Effects of TCDD on Differentiation of Osteoblasts Derived from Rat Mesenchymal Stem Cells

Anne Natunen[1], Merja Korkalainen, Anu Olkku[2], Joanna Ilvesaro[3], Juha Tuukkanen[3], Matti Viluksela[1], Anitta Mahonen[2]

[1]National Public Health Institute, Department of Environmental Health
[2]Department of Medical Biochemistry, University of Kuopio
[3]Department of Anatomy and Cell Biology, University of Oulu

Introduction

While broad range of dioxin toxicities have been well documented, their effects on bone are still poorly known. Only a small number of experimental studies have demonstrated that bone development and homeostasis could be critical targets of TCDD. Data from *in vitro* studies showed that TCDD suppresses differentiation of normal diploid rat osteoblasts [1]. Another AH receptor (AHR) ligand, 3-methylcholanthrene, also inhibited differentiation and proliferation in rat and mouse osteoblastic cells [2]. In adult rats, long-term exposure to TCDD was shown to dose-dependently interfere with bone growth, modeling, and mechanical strength [3]. In addition, AHR was suggested to play a role in modulating the effects of dioxins on bones, because dioxin-resistant rats carrying a deviant AHR were more resistant than wild-type rats. Interestingly, these changes were observed at doses of relevance for environmental health risk assessment [3]. Moreover, developing bones were shown to be more sensitive to TCDD than bones of adult animals [4].

In this study, we utilized rat osteoblasts derived from mesenchymal stem cells to study the effects of TCDD on bone. Activity of alkaline phosphatase and secretion of osteocalcin were used as markers of osteoblastic differentiation.

Materials and methods

Mesenchymal stem cells (MSC) were isolated from the bone marrow of femurs and tibias of adult male Wistar or line C rats. The rats were obtained from the breeding colonies of the National Laboratory Animal Centre, University of Kuopio, and the National Public Health Institute, Department of Environmental Health, Kuopio, Finland, respectively. Bones were first dissected free of adhering tissues and the bone ends were removed. The marrow cavity was flushed out by a syringe in *20* mM Hepes- buffered alpha-MEM supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Released cells were then plated at a density of 1 x 10^6 cells/cm^2 and maintained in humid atmosphere at 37°C with 5 % CO_2. The culture medium was enriched with 10 nM dexamethasone, 1 mM b-glycerol phosphate, and 50 mg/ml ascorbic acid, which favour the differentiation of osteoblastic cells. After reaching near-confluence the cells were seeded in 6- or 24-well plates at a density of 5000 cells/cm^2. Next day the medium was replaced with exposure medium containing 1 nM or 100 nM TCDD diluted in 0.1 % DMSO, or DMSO vehicle alone.

Alkaline phosphatase activity was assayed from the pelleted cells using a colorimetric technique with p-nitrophenyl phosphate as a substrate. The protein concentrations were measured by Bradford assay (Bio-Rad, Hercules, CA). Osteocalcin levels in culture medium were determined by immunoradiometric assay using Rat Osteocalcin IRMA Kit (Immutopics, Inc., San Clemente, CA). For AHR localization the cells were stained by polyclonal AHR antibodies (Biomol Research Laboratories, Plymouth Meeting, MA) and Alexa Fluor® 568 goat anti-rabbit IgG secondary antibodies (Molecular Probes, Leiden, The Netherlands).

The experiments were repeated independently two to three times with similar results. Statistical analysis was performed by the *ANOVA* followed by the Least Significant Difference test with the SPSS 11.5 program. The limit of statistical significance was set at $p<0.05$.

Results and Discussion

Osteoblasts are differentiated in three steps, which are proliferation, matrix maturation and mineralization. Alkaline phosphatase is generally used as a marker of matrix maturation and osteocalcin as a marker of mineralization. The effects of TCDD on these markers were measured in osteoblasts derived from both Wistar and line C rats, but the results showed no differences between these strains.

TCDD decreased the activity of alkaline phosphatase dose-dependently (Fig.1). However, this alteration was significant only after 4 days exposure with the highest TCDD-dose. The secretion of osteocalcin was significantly increased after 5 and 7 days of TCDD exposure (Fig.2). Interestingly, the lowest dose of TCDD was enough to produce the maximal increase.

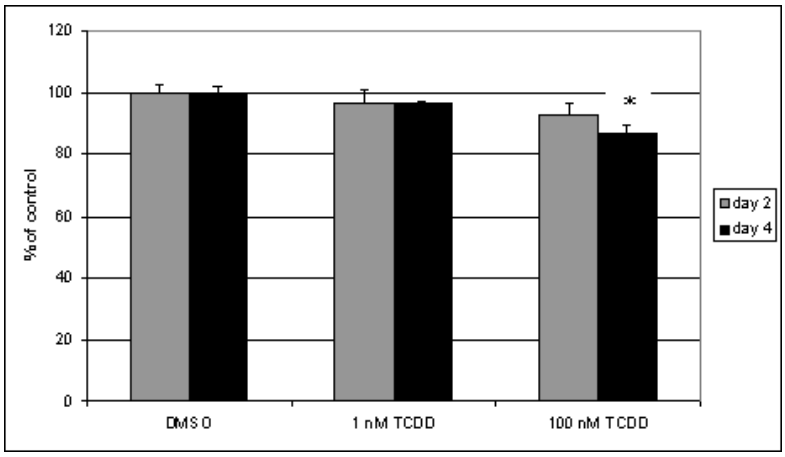

Figure 1. Effect of TCDD on alkaline phosphatase activity in osteoblasts of Wistar rats. The activity was measured after two and four days of TCDD-exposure. The columns represent means ± SEM of three replicates in two independent experiments. *Significantly different from DMSO control ($p<0.05$).

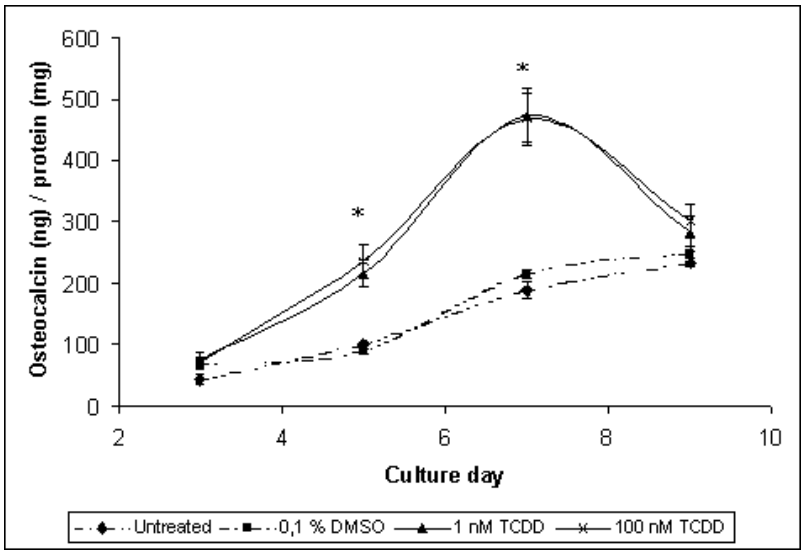

Figure 2. Secretion of osteocalcin in control and TCDD-treated osteoblasts of line C rats. Values represent means ± SEM of three replicates in two independent measurements. *Significant difference in both TCDD concentrations.

Our results indicate that TCDD affects both of the measured markers of osteoblastic differentiation *in vitro*. TCDD

caused a reduction in the activity of alkaline phosphatase consistently with earlier reports [1, 7]. Instead, the TCDD-induced increase in the secretion of osteocalcin has not been reported earlier. The mechanism of TCDD-induced alteration in the differentiation of osteoblasts is unknown. TCDD is known to mediate its effects mainly via AHR by binding to the dioxin response element (DRE) located upstream of the target genes. In this study, immunofluorescent staining indicated that the cells actually expressed AHR from the beginning of the exposure period and that upon TCDD exposure AHR was translocated into the nucleus.However, alkaline phosphatase and osteocalcin genes both lack DREs in their regulatory sequences [5, 6]. Therefore, the effect of TCDD on the expression of these genes is not likely a direct effect but rather a secondary response mediated by AHR.

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References

1. Gierthy, J.F., Silkworth, J.B., Tassinari, M., Stein, G.S. and Lian, J.B. (1994) *J Cell Biochem*, 54, 231-238.

2. Naruse, M., Ishihara, Y., Miyagawa-Tomita, S., Koyama, A. and Hagiwara, H. (2002) *Endocrinology*, 143, 3575-3581.

3. Jämsä, T., Viluksela, M., Tuomisto, J.T., Tuomisto, J. and Tuukkanen, J. (2001) *J Bone Miner Res*, 16, 1812-1820.

4. Miettinen, H.M., Pulkkinen, P., Jämsä, T., Koistinen, J., Simanainen, U., Tuomisto, J., Tuukkanen, J. and Viluksela, M. (2005) *Toxicol Sci*, 85, 1003-1012.

5. Terao, M., Studer, M., Gianni, M. and Garattini, E. (1990) *Biochem J*, 268, 641-648.

6. Desbois, C., Hogue, D.A. and Karsenty, G. (1994) *J Biol Chem*, 269, 1183-1190.

7. Singh, S.U., Casper, R.F., Fritz, P.C., Sukhu, B., Ganss, B., Girard, B., Jr., Savouret, J.F. and Tenenbaum, H.C. (2000) *J Endocrinol*, 167, 183-195.