

Inhibition of dioxin effects on bone formation *in vitro* by a newly described aryl hydrocarbon receptor antagonist, resveratrol

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Abstract

Aryl hydrocarbon receptor (AhR) ligands are environmental contaminants found in cigarette smoke and other sources of air pollution. The prototypical compound is TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), also known as dioxin. There is an increasing body of knowledge linking cigarette smoking to osteoporosis and periodontal disease, but the direct effects of smoke-associated aryl hydrocarbons on bone are not well understood. Through the use of resveratrol (3,5,4'-trihydroxystilbene), a plant antifungal compound that we have recently demonstrated to be a pure AhR antagonist, we have investigated the effects of TCDD on osteogenesis. It was postulated that TCDD would inhibit osteogenesis in bone-forming cultures and that this inhibition would be antagonized by resveratrol. We employed the chicken periosteal osteogenesis (CPO) model, which has been shown to form bone *in vitro* in a pattern morphologically and biochemically similar to that seen *in vivo*, as well as a rat stromal cell bone nodule formation model. In the CPO model, alkaline phosphatase (AP) activity was reduced by up to 50% ($P < 0.01$ vs control) in the presence of 10^{-9} M TCDD and these effects were reversed by 10^{-6} M resveratrol ($P < 0.05$ vs TCDD alone). TCDD-mediated inhibition of osteogenesis was restricted primarily to the

osteoblastic differentiation phase (days 0–2) as later addition did not appear to have any effects. Message levels for important bone-associated proteins (in the CPO model) such as collagen type I, osteopontin, bone sialoprotein and AP were inhibited by TCDD, an effect that was antagonized by resveratrol. Similar findings were obtained using the rat stromal bone cell line. TCDD (at concentrations as low as 10^{-10} M) caused an approximately 33% reduction in AP activity, which was abrogated by 3.5×10^{-7} M resveratrol. TCDD also induced a marked reduction in mineralization (~75%) which was completely antagonized by resveratrol. These data suggest that AhR ligands inhibit osteogenesis probably through inhibition of osteodifferentiation and that this effect can be antagonized by resveratrol. Since high levels of AhR ligands are found in cigarette smoke, and further since smoking is an important risk factor in both osteoporosis and periodontal disease, it may be postulated that AhR ligands are the component of cigarette smoke linking smoking to osteoporosis and periodontal disease. If so, resveratrol could prove to be a promising preventive or therapeutic agent for smoking-related bone loss.

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Introduction

Cigarette smoking is an important risk factor for osteopenia in men (Hollenbach *et al.* 1993, Nguyen *et al.* 1994, Egger *et al.* 1996, Kiel *et al.* 1996, Grisso *et al.* 1997) and women (Hopper & Seeman 1994, Egger *et al.* 1996, Franceschi *et al.* 1996). Further, a dose–response relation-

ship between the number of cigarettes smoked per day and decreased bone mineral density has been identified (Hollenbach *et al.* 1993, Franceschi *et al.* 1996), as has an association between smoking and increased fracture risk (Williams *et al.* 1982, Aloia *et al.* 1985, La Vecchia *et al.* 1991, Hollenbach *et al.* 1993, Johansson & Mellstrom 1996, Grisso *et al.* 1997).

Similarly, smoking is accepted as one of the stronger risk factors for periodontal disease (Genco & Kinane 1999), a condition that is characterized by the loss of alveolar bone. Cross-sectional and longitudinal studies have detailed the consistent, positive association between smoking and periodontal attachment loss (Bergstrom 1989, Preber & Bergstrom 1990, Haber & Kent 1992, Horning *et al.* 1992, Haber *et al.* 1993, Ah *et al.* 1994). A meta-analysis of the literature conducted by Papananou (1996) for the 1996 World Workshop in Periodontics revealed that smokers have an almost 3-fold increase in their risk for severe periodontal disease vs non-smokers. Evidence also indicates that failures of endosseous dental implants (relating to osseointegration) are increased in smokers vs non-smokers (Bain & Moy 1993, De Bruyn & Collaert 1994).

Potential role for aryl hydrocarbons on bone

Among products of cigarette smoke which warrant particular attention with respect to bone metabolism, are the aryl hydrocarbon receptor (AhR) ligands. AhR ligands such as dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)), benzo-a-pyrene (BaP) and polychlorinated biphenyls are environmental contaminants that bind to the AhR, which is present in the cytosol of mammalian cells of almost all organs and tissues. The presence of these detrimental compounds (i.e. BaP) in cigarette smoke, combined with the confirmed association between smoking and bone loss, establishes AhR ligands as prime candidates for investigation.

While few studies have been undertaken to investigate the possible direct effects of AhR ligands on bone, it has been shown that during embryonic development of bone tissue in mice both AhR and AhR nuclear translocator are expressed (Abbott & Probst 1995) and that TCDD suppresses differentiation of rat osteoblasts *in vitro* (Gierthy *et al.* 1994). This latter study showed that upregulation of alkaline phosphatase (AP) and osteocalcin message was inhibited in calvarial-derived osteoblasts exposed to TCDD. The authors suggested, appropriately, that TCDD-mediated inhibition of the formation of bone tissue-like multicellular nodules *in vitro* intimates a direct association between TCDD and reduced bone mass *in vivo*. AhR ligands are known to increase the expression of interleukin-1 β in rat liver keratinocytes (Sutter *et al.* 1991) and the endometrial cell line RL95-2 (Charles & Shiverick 1997), which could also have a negative impact on bone formation or metabolism.

In light of the above, TCDD or similar AhR ligands could prove to be among the most important compounds linking cigarette smoking to bone loss. In relation to this, we, and others (Ciolino *et al.* 1998, Casper *et al.* 1999), have shown that resveratrol (3,5,4'-trihydroxystilbene) is an AhR antagonist and may actually inhibit TCDD effects on cells. This antifungal compound, which is found in high levels in red wine, has been demonstrated to inhibit

TCDD-mediated upregulation of gene transcription dependent on a dioxin response element (Casper *et al.* 1999).

In view of the foregoing, the goals of this investigation were two-fold. First, we wanted to investigate in more detail how the prototypical AhR ligand, TCDD, might affect bone cell differentiation and bone formation *in vitro* using previously characterized osteogenic model systems (Maniatopoulos *et al.* 1988, Tenenbaum *et al.* 1995, Sukhu *et al.* 1997, S Kotev-Emeth, S Pitaru, S Pri-Chen & N Savion, unpublished observations). Secondly, presuming that TCDD did inhibit osteodifferentiation and/or osteogenesis, we also wanted to determine whether these putative effects could be inhibited by resveratrol, thereby 'protecting' bone from the potentially harmful actions of TCDD. Our data suggest that TCDD does inhibit osteodifferentiation, that resveratrol inhibits these effects and that these regulatory events are mediated through the AhR in bone cells.

Materials and Methods

Rationale for use of proposed in vitro models

In order to address the objectives outlined above, we chose to utilize two different models of osteogenesis, the chick periosteal osteogenesis (CPO) model and the stromal bone marrow cell (SBMC) (courtesy of Dr S Pitaru, Tel Aviv University, Israel) model (Pitaru *et al.* 1993, S Kotev-Emeth, S Pitaru, S Pri-Chen & N Savion, unpublished observations). The CPO model has been described in detail previously (Nijweide 1975, Tenenbaum & Heersche 1982, 1985, 1986, Tenenbaum *et al.* 1986) and has been shown to produce morphotypical bone reliably within a 6-day culture period. Of course, cell culture systems also have unique advantages that may be exploited as outlined below (Tenenbaum *et al.* 1986). The SBMC system has also been described in detail (Pitaru *et al.* 1993, S Kotev-Emeth, S Pitaru, S Pri-Chen & N Savion, unpublished observations) and is similar to the rat bone marrow cell (RBMC) system (Grigoriadis *et al.* 1988, Maniatopoulos *et al.* 1988, Kasugai *et al.* 1991, McCulloch *et al.* 1991) in which distinct mineralized bone-like nodules are formed *in vitro* under appropriate culture conditions. These stable long-term cultures can be analyzed biochemically, and are also adaptable to receptor binding studies, unlike the CPO model. The rationale for using the two systems was that with both, detailed assessment of a much wider variety of outcome measures could be performed than if only one system were used. Moreover, although many findings produced in the CPO model have been replicated in mammalian systems *in vitro* and *in vivo* (Tenenbaum 1981, McCulloch & Tenenbaum 1986, Kamalia *et al.* 1992, Letic *et al.* 1997, Sukhu *et al.* 1997), it was still thought appropriate to utilize a mammalian model system like the SBMC model. Finally,

it was thought that if the data produced in both systems were parallel to one another, the validity of those findings would be strengthened and this would reduce the likelihood that the data were related to species or a culture system artifact.

Culture systems

CPO culture Folded periosteal explants from 17-day-old embryonic chicks were cultured as described previously in detail (Nijweide 1975, Tenenbaum & Heersche 1982). Media changes occurred on days 2 and 4, and the medium comprised BGJ_b medium (Fitton-Jackson modification) with L-glutamine (Gibco, Grand Island, NY, USA) and supplemented with 2% antibiotic (10 000 units/ml penicillin G sodium, 10 000 µg/ml streptomycin sulfate in 0.85% saline) (Gibco), 10% fetal calf serum (Gibco), 10^{-7} M dexamethasone (Sigma, St Louis, MO, USA) (Tenenbaum & Heersche 1985, McCulloch & Tenenbaum 1986), 10 mM β-glycerophosphate (Tenenbaum *et al.* 1989), and 300 µg/ml L-ascorbate (Gibco) (Tenenbaum & Heersche 1986). The compounds under investigation were added to the media in the appropriate concentrations (see below).

Rat SBMC line culture Cells for the SBMC model were obtained from Dr Sandu Pitaru (Tel Aviv University, Israel) and maintained in T₂₅ tissue culture flasks (Sarstedt, Newton, NC, USA) in a medium composed of MEM+RNA+DNA+antibiotic supplemented with 10% fetal calf serum, 10 mM β-glycerophosphate, 10^{-8} M dexamethasone, 50 µg/ml vitamin C and 3 ng/ml fibroblast growth factor. The media were changed every 48 h and the cells subcultured when almost confluent (approximately every 72 h). Cells were grown for a period of 1–2 weeks to allow for formation of mineralized bone nodules.

Biochemical analyses

CPO and SBMC cultures were assayed for AP activity using paranitrophenol-phosphate (pN-p) as the substrate (Tenenbaum *et al.* 1995, Demetriou *et al.* 1996). The intensity of the color change was evaluated using a Titertek Multiskan MCC/340 Spectrophotometer (Flow Laboratories, Mississauga, ON, Canada) and compared with a standard curve obtained from a serial dilution of 10 mM pN with bicarbonate buffer (pH 7.4) in flat bottomed Titertek 96-well plates as described previously (Demetriou *et al.* 1996). Calcium incorporation was assayed in the pellets produced from centrifugation of the homogenized CPO cultures following hydrolysis in 500 µl 0.5 M HCl and then measured using atomic absorption spectrophotometry (Sukhu *et al.* 1997). In some cases, AP activity was also measured using direct staining *in situ* of SBMC cultures in 96-well plates as described previously (Tenenbaum *et al.* 1995, Demetriou *et al.* 1996). Miner-

alization in the SBMC cultures was determined using Alizarin Red staining also *in situ*, which was assessed with the Titertek spectrophotometer set at 525 nm. In the case of direct *in situ* staining, only relative units were used based on 'zeroing' the Titertek spectrophotometer with empty wells.

Radiolabeled collagen was measured in CPO cultures incubated with [¹⁴C]glycine (Amersham Pharmacia Biotech Inc., Quebec City, Canada, 59 mCi/mmol) at a concentration of 10 µCi/ml media in each group over days 4–6 as described previously (Tenenbaum *et al.* 1995). Fluorography was used to analyze the amount of newly synthesized ¹⁴C-labeled collagen in each sample. The intensity of the resultant bands on the film was quantified using a laser densitometer to give a measure of the amount of newly synthesized collagen in each sample.

Competitive binding assay for TCDD and resveratrol

Four replicates were used in conducting the live whole-cell competition binding assay to demonstrate specific receptor-mediated uptake of radiolabeled TCDD. This technique has been described previously (Tenenbaum *et al.* 1995, Sukhu *et al.* 1997) and demonstrates results that are comparable to more traditional binding assays relying on cytosol preparations. However, it should be pointed out that the binding curves produced using vital cell assays may be different from those produced using more standard cytosol binding assays. Thus, it is not possible to calculate K_d in these studies. However, this vital cell uptake/binding can be accomplished with far fewer cells than would otherwise be required to generate appropriate amounts of cytosol for more conventional assays. Cells were plated at a density of 10 000 per well in Titertek 96-well plates, and left overnight to attach in medium. The medium was removed by aspiration and the various concentrations of 'cold' drug (TCDD, resveratrol) were prepared and added to the wells in a volume of 90 µl. Cells were preincubated with cold drug 1 h prior to addition of radiolabeled TCDD (specific activity 33.4 Ci/mmol; Chemsyn Lab., Lenexa, KS, USA), pilot studies having shown that preincubation was required to detect competition. One set of wells was incubated without cold drug to determine total binding. [³H]TCDD (10 000 c.p.m./well in a volume of 10 µl) was added and incubated for 4 h at room temperature. The media were aspirated and the cells washed four times with 100 µl PBS. One hundred microliters of 0.05 M sulfuric acid were added and the cells were incubated at 37 °C overnight to break the cells open. Eighty microliters of the lysate were added to 5 ml of scintillation fluid and ³H counted in a beta counter.

Semiquantitative RT-PCR

The isolation of total RNA from periosteal cultures was accomplished using the QIAGEN RNeasy kit and

QIASHredder (Hilden, Germany) according to the manufacturer's instructions. Total RNA (2 µg) was reverse transcribed using SuperScript II RNase H⁻ Reverse Transcriptase (Gibco BRL). PCR reactions were performed in 20 µl volumes at a cycle number ensuring a linear amplification profile (bone sialoprotein (BSP), 5 min at 94 °C, 30 cycles of 20 s at 94 °C, 20 s at 55 °C, 20 s at 72 °C; collagen 5 min at 94 °C, 30 cycles of 20 s at 94 °C, 20 s at 58 °C, 20 s at 72 °C; AP 5 min at 94 °C, 30 cycles of 20 s at 94 °C, 20 s at 59 °C, 20 s at 72 °C; osteopontin (OPN) 5 min at 85 °C, 5 min at 94 °C, 25 cycles of 20 s at 94 °C, 20 s at 60 °C, 20 s at 72 °C, and 7 min at 72 °C). The oligonucleotides for BSP (sense: 5'-GAGCGGGCACC GGTTACTA-3'; antisense: 5'-CTCTAGACACTGACATCCTGCTC-3'), AP (sense: 5'-ACCGTGCAAC ACCACCA-3'; antisense: 5'-TCCCCGCAGGCTTAGTGT-3'), collagen (sense: 5'-ACCCGACCCTAAGACAAA-3'; antisense: 5'-TCGGCGTTGGGGCAGT-3') and OPN (sense 5'-AGGCCGGGGTGACAGTGT-3'; antisense 5'-CCCCGCAGGCAGCACTC-3') were synthesized by Life Technologies (Gibco BRL, Rockville, MD, USA).

PCR products were electrophoresed on 1.5% (w/v) agarose gels with 0.1 µg/ml ethidium bromide. The gels were visualized on a UV transilluminator and photographed using 667 Polaroid film. The inverse images of the photographs were analyzed by densitometry. All values were normalized for the internal control, β-actin.

Temporal experimental conditions in the CPO model

As alluded to above, the CPO model traverses a number of phases of osteogenesis. Therefore, it was possible to design temporal or 'window' experiments in order to determine the phases of osteogenesis that were sensitive to TCDD and/or resveratrol. CPO cultures were treated with TCDD 10⁻⁹ M, resveratrol 10⁻⁶ M, TCDD 10⁻⁹ M or vehicle. All cultures were maintained for the entire 6-day period. The compounds were administered over four different temporal phases, i.e. days 0–6; days 0–2; days 2–4 or days 4–6. (All cultures were stopped at 6 days.) Results were compared with vehicle alone over days 0–6.

The cultures received vehicle control when not receiving resveratrol or TCDD and the media were changed every 48 h. When given together, TCDD and resveratrol were added simultaneously to assess their effects in direct combination.

Statistical methods

All CPO experiments were carried out with nine cultures per group except for the mRNA extraction in which 12 cultures per group were utilized. The rat SBMC line experiments were carried out with three replicates per group in all cases. In all experiments the results from each treatment group were compared only against the group

which received vehicle-containing media (denoted as 'vehicle' in the figures). In the RT-PCR experiments, multiple comparisons between groups were performed using one-way ANOVA, and Student–Newman–Keul's test for post-hoc analysis.

Results

Bone formation in the CPO model

As shown in Fig. 1, there were obvious changes in bone formation in CPO cultures treated with TCDD. Although morphometric measurements were not made, the TCDD-treated specimens (Fig. 1B) had to be sectioned completely to visualize any bone at all. In those cultures, however, the small amount of bone that did form appeared to be more or less normal in that there was recognizable bone matrix containing osteocytic cells that was also surrounded by osteoblast-like cells. There appeared to be a complete reversal of the TCDD effect in cultures treated with both TCDD and resveratrol (Fig. 1D). Large seams of bone were visualized readily and the bone, which was surrounded by a multilayer of osteoblasts, contained numerous osteocytes. The cultures treated with both TCDD and resveratrol were indistinguishable from control cultures (Fig. 1A) or cultures treated with resveratrol alone (Fig. 1C), which also demonstrated large deposits of bone.

Biochemical markers of osteogenesis in the CPO model

Cultures treated with 10⁻⁹ M TCDD over a 6-day period showed 80% reduction in AP activity vs cultures with vehicle alone. Resveratrol, at doses of 10⁻⁵ M, 10⁻⁶ M and 10⁻⁷ M partially prevented TCDD's inhibitory effects by increasing AP activity 2- to 4-fold in comparison to TCDD alone (Fig. 2a). Administration of 10⁻⁹ M TCDD alone during days 2–4 or 4–6 did not significantly alter AP activity, but administration during days 0–2 reduced AP activity by 53%, similar to that of 6-day treatment (*P*<0.05 vs vehicle) (Fig. 2b). No significant change in AP activity was noted when 10⁻⁶ M resveratrol alone was added over days 0–6, days 0–2, days 2–4, or days 4–6 (Fig. 2c). As shown in Fig. 3, message levels for AP were also reduced about 50% (*P*<0.05) in cultures treated with TCDD in comparison with control. In cultures treated with both TCDD and resveratrol, the mRNA levels were similar to those in control. In parallel with AP, mRNA levels for OPN were reduced by about 40% (*P*<0.05) in the presence of TCDD and restored in cultures treated with both TCDD and resveratrol. A similar profile was demonstrated for BSP in that there was about a 4-fold reduction in mRNA for BSP in the presence of TCDD (*P*<0.05) as shown in Fig. 3. This inhibition was reversed completely with resveratrol.

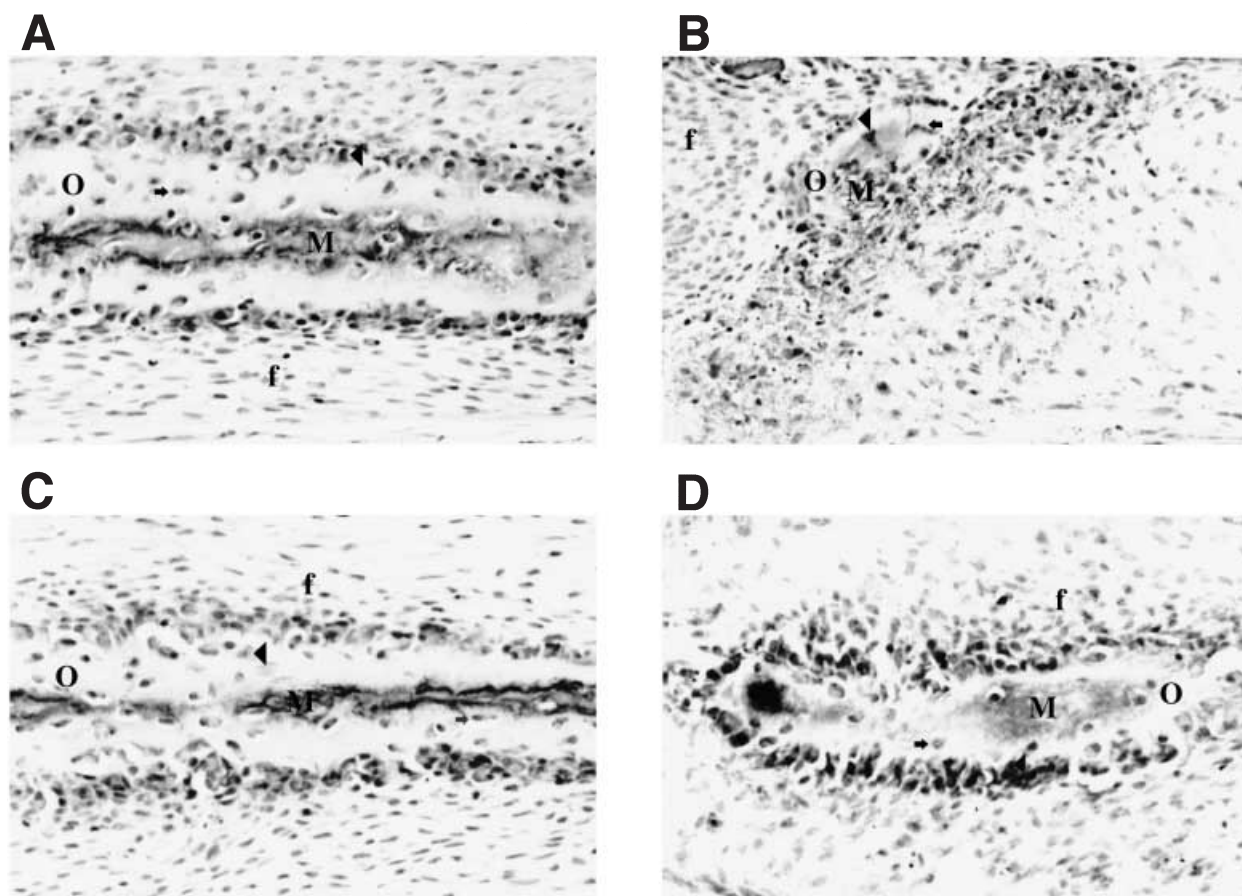


Figure 1 Histological evaluation of 5 µm thick paraffin-embedded sections obtained from CPO cultures stained with hematoxylin and eosin. (A) Control cultures treated with vehicle only contained easily recognizable seams of mineralized bone (M) surrounded by unmineralized osteoid (O). The layer of bone is surrounded by a multilayer of osteoblasts (arrowhead) and contains numerous osteocytes (small arrow). The bone seam and osteoblasts are surrounded by a collagen-rich layer of fibrous periosteum (f). (B) In TCDD-treated cultures (10^{-9} M), there were only small islands of bone. In fact, the TCDD-treated cultures had to be sectioned completely through to visualize any bone (if it was present at all). Otherwise, the appearance of the bone in both other groups (C, D) was indistinguishable from vehicle-treated control (A) and could be detected early on and throughout the sectioning process. Original magnification in all sections: 480 × .

Interestingly, resveratrol alone appeared to partially down-regulate message for collagen and BSP but not other transcripts.

Matrix synthesis in the CPO model

Newly synthesized collagen type I levels (reported as absorbance units (AU) × mm) were decreased by about 30% in the treatment groups receiving 10^{-9} M TCDD alone ($P > 0.05$), while 10^{-6} M resveratrol alone had no effect compared with vehicle (Fig. 4). Co-treatment with TCDD and resveratrol increased the newly synthesized collagen type I level (Fig. 4a), but this was not significantly different from the collagen levels shown for cultures treated with TCDD alone. However, as shown in Fig. 3, mRNA levels for type I collagen were reduced signifi-

cantly (50%, $P < 0.05$) in the presence of TCDD in comparison to control, while cultures treated with both TCDD and resveratrol had identical levels of mRNA for type I collagen.

SBMC line

AP As shown in Fig. 5, TCDD (3.3×10^{-9} M and higher) induced a 33% reduction in AP activity vs control but this reduction was not reversed by administration of resveratrol at various concentrations, suggesting that the TCDD levels were probably too high to be attenuated with resveratrol doses used. A 33% reduction in AP activity was also shown following treatment with a lower concentration of TCDD (1.1×10^{-9} M). In this case, however, increasing concentrations of resveratrol

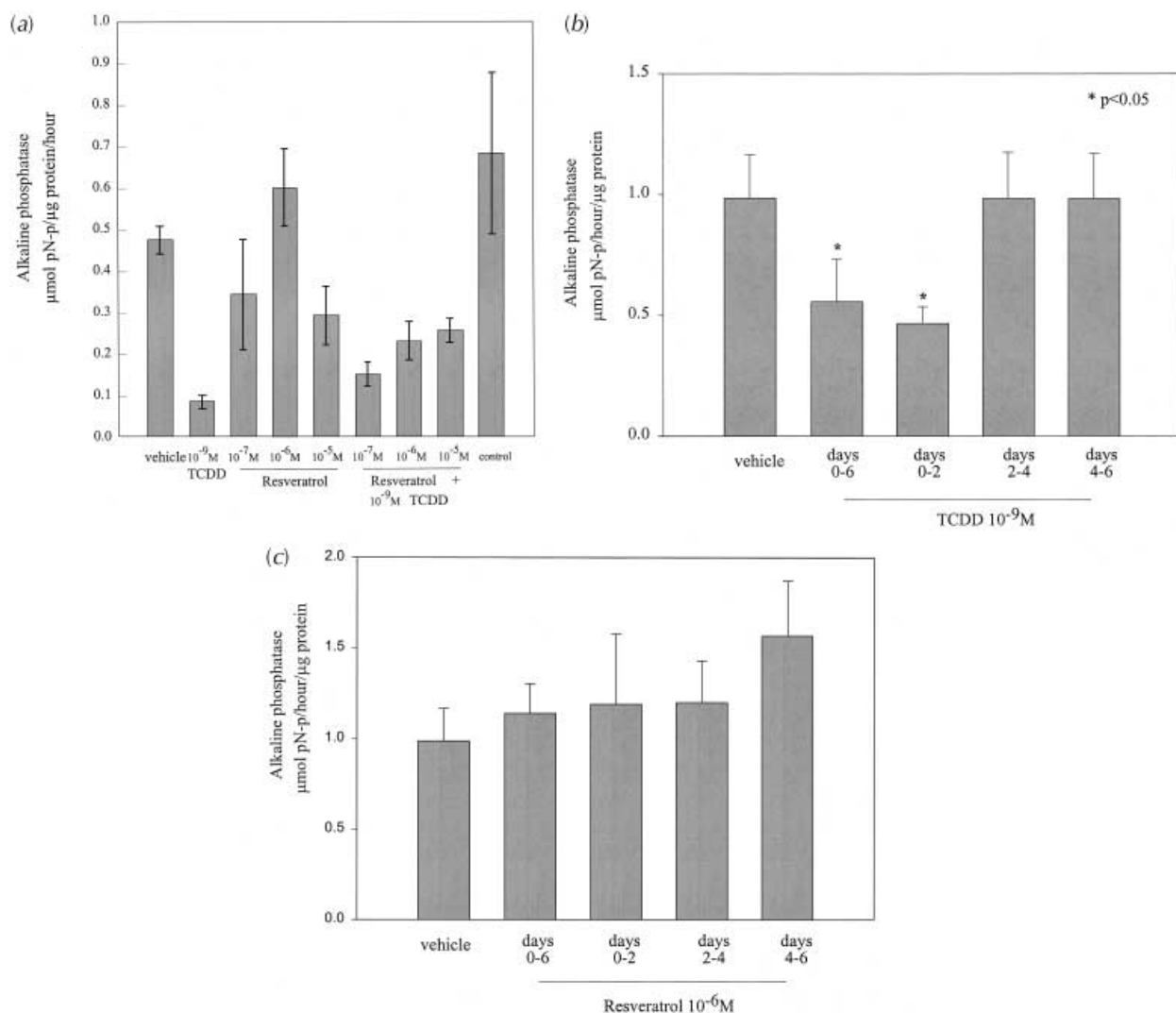


Figure 2 (a) AP activity in 6-day CPO cultures was reduced dramatically ($P < 0.05$) with TCDD (10^{-9}M) treatment in comparison to complete control (nothing added to medium) or vehicle control. AP activity was increased by 2- to 3-fold in cultures treated with both TCDD and resveratrol (10^{-6}M) as compared with cultures treated with TCDD alone ($P < 0.05$), although in this experiment AP activity was not totally restored to control levels. (b) TCDD (10^{-9}M) had its most profound inhibitory effects on AP activity when CPO cultures were treated either over the entire 6-day culture period or during days 0–2 (differentiation phase). When cultures were treated with TCDD after the differentiation phase, there was little to no inhibitory effect on AP activity. In this series of experiments, AP activity was reduced by only about 50% in comparison with control, whereas in (a) there appeared to be at least an 80% reduction. It should be noted that overall AP activity was also different in both experiments. This variation is not unusual for this type of tissue culture system, and so direct statistical comparisons are only made within and never between experiments. Resveratrol alone (10^{-6}M) had no major effects on AP activity (c) no matter when it was added. However, there was a mild, but non-significant ($P > 0.05$) stimulatory effect on AP activity when resveratrol was added over days 4–6.

($4 \times 10^{-8}\text{M}$ to $1 \times 10^{-5}\text{M}$) abrogated the effects of TCDD and brought AP activity back to, and even above that seen in control cultures, which is quite different from that observed in the CPO model but nonetheless depicts a parallel trend. This pattern was repeated in the cultures receiving lower levels of TCDD. The initial reduction in AP activity due to TCDD was dose dependent

($3.3 \times 10^{-9}\text{M}$ to $4.12 \times 10^{-11}\text{M}$). The abrogation of the effect of TCDD by increasing resveratrol concentrations was exhibited at each level of TCDD administration until a concentration of $4.12 \times 10^{-11}\text{M}$ TCDD was reached. At this concentration, AP activity was not affected (i.e. similar to control) and the increasing concentration of resveratrol had no effect either. At several of the

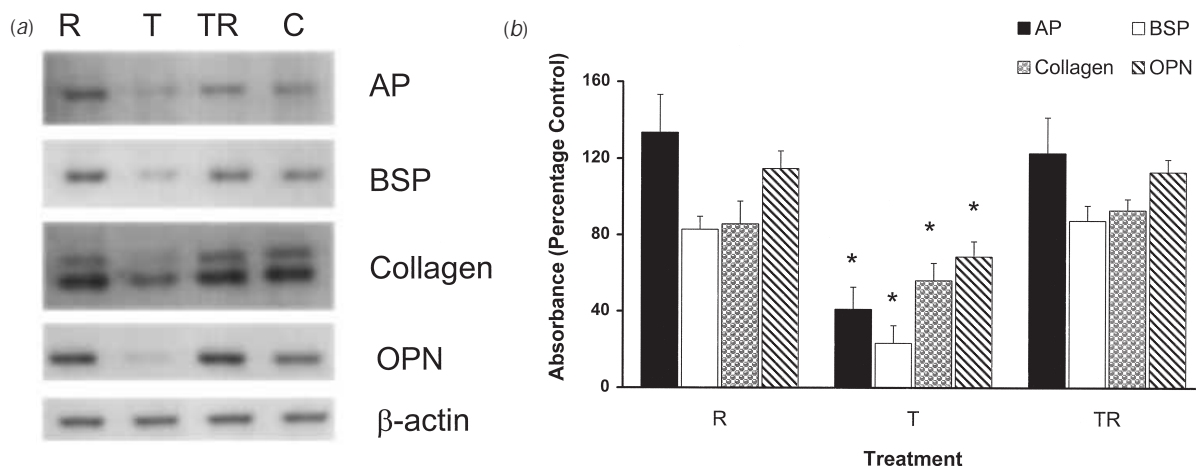


Figure 3 A semiquantitative RT-PCR method was used to demonstrate changes in mRNA expression for bone proteins in CPO cultures treated with TCDD (T) and resveratrol (R). (a) Agarose gel electrophoresis patterns for RT-PCR products demonstrate obvious reductions in mRNA bands for bone proteins, AP, BSP, OPN and collagen in cultures treated with TCDD. Band intensity was increased to control levels in cultures treated with TCDD plus resveratrol (TR). β -actin bands were consistent in all groups, as would be expected for this 'housekeeping gene'. (b) Band intensities were estimated using densitometric measurements. The numbers are expressed as the means \pm S.E.M. of triplicate measurements. All values were normalized against β -actin. Statistical significance was set at $P < 0.05$ by ANOVA and is denoted on the bars by an asterisk (*).

lower TCDD concentrations, the addition of the highest levels of resveratrol caused an increase in AP activity above that found in control, suggesting that resveratrol could have its own stimulating effects, or perhaps other co-operative AhR interactions, as will be discussed below.

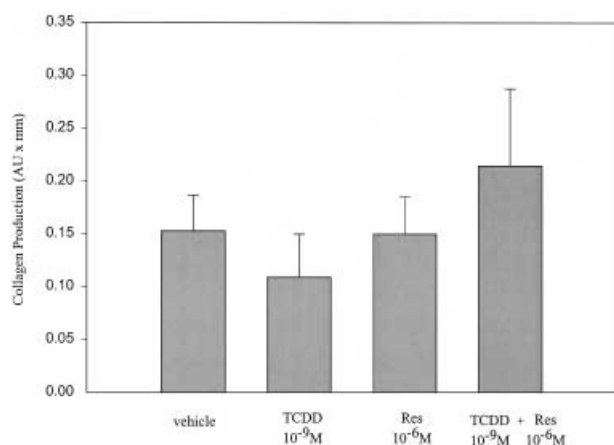


Figure 4 Radiolabeled (^{14}C) collagen type I levels in 6-day cultures treated with 10^{-9} M TCDD with or without 10^{-6} M resveratrol (Res) co-incubation over the entire culture period. There was a trend suggesting inhibition of newly synthesized collagen in the presence of TCDD, which was reversed by resveratrol. However, these differences were not found to be significantly different despite the obvious changes in bone formation suggested in Fig. 1 and changes in collagen mRNA shown in Fig. 3.

Interestingly, this was also suggested in the CPO model where mRNA for AP also demonstrated a tendency to be higher than control (see Fig. 3).

Mineralization in the rat SBMC line There was a dose-dependent reduction in mineralization (i.e. Alizarin Red staining) in the presence of 4.12×10^{-11} M to 1.52×10^{-12} M TCDD (Fig. 6). Mineralization was decreased by approximately 75% in the presence of TCDD, which was attenuated by resveratrol. Interestingly, at the highest concentration of resveratrol, not only were TCDD effects attenuated but there appeared to be a 3-fold increase relative to control levels, further suggesting a direct stimulatory effect or some form of cooperative interactions between TCDD and resveratrol.

Specific binding of TCDD and resveratrol Increasing concentrations of TCDD effectively competed with [^3H]TCDD for its cognate receptor and reduced binding to background levels at 3.3×10^{-8} M, thus suggesting the existence of the AhR in SBMCs despite the fact that 'classic' competition curves were not demonstrated. Approximately 50% competition was achieved at 3.7×10^{-9} M TCDD (Fig. 7a). Resveratrol competed with [^3H]TCDD in a similar fashion. Resveratrol (1.37×10^{-9} M) also reduced radiolabeled TCDD binding to background levels, while 50% competition occurred at $\sim 1.8 \times 10^{-10}$ M resveratrol, a dose 20 times lower than the dose of unlabeled TCDD required to produce this level of competition (Fig. 7b).

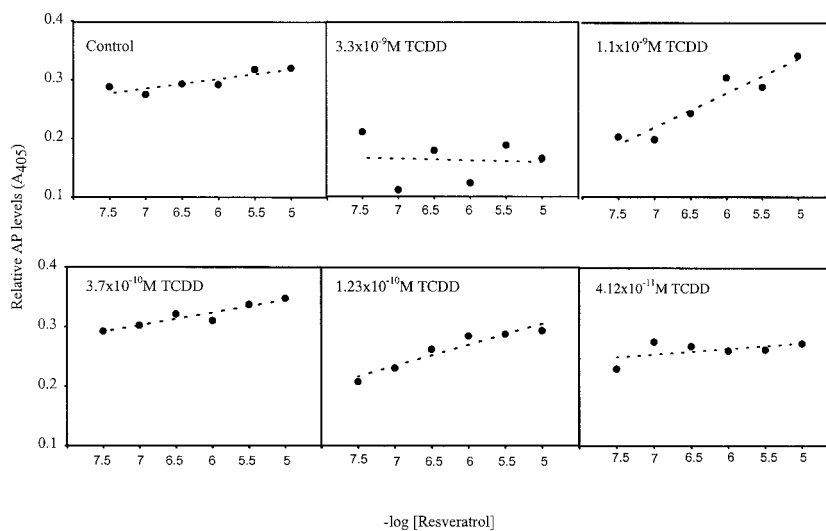


Figure 5 Relative (to blank wells) AP levels in the rat SBMC line. Each panel represents one TCDD concentration (or control as in the first panel) in the presence of increasing levels of resveratrol (4×10^{-8} M to 1×10^{-5} M). TCDD appeared to reduce AP levels by up to about 33% ($P < 0.05$), an effect that was antagonized and even stimulated above control by increasing concentrations of resveratrol except at the highest levels of TCDD where resveratrol, even in higher doses, had no antagonistic effect.

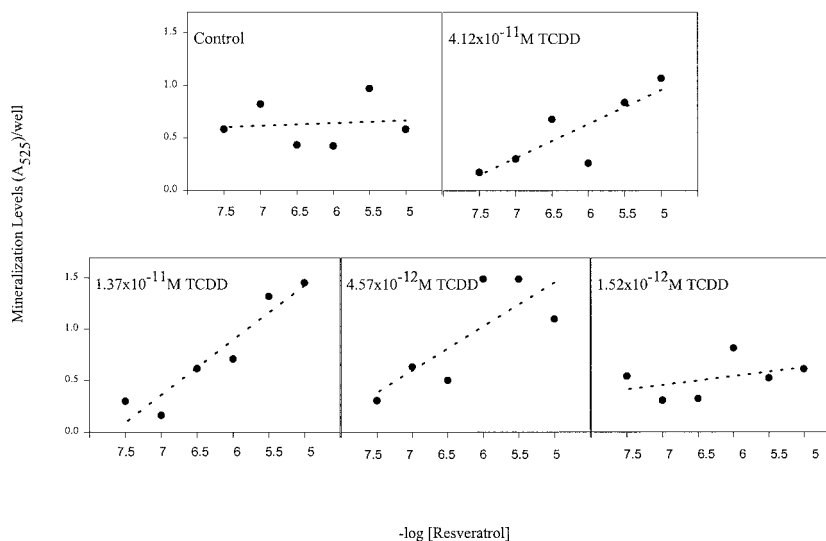


Figure 6 Relative mineralization levels in the rat SBMC line. Each panel represents either control or a single dose of TCDD in the presence of increasing concentrations of resveratrol as outlined in Fig. 5. As shown for AP, TCDD inhibited mineralization (Alizarin Red staining) ($P < 0.05$) and this effect was antagonized by increasing doses of resveratrol, which at the highest levels stimulated mineralization above control levels. Relative absorbance refers to absorbance compared with blank wells.

Discussion

The studies we employed were designed to test the effects of a prototypical AhR ligand, TCDD (Rowlands & Gustafsson 1997), on bone formation *in vitro*. Using two different *in vitro* bone-forming models it was shown that

TCDD inhibits bone formation and that a novel AhR antagonist, resveratrol, partially and in some cases completely reverses this inhibition. The competition studies confirm that these effects are mediated through the AhR as shown in other systems (Birnbaum 1994, Sukhu *et al.* 1997, Lavin *et al.* 1998). Currently no endogenous ligand

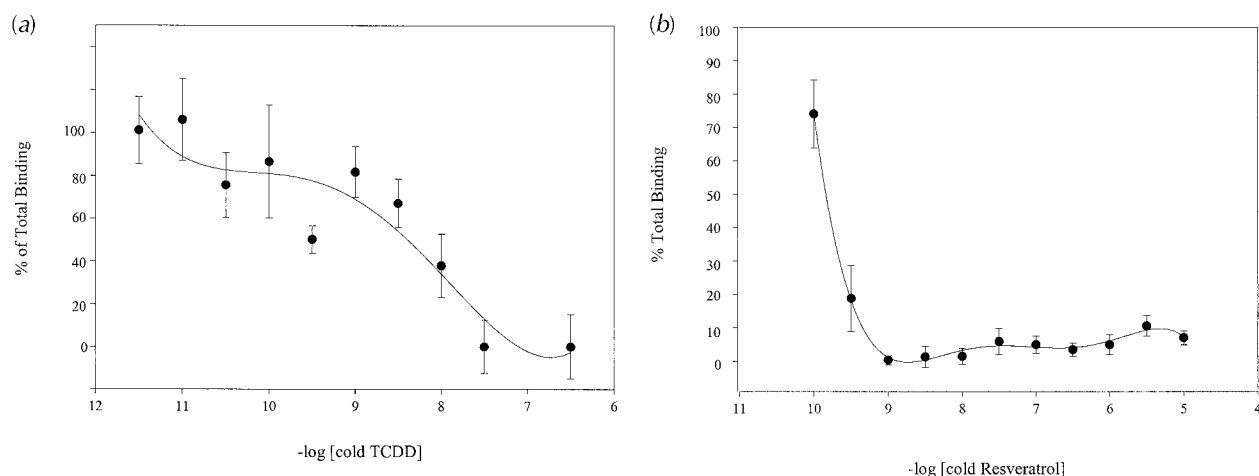


Figure 7 A live cell (SBMC) competition assay demonstrating competitive uptake/binding between radiolabeled TCDD and either unlabelled TCDD (a) or resveratrol (b). Background uptake/binding was subtracted for these calculations. Binding/uptake of radiolabeled TCDD was reduced to background levels at a dose of 3.3×10^{-8} M TCDD and 50% competition was produced with a dose of approximately 3.7×10^{-9} M TCDD. A dose of 1.37×10^{-9} M resveratrol reduced uptake/binding of radiolabeled TCDD to background levels while 50% competition occurred at a dose of approximately 1.8×10^{-10} M resveratrol. All cultures were pre-treated for a period of 1 h with 'cold' TCDD or resveratrol prior to adding the labeled TCDD. Although 'classic' competition curves were not produced in these studies, there was still, nonetheless, clear competitive binding. However, binding kinetics were not calculated as a result.

has been identified for the AhR and the only known natural ligands for this 'orphan receptor' are methyleneindole condensation products formed after ingestion of cruciferous vegetables such as cabbage, broccoli and cauliflower (Bjeldanes *et al.* 1991). In addition to being linked to the pathoses listed above, TCDD and other AhR ligands may have anti-estrogenic effects as demonstrated by their ability to inhibit a broad spectrum of estrogen-induced responses in rodents and breast cancer cell lines (Harris *et al.* 1990, Safe *et al.* 1991, Wang *et al.* 1993, Gierthy *et al.* 1994, Harper *et al.* 1994, Liu *et al.* 1994, Krishnan *et al.* 1995, Safe & Krishnan 1995, Nodland *et al.* 1997). However, as discussed below, resveratrol might also possess estrogen-like activity, although this is less likely.

Differentiation

AP activity and OPN Utilizing the CPO model, it was shown that 10^{-9} M TCDD administration over days 0–6 (i.e. the entire culture period) consistently caused a significant reduction in AP activity and transcripts. Similarly, 10^{-6} M resveratrol antagonized the effects of TCDD on AP activity consistently. AP has a number of functions and may act as an inorganic phosphate provider or as a modulator of matrix macromolecule turnover (Golub 1996), as well as in other diverse manners including regulation of mineralization (see below). In any case, synthesis of AP is accepted as a hallmark of the osteoblastic phenotype and bone formation (Termine 1993). The doses of TCDD and resveratrol shown to have a significant effect in the CPO model are consistent with doses used by

other investigators employing other systems (Gierthy *et al.* 1994, Mizutani *et al.* 1998) and in a breast cancer cell line (Casper *et al.* 1999). As well, the findings obtained for AP activity using the CPO model were paralleled in the rat SBMC line. However, as will be pointed out below, resveratrol alone may have stimulatory effects on its own or in combination with TCDD. The effects of TCDD on AP activity were dose dependent, and the ability of resveratrol to antagonize these effects was likewise shown to be dose dependent. Given similar findings in the two models it appears that TCDD inhibits osteoblastic AP activity and probably osteodifferentiation, and that resveratrol antagonizes this effect in a manner that is neither species nor model specific. Similar effects were shown with OPN, another marker for osteoblastic differentiation, further suggesting that TCDD inhibits osteodifferentiation. To investigate TCDD (and resveratrol) effects on differentiation further, we also carried out a series of temporal studies.

The temporal or 'window' experiments suggested that TCDD's inhibitory effects on osteodifferentiation, as reflected by AP activity, were exerted mainly during the early osteodifferentiation phase of culture in the CPO model (i.e. over days 0–2). Since TCDD inhibited parameters of osteogenesis when present at days 0–2, and not when added later, it may be suggested that while this agent interferes with osteoblastic differentiation, it has fewer effects on already differentiated osteoblasts. Consistent with our previous results, resveratrol administration antagonized the effects of TCDD when the two drugs were given together over days 0–2, confirming resveratrol's ability to reverse the effects of TCDD.

A second interesting finding in the CPO temporal studies was a trend suggesting an increase in AP activity induced by administration of resveratrol alone over days 4–6 (Fig. 2c; $P>0.05$). These data, combined with the finding that resveratrol induces a dose-dependent increase in AP activity and mineralization above control levels in the rat SBMC line, a mature osteoblastic cell line that is capable of producing mineralized bone-like tissue (Pitaru *et al.* 1993, S Kotev-Emeth, S Pitaru, S Pri-Chen & N Savion, unpublished observations), intimate a potentially stimulatory effect of this drug on more differentiated osteoblasts. This effect was observed only when resveratrol was administered during the late stages of osteogenesis (i.e. days 4–6 in the CPO model), pointing toward the presence of a resveratrol-responsive cell population that is more fully differentiated (Tenenbaum 1981, Tenenbaum & Heersche 1982, 1985). This phenomenon has recently been observed and reported elsewhere. For example, it has been shown that resveratrol alone causes a dose-dependent increase in AP activity when administered to a mature osteoblastic cell line (osteoblastic MC3T3-E1 cells) (Mizutani *et al.* 1998). Further, this effect was blocked by tamoxifen, an estrogen antagonist, suggesting that some of resveratrol's stimulatory actions may be mediated through the estrogen receptor. In relation to this, others have also shown that resveratrol exhibits various degrees of estrogenic effects (Gehm *et al.* 1997). However, there are other more recent investigations that question whether resveratrol has any significant estrogenic activity at all, so this issue is still open to some debate (Ashby *et al.* 1999, Lu & Serrero 1999, Turner *et al.* 1999) and warrants further investigation. Furthermore, as shown in models similar to the SBMC model, prostaglandins may inhibit osteogenesis (Ogiso *et al.* 1992). In other studies it has been shown that resveratrol may possess inherent anti-inflammatory properties (Subbaramaiah *et al.* 1998, 1999). Hence, it is conceivable that resveratrol's apparent stimulatory effects on some aspects of osteogenesis, especially in the SBMC model, could be related to inhibition of prostaglandin synthesis. In a related sense it was also apparent, especially in the SBMC model, that in some cases when lower levels of TCDD were used, resveratrol not only antagonized TCDD effects, but also promoted even higher AP levels than in control. In addition to resveratrol's putative inhibitory effects on prostaglandin synthesis, it is also conceivable that co-operative (i.e. between TCDD and resveratrol) stimulation of cells is occurring. It might also be postulated that resveratrol could be antagonizing environmentally derived aryl hydrocarbons that may already be present (i.e. constitutive) in the serum supplements used for culture, thereby stimulating osteogenic parameters above control levels.

Collagen synthesis (matrix formation)

Changes in levels of collagen type I were expected to follow the pattern of changes seen in AP activity and,

as expected, a trend in this direction was observed in the level of ^{14}C -labeled collagen type I in the CPO model treated with both TCDD and resveratrol. However, these effects (i.e. resveratrol antagonism of TCDD effects) were not statistically significant. It is possible that in addition to its ability to inhibit collagen synthesis and mRNA formation TCDD may also have inhibited collagen degradation. This could conceivably allow an accumulation of some collagen over the culture period even with less synthesis, which could partially mask a reduction in newly synthesized collagen levels. Turnover and remodeling of collagen is known to occur continuously during both physiological and pathological conditions and appears to be accomplished through the action of a number of proteolytic enzymes and lysosomal phagocytosis (Everts *et al.* 1996). In this regard, we have shown previously that collagen phagocytosis, the principal pathway of collagen turnover, is regulated by AP (Hui *et al.* 1997). As TCDD decreases AP activity and presumably AP protein due to mRNA reductions, it is conceivable that AP-dependent collagen phagocytosis could also be inhibited, thereby decreasing the impact of reductions in synthesis of collagen in the TCDD-treated cultures despite obvious reductions in mRNA for this protein. In any case, the histological observations of the CPO model suggested that bone matrix formation (i.e. related to bone collagen production) is almost certainly reduced by TCDD and then brought back to more normal levels in TCDD/resveratrol-treated cultures.

Mineralization

TCDD mediated substantial reductions in mineralization as shown by decreases in phosphate and calcium accumulation. Resveratrol antagonized the TCDD-mediated inhibition of mineralization in SBMC cultures and this occurred in a dose-dependent fashion, similar to its effect on TCDD-mediated inhibition of AP activity. However, when the concentration of TCDD exceeded 4.12×10^{-11} M, resveratrol could not antagonize its effect. Inasmuch as TCDD has the highest binding affinity to the AhR of all known compounds (Rowlands & Gustafsson 1997), it would appear that resveratrol could not compete with TCDD for the latter's cognate receptor, at these high doses. Interestingly, mineralization in the CPO model was inhibited by TCDD, but only partially (not shown; $P>0.05$). These findings are difficult to explain, especially in light of the data obtained using the SBMC model. One explanation may be related to the fact that although the rat SBMC line and CPO models produced comparable results they were not always identical. Similarly, the two models are not identical either. For example, whereas the rat SBMC line, like the RBMC model, relies absolutely on the presence of a glucocorticoid such as dexamethasone for osteodifferentiation (Grigoriadis *et al.* 1988, Maniopoulos *et al.* 1988, Kasugai *et al.* 1991, Shalhoub *et al.* 1992,

Rickard *et al.* 1994, Herbertson & Aubin 1995), the CPO model does not (McCulloch & Tenenbaum 1986) suggesting the presence of glucocorticoid-dependent and -independent osteogenic cells. TCDD may not affect the glucocorticoid-independent osteogenic cells in the same manner as the glucocorticoid-dependent ones. That having been said, it is also important to note that BSP mRNA levels were significantly reduced in the CPO model (an effect that was antagonized by resveratrol). BSP is thought to play an important, although perhaps not an exclusive or obligatory role in mineralization (Sodek *et al.* 1995). This could explain in part how TCDD inhibits some mineralization in the CPO model, while non-BSP-mediated mineralization could still occur.

Conclusions

The evidence gathered from these experiments supports the hypothesis that TCDD, a prototypical AhR ligand, inhibits bone formation and could explain how similar hydrocarbons may predispose smokers to both osteoporosis and periodontal bone loss. This inhibition appears to be receptor mediated and occurs during cellular proliferation and osteoblastic differentiation. Resveratrol, a novel AhR antagonist, competes for the AhR and partially or fully antagonizes AhR ligand effects depending on the model or parameters being used. The clinical implications of these findings could be important because it is possible that resveratrol may be considered as a therapeutic agent which might protect bone and periodontal tissues from some of the deleterious effects of environmental hydrocarbons in general and cigarette smoking in particular.

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