OSTEOCLAST DIFFERENTIATION IS AFFECTED BY TCDD

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Introduction

Environmental contaminants may affect bone cells and thereby bone remodeling. We have previously reported that TCDD as a model compound does not affect bone resorption *in vitro* in the resorption pit assay [1]. In that assay we used mature rat osteocasts disaggregated from long bones of newborn rats. However, we have shown *in vivo,* that TCDD can affect bone growth, remodeling and mechanical strength [2]. This discrepancy led us to study the initial steps of bone remodeling i.e. the induction of bone resorption. Bone remodeling starts with recruitment and activation of osteoclast stem cells from bone marrow or circulating blood. After formation of osteoclasts and their eating a package of bone the osteoclasts die with apoptosis and the process is followed by formation of equal package of new bone. This cycle of activation, resorption and formation is called ARF of bone remodeling.

In this study, we utilized the *in vitro* differentiation of bone marrow cells of monocyte-macrophage lineage to multinuclear osteoclasts as a model to study the effects of TCDD on osteoclast differentiation. The formation of multinuclear osteoclasts and their ability to resorb bone was evaluated in 7 and 10 days cell cultures on bovine bone slices.

Materials and Methods

The procedure for osteoclast differentiation was modified from Takahashi et al. [3]. Bone marrow cells from C57BL/6 mice (8-12 weeks) were isolated from long bones by using syringe. After incubation at +37 °C for 2 hours non-attached cells were collected from petri dish. Cells were seeded on ultrasonicated bovine cortical bone slices at a concentration of 1 x 10^6 cells/slice. The control group was cultured in α-MEM medium (Sigma Chemicals) with 10 % heat-inactivated fetal calf serum (FCS) (Promo Cell), 2 mM L-glutamin (Sigma Chemicals), 100 IU/ml penicillin, 10 µg/ml streptomycin (Sigma Chemicals) and 20 mM Hepes (Sigma Chemicals). Ethanol was used to dissolve TCDD with a final concentration of 0.2 % ethanol in α-MEM-FCS. TCDD was investigated at concentrations of 10-9 and 10-7 M in α-MEM-FCS. Cells were cultured in 500 µl α-MEM containing 30 ng/ml RANKL (Peprotech Ec.) and 10 ng/ml M-CSF (R&D Systems). Half of the medium was replaced every 3rd day. Cells were cultured at +37 °C (5 % CO2, 95 % air) for 10 days, after which the cultures were stopped by fixing the cells with 3 % paraformaldehyde (PFA) / 2 % sucrose in PBS.

Mouse bone marrow cells were cultured for 7 days in the presence of RANKL and M-CSF. After fixation the cells were stained for tartrate-resistant acid phosphatase (TRACP), a commonly accepted marker of osteoclasts (Analysis kit no. 386-A, Sigma Chemicals, St. Louis, MO). For each bone slice, ten random fields were chosen to estimate the number of osteoclasts (TRACP-positive cells with two or more nuclei).

The resorption pits were stained and visualized as described earlier [4]. The pits were stained with peroxidase-conjugated wheat germ agglutin-lectin (WGA; 20 mg/ml; Sigma Chemicals) for 20 minutes at room temperature, washed with PBS, and incubated for 10 minutes at room temperature in diaminobenzidine (DAB) (0,5 mg/ml) and 0,03 % H_2O_2. For each bone slice, ten random areas were chosen to estimate the resorbed area.

All statistical analyses were performed by using the computer program Microcal Origin Version 6.0 (Microcal Software Inc., Northampton, MA). After one way ANOVA, Student's t-test was used to determine which means were significantly different ($p < 0.05$). Data presented are the result of two independent experiments performed in triplicate.

Results and Discussion

When mouse bone marrow stem cells were cultured with M-CSF and RANKL most of the cells differentiated into cells of osteoclast lineage and became mature osteoclasts at 7 day culture. At the beginning the plated cells do not express TRACP. Along osteoclast differentiation mononuclear TRACP-positive cells are detected, which fuse to form multinucleated osteoclasts.
The addition of TCDD decreased the number of TRACP-positive multinucleated cells in these cultures. TCDD at 10^{-9} M ($p < 0.001$) and 10^{-7} M ($p < 0.001$) decreased the number of TRACP-positive multinucleated cells, when compared to control. At 10^{-9} M, the number of TRACP-positive multinucleated cells was 3-fold lower and at 10^{-7} M 3.5-fold lower, when compared to control. TCDD at 10-9 and 10-7 M decreased the area of bone resorption ($p < 0.001$) at the day 7. At 10^{-9} M, the resorption area was 21-fold lower and at 10^{-7} M 27-fold lower when compared to control.

In our previous pit assay study [1] TCDD did not interfere with the activation of osteoclasts, nor did the number of TRAP-positive multinucleated cells change after TCDD treatment. This was somewhat unexpected since dioxin-treated animals have smaller bones with relatively weaker mechanical strength [2]. On reason may be the effects on cells of osteoblastic lineage, as Naruse et al. [5] demonstrated with 3-methylcholantrene (3MC). They found out that 3MC inhibited osteoclastogenesis through inhibiting RANKL expression on bone marrow stromal cells. 3MC is, nevertheless, also able to activate AHR-mediated events.
In the present study the only cells in culture were the hematopoietic stem cells and osteoclasts differentiated from them. Our results indicate that Dioxin has direct effect on osteoclast stem cells and thereby on osteoclastogenesis and finally on bone remodeling.

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