling in osteogenic differentiation. Although, in this study, DPH effects on Hox and RA signaling molecules were not determined, the increased expression of RA receptors in mouse fetuses exposed to DPH (Waes, 1999) has been reported. Thus DPH may possibly alter the expression of Hox genes and RA signaling molecules directly or indirectly via members of the TGF-β superfamily in tissues of adults and embryos. However, we show no change here in any parameters for chondrogenic or osteogenic differentiations in ATDC5 and MC3T3-E1 cells cultured with the other antiepileptics, PBT and VPA, which have teratogenic effects similar to DPH on fetal skeletons. Therefore, further evaluations to clarify the molecular target of antiepileptics on skeletogenesis for adults and embryos are necessary. In conclusion, this study demonstrates for the first time that DPH could have a positive chondrogenic effect on bone formation, and that DPH-promoted chondrogenic differentiation could be in part mediated by Ihh, Ptc, TGF-β1, and BMP-4 in ATDC5 cells.

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