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2,3,7,8-Tetrachlorodibenzo-P-Dioxin Inhibits Differentiation of Normal Diploid Rat Osteoblasts In Vitro

John F. Gierthy, J.B. Silkworth, Melissa Tassinari, Gary S. Stein, and Jane B. Lian

Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201-0509 (J.F.G., J.B.S.) and Department of Cell Biology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655 (M.T., G.S.S., J.B.L.)

Abstract The influence of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a potent halogenated aromatic hydrocarbon, on the development of bone tissue-like organization in primary cultures of normal diploid calvarial-derived rat osteoblasts was examined. Initially, when placed in culture, these cells actively proliferate while expressing genes associated with biosynthesis of the bone extracellular matrix. Then, post-proliferatively, genes are expressed that render the osteoblast competent for extracellular matrix mineralization and maintenance of structural as well as functional properties of the mature bone-cell phenotype. Our results indicate that, in the presence of TCDD, proliferation of osteoblasts was not inhibited but post-confluent formation of multicellular nodules that develop bone tissue-like organization was dramatically suppressed. Consistent with TCDD-mediated abrogation of bone nodule formation, expression of alkaline phosphatase and osteocalcin was not upregulated post-proliferatively. These findings are discussed within the context of TCDD effects on estrogens and vitamin D-responsive developmental gene expression during osteoblast differentiation and, from a broader biological perspective, on steroid hormone control of differentiation.

Key words: bone, osteocalcin, alkaline phosphatase, differentiation, halogenated hydrocarbons

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) has been studied extensively as the most biologically potent member of a class of widespread and persistent halogenated aromatic hydrocarbons that are environmental contaminants. These compounds induce a broad spectrum of toxic effects dependent on the particular compound, animal species, and tissue examined. In animals, effects of TCDD exposure include carcinogenic, immunological, teratogenic, fetotoxic, and biochemical manifestations, as well as a wasting syndrome leading to death [Safe, 1986]. It is apparent that many of the distinct biological effects of TCDD exposure include alteration of differentiation pathways such as those associated with teratogenicity [Couture et al., 1990]. The most consistent manifestation of human TCDD exposure is the development of chloracne, which is caused by an alteration of epithelial differentiation (hyperkeratization) [Poland and Knutson, 1982; Safe, 1986]. In animals, modulated differentiation in response to TCDD exposure is also seen as thymic involution [Blaylock et al., 1992; DeWaal et al., 1992] and hyperplasia of the bladder endothelium [Poland and Knutson, 1982]. The specific risk to humans from TCDD exposure is unclear and the mechanism of TCDD toxicity is unknown.

The activity of TCDD is generally considered to involve high-affinity saturable binding of TCDD to the cytosolic Ah receptor. The receptor-ligand complex, in concert with an Ah receptor nuclear translocation (Arnt) protein, is relocated to the nucleus, followed by sequence-specific interaction with the xenobiotic response elements of the Ah locus resulting in multigene expression [Reyes et al., 1992].

Previous investigations have provided evidence that an incidence of skeletal abnormalities in fetal rats was a result of maternal expo-

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Melissa Tassinari's present address is Pfizer Central Research, Groton, CT 06341.

Address reprints requests to John F. Gierthy, Wadsworth Center for Laboratories and Research, New York State Department of Health, P.O. Box 509, Albany, NY 12201-0509.

sure to the TCDD component of a natural soil leachate from the Love Canal chemical dump site in Niagara Falls, New York [Silkworth et al., 1986]. In these studies the effects of a solvent extract of a TCDD-contaminated soil sample were compared to those of a natural leachate which contained 18 times as much TCDD. The only developmental skeletal effect observed in the offspring of rats given 150 mg/kg of the solvent extract for 10 days (a cumulative dose of 0.25 μg TCDD/kg) was an increase in the percentage of undersized chest bones including the sternebrae, manubria, or xyphoid processes. However, offspring of rats given 10–250 mg/kg of the natural leachate for 10 days (a cumulative dose of 30–750 μg TCDD/kg) exhibited significant increases in the percentage of fetuses per litter with signs of retarded or altered bone development, such as the continued presence of ossification centers, bones with incomplete ossification, or bipartite formations. On the basis of comparisons with the values from the published literature, the authors concluded that the TCDD component alone could account for the delayed fetal bone development. Since many of the effects of TCDD are thought to be mediated through alterations in the normal rates of differentiation, it is possible that TCDD also affects the early stages of differentiation and growth of embryonic or fetal bone.

Cultured osteoblasts [Aronow et al., 1990; Bellows et al., 1986; Bhargava et al., 1988; Escarot-Charrier et al., 1983] have been used to study the bone differentiation pathway and have yielded results which indicate reciprocal relationships between cell growth-related gene expression and gene expression that immediately follows completion of proliferative activity [Bellows et al., 1986]. Control mechanisms are suggested by which genes expressed in proliferating osteoblasts may downregulate gene expression associated with postproliferative development of the fully differentiated bone-cell phenotype [Owen et al., 1990a].

Bone development is a consequence of a cascade of differentiation events which involve sequential osteoblast gene expression [Stein et al., 1990, 1992; Lian et al., 1992; Owen et al., 1990b; Shalhoub et al., 1991]. This sequence begins with expression of genes regulated by cell cycle (e.g., histone) and cell growth (e.g., c-myc, c-fos, and c-jun) in the proliferating osteoblast. The expression of extracellular matrix-forming genes (collagen, fibronectin, and TGF-β) essential for the development of the bone phenotype is also evident in the proliferative phase. Following cessation of proliferation, expression of a series of genes that include alkaline phosphatase are upregulated, rendering the developing bone extracellular matrix competent for mineralization. Extracellular matrix mineralization is associated with increased expression of osteocalcin, a bone-specific protein, and osteopontin.

In this study, primary cultures of normal diploid calvarial-derived rat osteoblasts that develop a bone-tissue-like organization in vitro are used to determine whether TCDD treatment could result in modification of specific markers of the proliferation/differentiation cascade that supports progressive development of the mature osteocytic phenotype. The results indicate that TCDD induces major alterations in biochemical parameters of osteoblast differentiation and development of bone tissue organization. Our findings suggest that altered bone development may be another manifestation of TCDD-altered differentiation and may, at least in part, explain observations made in the earlier in vivo studies with samples from the Love Canal.

MATERIALS AND METHODS

TCDD was obtained from Cambridge Isotope Laboratories, Woburn, MA. Its purity was determined by mass spectroscopy to be >99%. Dimethyl sulfoxide (DMSO) was from the Aldrich Chemical Company, Milwaukee, WI.

Osteoblast Cultures

Calvaria from fetal rats of 21 days gestation were isolated and subjected to sequential digestions of 20, 40, and 90 min at 37°C in 2 mg/mL collagenase A (Boehringer-Mannheim, Indianapolis, IN) and 0.25% trypsin (Gibco, Grand Island, NY) [Aronow et al., 1990, Owen et al., 1990b]. The cells of the first two digests were discarded and those released from the third digestion were plated in minimal essential medium (MEM: Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) in six-well culture dishes (Corning, Corning, NY) at a density of 5×10^5 or 3.6×10^5 cells/dish, respectively. At confluence (day 7), the time course of mineralization was accelerated by the addition of BGJb medium (Gibco, Grand Island, NY) supplemented with 10% FCS, 50 μg/mL ascorbic acid, and 10 mM β-glycerol phosphate. Cell number was determined for points after plating by extensively incubating three independent

wells of cells with 0.25% trypsin and counting the cells in a hemocytometer.

Histochemistry

Cells growing in six-well culture dishes were rinsed twice with ice-cold phosphate-buffered saline (PBS) and fixed for 10 min in absolute methanol (−20°C). Alkaline phosphatase activity was visualized by incubating the cells for 30 min at room temperature with shaking in 10 mM Tris HCL, pH 8.4, containing 20 mg/mL disodium naphthol AS-MX phosphate and 40 mg/mL fast red TR salt (Sigma Chemical Co., St. Louis, MO). Mineral deposition was assessed by von Kossa staining of the cultures (30 min in 3% $AgNO_3$) [Clark, 1981].

Biochemical Determinations

Total DNA was assayed by a fluorometric procedure using diaminobenzoic acid reagent as described by Vysatek [1982]. Alkaline phosphatase activity was determined spectrophotometrically as described by measuring the amount of p-nitrophenol formed at 37°C after 30 min [Lowry et al., 1954]. The medium from all these cell layers was simultaneously analyzed for osteocalcin by radioimmunoassay as previously described [Gundberg et al., 1984]. For all biochemical determinations, the value represents the mean of three independent samples.

Quantitation of Nodules

Nodules in fixed and stained 21-day cultures were counted using a stereomicroscope at 20× magnification. Mean values were determined for 3–35 mm diameter culture plates treated with 10^{-8} M TCDD in 0.1% DMSO, 0.1% DMSO alone, or untreated control cultures.

RESULTS

The effect of TCDD exposure on principal parameters of proliferation and bone-cell differentiation were examined in primary cultures of calvarial-derived rat osteoblasts. Treatment of calvarial osteoblast cultures with 10^{-8} M TCDD was initiated on day 3 and was maintained throughout the 21-day culture period. Osteoblast phenotypic properties were assayed on days 6, 8, 10 (proliferation period), 16 (extracellular matrix maturation characterized by alkaline phosphatase activity), and 21 (extracellular matrix mineralization reflected by nodule formation and osteocalcin synthesis [Owen et al., 1990b; Aronow et al., 1990 and reviewed in Stein et al., 1990; 1992; Lian et al., 1992; Lian and Stein, 1992; Stein and Lian, 1993].

The influence of TCDD on nodule formation was established by microscopic examination and quantitated by visual enumeration as described. The TCDD-treated cultures developed significantly fewer and smaller nodules (Fig. 1). A greater than 90% reduction in the number of nodules was observed on day 21 (Fig. 2). Direct observations and measurement using an ocular micrometer indicated that the reduced nodules of the TCDD-treated and untreated cultures exhibit the same size range (0.25–1 mm^2). However, the cell density of these nodules was reduced compared with the control nodules.

An increase in alkaline phosphatase activity normally occurs after day 15 of culture as a primary postproliferative component of the osteoblast developmental sequence (Fig. 3). TCDD

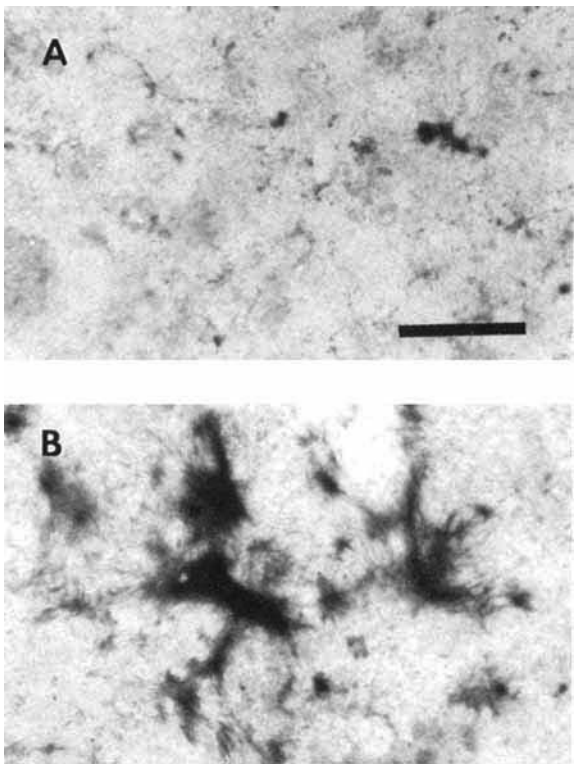

Fig. 1. Effect of TCDD on primary rat osteoblast cultures. Rat osteoblast cultures were maintained for 21 days in (**A**) 10^{-8} M TCDD in 0.1% DMSO or (**B**) 0.1% DMSO, fixed with absolute methanol, and stained with Giemsa. Note the heavily stained multilayered nodules on the lightly stained monolayer background, and the suppression of nodule formation in the TCDD-treated cultures of these representative fields. Untreated controls were similar to the 0.1% DMSO treated cultures. Bar, 1 mm.

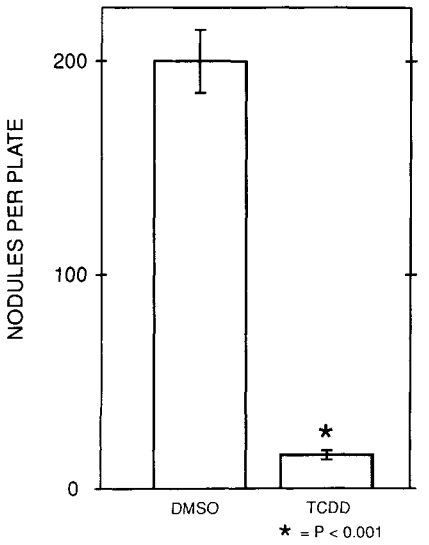

Fig. 2. Effect of TCDD on nodule formation in primary rat osteoblast cultures. Nodules per plate in 21-day rat osteoblast cultures treated with 10^{-8} M TCDD in 0.1% DMSO or 0.1% DMSO as indicated. Discernable nodules from coded plates were counted at 20× magnification. Each point represents the mean of three 35 mm plates ± S.D.; $*P < 0.001$ compared with DMSO treatment by the Student's t test. Untreated controls were not significantly different from the 0.1% DMSO-treated cultures.

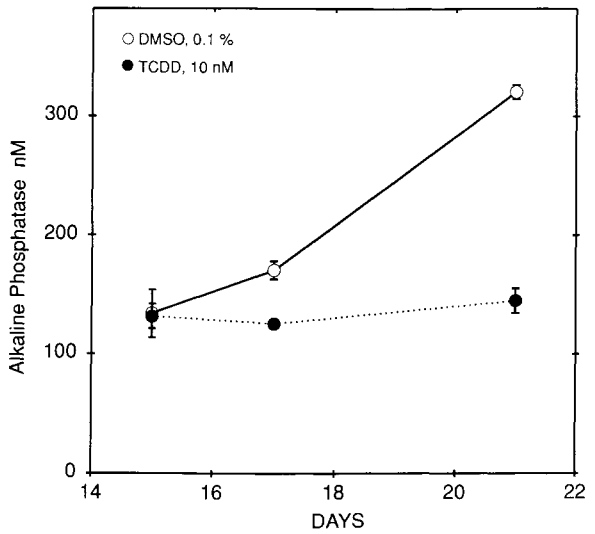

Fig. 3. Effect of TCDD on alkaline phosphatase activity in primary rat osteoblast cultures maintained for 21 days in medium containing 10^{-8} M TCDD in 0.1% DMSO (●) or in 0.1% DMSO alone (○). Each point represents the mean of three cultures ± S.D. Untreated controls were not significantly different from 0.1% DMSO-treated cultures.

treatment completely suppressed this increase. In day-21 cultures, alkaline phosphatase activity in the presence of TCDD was not significantly different from the day-15 values, while control levels increased greater than 250%.

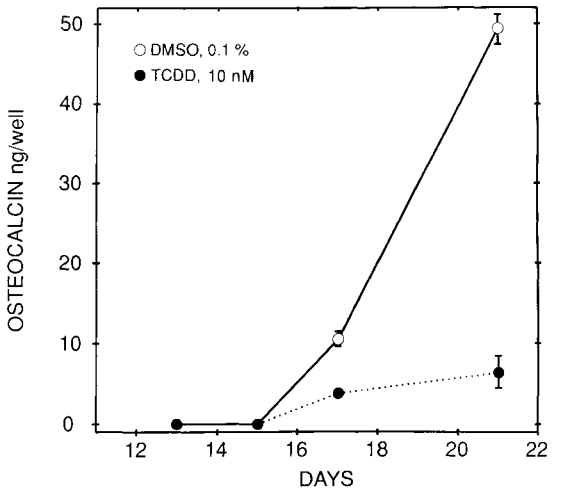

Fig. 4. Effect of TCDD on osteocalcin in primary rat osteoblast cultures maintained for 21 days in medium containing 10^{-8} M TCDD in 0.1% DMSO (●) or in 0.1% DMSO alone (○). Each point represents the mean value of three cultures ± S.D. Untreated controls were not significantly different from 0.1% DMSO-treated cultures.

Examination of TCDD-mediated alteration of osteocalcin levels was examined in 21-day osteoblast cultures. At this time, osteocalcin is expressed at maximal levels in postproliferative rat osteoblasts undergoing extracellular matrix mineralization [Owen et al., 1990b; Aronow et al., 1990]. This relationship between osteocalcin expression and bone extracellular matrix mineralization is supported by in situ hybridization studies, which indicate that osteocalcin mRNA is restricted to cells within maturing nodules [Pockwinse et al., 1992]. It is clear from the data presented in Figure 4 that TCDD causes dramatic suppression of osteocalcin biosynthesis to 13% of the level observed in control cultures on day 21.

Therefore, TCDD-mediated effects on osteoblast differentiation prior to the mineralization stage are indicated by the decreased formation of multilayered cellular nodules reflecting inhibited development of bone-tissue-like organization. This was accompanied by dramatic suppression of both alkaline phosphatase (Fig. 3) and osteocalcin levels (Fig. 4).

Cell proliferation of the osteoblast cultures, as measured by total DNA content per culture well throughout the 21-day culture period, was not markedly changed by TCDD treatment compared with the DMSO-treated controls (Fig. 5). The kinetics of cell growth in both cases was similar to that previously observed [Owen et al., 1990b; Aronow et al., 1990] and is characterized by an increase during the first week of culture

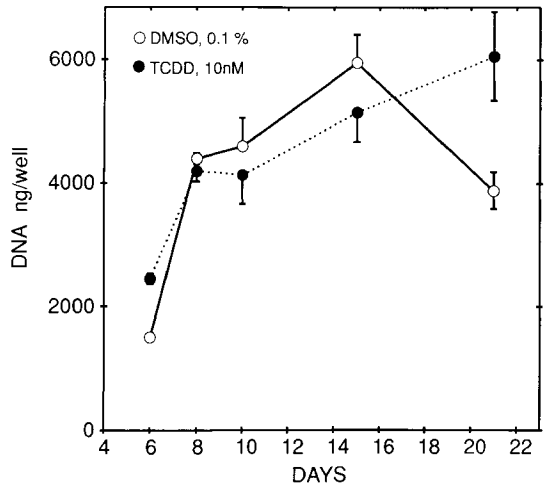

Fig. 5. Effect of TCDD on total DNA from primary rat osteoblast cultures maintained for 21 days in medium containing 10^{-8} M TCDD in 0.1% DMSO (●) or 0.1% DMSO alone (○). Each point represents the mean of three cultures ± S.D. Untreated controls were not significantly different from the 0.1% DMSO-treated cultures.

followed by relatively constant levels of total DNA per culture during the following 2 weeks. The decreased DNA levels in control cultures at day-21 reflect cell loss often observed in mature nodules during advanced stages of mineralization.

DISCUSSION

Results of animal studies and human environmental exposure to TCDD have shown that many of the toxic effects involve altered differentiation. Previous studies indicate that maternal exposure to TCDD-containing extracts of soil and leachate from Love Canal alters rat fetal bone development [Silkworth et al., 1986]. The results of this study indicate that TCDD alters various manifestations of rat osteoblast differentiation with possible consequence on bone development and maintenance of structural and functional integrity at the cell and tissue levels. Here it is demonstrated that, while TCDD does not cause significant changes in osteoblast proliferation, which is an early event in bone development, TCDD exposure does suppress the postproliferation parameters reflected by alkaline phosphatase and osteocalcin levels, and nodule formation, which are characteristics of the later stages of osteoblast differentiation essential for both bone development and maintenance of osteocytic activity. The lack of significant effects of TCDD on cell number suggests these effects were not due to overt cytotoxicity.

The mechanism of this TCDD-induced alteration in osteoblast differentiation is unknown. However, there are similarities between the TCDD-induced suppression of osteoblast differentiation and TCDD-mediated alteration of 17β-estradiol responses that have been reported in non-osseous cells and tissues. Extensive studies show that TCDD is a potent antiestrogen in animals and in in vitro systems using human estrogen-dependent MCF-7 breast cancer cells. TCDD inhibits many of the effects elicited by 17β-estradiol (E_2) in the MCF-7 cells, which include increased preconfluent cell proliferation [Safe et al., 1991] and postconfluent cell proliferation, which results in formation of three-dimensional multicellular foci [Gierthy et al., 1991; Spink et al., 1990], tissue plasminogen activator synthesis and secretion [Gierthy et al., 1987], and cathepsin D synthesis [Biegel and Safe, 1990]. TCDD treatment suppresses E_2-dependent growth of MCF-7 tumors in immunosuppressed mice [Gierthy et al., 1993]. This human E_2-responsive tumor has been suggested as a surrogate human E_2-responsive target tissue for in vivo examination of the antiestrogenic effects of TCDD and related compounds [Gierthy et al., 1993].

While the mechanism of TCDD antiestrogenicity in MCF-7 cells is unknown, various hypotheses have been considered. TCDD-mediated transcriptional down-regulation of the estrogen receptor has been suggested on the basis of a reduction of occupied nuclear estrogen receptor using ligand binding and immunological evaluation [Harris et al., 1990]. However, a recent report suggests that ER transcription in MCF-7 cells is not affected by TCDD [Porter et al., 1993] and substantial evidence has been presented which describes and characterizes extensive TCDD-induced increases in E_2 metabolism in MCF-7 cultures [Gierthy et al., 1988; Spink et al., 1990, 1992a,b]. This leads to another hypothesis that TCDD's antiestrogenic activity is based on an intracellular depletion of E_2 in the target tissue resulting in less E_2 reaching and occupying the ER, which is almost exclusively located in the nucleus. This hypothesis is consistent with ligand-binding studies using MCF-7 cells, which demonstrate that TCDD has no effect on total cellular ER levels under conditions of estrogen deprivation [Gierthy et al., 1987]. Whatever roles E_2 metabolism, ER alteration, or other transcription factors have in this mechanism, it has been noted that many of the toxic effects of TCDD exposure are similar to those of E_2 and

perhaps other steroid modulation [Umbreit and Gallo, 1988].

The modulation of bone turnover by estrogens is well established [Turner et al., 1988; Wronsky et al., 1988]. The presence of estrogen receptor has been demonstrated in human osteoblasts and osteoblastic cells [Eriksen et al., 1988; Komm et al., 1988]. Direct estrogen modulation of bone-cell development has been observed in vitro and in vivo by transcriptional activation [Gray et al., 1987; Arnett and Colston, 1988; Turner et al., 1990]. The estrogen receptor-binding antiestrogen tamoxifen has been shown to inhibit the effect of E_2 in vivo and in chondroblasts and osteoblasts in vivo [Somjen et al., 1991; Tobias and Chambers, 1991]. These findings suggest that bone is an estrogen target tissue and that E_2-dependent bone development and homeostasis may be altered by the antiestrogenicity of TCDD exposure. This could occur by a combination of altered ER through an E_2-modulated feedback loop, E_2 depletion through metabolism, or another undertermined mechanism.

Analogous mechanisms may be operative in other skeletal tissue responses to TCDD. In the case of bone, the steroidal hormone Vitamin D plays a major role in osteoblast differentiation. Vitamin D-mediated influences on expression of genes are developmental stage-specific and selectively influence cellular and molecular parameters of the bone-cell phenotype [Owen et al., 1991]. The vitamin D modulation of proliferation-dependent and postproliferative events has led to the proposal of a novel mechanism for the control and regulation of the concerted gene expression necessary for development of this tissue [Owen et al., 1990a, 1991]. It is possible that, as with E_2 in MCF-7 cultures, the depression of osteoblast differentiation by TCDD described in this report may at least in part be due to altered vitamin D efficacy. Like E_2, vitamin D also has a nuclear receptor-dependent mechanism. However, unlike E_2, vitamin D requires metabolic alterations by organ-specific cytochrome P450s to the 1,25-dihydroxy vitamin D_3 [$1,25(OH)_2D_3$] form to be active. This is then further metabolized to an inactive form. As a result, TCDD effects on vitamin D-mediated parameters of bone-cell growth and differentiation may operate at multiple levels.

From the perspective of perturbations in developmental mechanisms operative during progressive expression of the bone-cell phenotype, our results indicate the absence of a TCDD effect on osteoblast proliferation. However, a pronounced inhibition of genes expressed postproliferatively is evident and bone nodule formation is dramatically inhibited. Together with the pronounced reduction in size and altered morphology of those limited nodules that develop in the presence of TCDD, an influence of TCDD on regulatory mechanisms that mediate establishment of bone tissue-like organization must be considered. A key transition point during progressive development of the bone-cell phenotype is when the proliferative period is completed and genes associated with organization, maturation, and mineralization of the bone extracellular matrix initiate expression [Owen et al., 1990b]. Understanding effects of TCDD on those genes which are responsible for this transition can provide valuable insight into molecular parameters of skeletal development impinged upon by other environmentally significant halogenated aromatic hydrocarbons.

The current study demonstrates that TCDD alters an effect presumed to be regulated by the endogenous E_2 and/or vitamin D levels in the medium. These results suggest future studies on the effect of TCDD on E_2 and vitamin D-induced bone differentiation, effects on E_2 and vitamin D receptor regulation and on E_2 and vitamin D metabolic activation and deactivation pathways that will elucidate the mechanistic role of TCDD in suppression of osteoblast differentiation. It is possible that chronic exposure to low environmental levels of TCDD and related compounds could result in altered fetal bone development or increased magnitude of osteoporosis in the adult.

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