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Expression of osteoblast marker genes in rat calvarial osteoblast-like cells, and effects of the endocrine disrupters diphenylolpropane, benzophenone-3, resveratrol and silymarin

Agnieska Ziolkowska [a], Marcin Rucinski [a], Andrzej Pucher [b], Cinzia Tortorella [c], Gastone G. Nussdorfer [c,*], Ludwick K. Malendowicz

[a] *Department of Histology and Embryology, Poznan University of Medical Sciences, PL-60781 Poznan, Poland*
[b] *Department of Orthopedy and Traumatology, Poznan University of Medical Sciences, PL-60781 Poznan, Poland*
[c] *Department of Human Anatomy and Physiology, Section of Anatomy, School of Medicine, University of Padua, Via Gabelli 65, I-35121 Padua, Italy*

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Abstract

Compelling evidence indicates that some endocrine disrupters (EDs), acting as selective estrogen-receptor modulators, interfere with osteoblast differentiation and function. Hence, we investigated whether four EDs [bisphenol-A (BSP), benzophenone-3 (BP3), resveratrol and silymarin] affect differentiation and growth of rat calvarial osteoblast-like (ROB) cells in primary in vitro culture. ROB cells were cultured for up 30 days in a medium supplemented with fetal calf serum (FCS), and conventional RT-PCR detected the expression of collagen-1α and osteonectin mRNAs throught the entire culture period. Real time-PCR demonstrated that at days 2 and 7 of culture the expressions of collagen-1α and osteonectin were very low, and underwent a 192- and a 334-fold increase, respectively, at day 21 of culture. In contrast, osteocalcin expression remained unchanged from days 2 to 21 of culture. EIA showed that ROB cells secreted sizeable amounts of osteocalcin and osteopontin between days 13 and 15 of culture. EDs were added at day 13 of culture at concentrations ranging from 10^{-10} to 10^{-6} M, being the culture medium deprived of FCS, and their effects were tested 48 h later. None of EDs was found to affect osteocalcin and osteopontin secretion from ROB cells, suggesting that their effects were tested at a relatively earlier stage of culture, when ROB cell differentiation into osteoblats is not fully accomplished, and/or the presence of estrogens contained in FCS is needed for EDs to exert their osteoblast-differentiation modulating action. BSP and BP3, but not resveratrol and silymarin, decreased proliferative activity of cultured ROB cells, a cytotoxic effect conceivably independent of their estrogen-receptor modulating activity.

Keywords: Rat calvarial osteoblast-like cells; Diphenylolpropane; Benzophenone-3; Resveratrol; Silymarin; Osteopontin; Osteocalcin; Osteonectin; Collagen-1α

1. Introduction

Data accumulated during the last two decades draw our attention on the adverse effects of endocrine disrupters (EDs) on human health and wildlife. Since the original finding of the estrogenic activity of DDT

* Corresponding author. Tel.: +39 049 827 2317; fax: +39 049 827 2319.
E-mail address: gastone.nusdorfer@unipd.it (G.G. Nussdorfer).

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in cockerels [1], numerous EDs have been identified, sharing the ability to alter various components of the endocrine system [2,3]. Numerous EDs, either synthetic or natural compounds, have been shown to be ligands of a variety of steroid-hormone receptors, and their best recognized disrupting properties are those connected with their estrogenic activities. Several EDs act as selective estrogen receptor (ER) modulators (SERMs): they may act via both ERα and ERβ isoforms, and frequently exert estrogenic effects in some but not all organs [3,4].

The high frequency of osteoporosis prompted investigation on bone metabolism and the effects of EDs on differentiation, growth and specialized functions of bone cells. Osteoblasts and osteoclasts were found to express the ERα and ERβ, androgen receptor (AR), glucocorticoid receptor and both subtypes of thyroid-hormone receptor (TRα and TRβ) [5–8]. Evidence indicates that EDs exert adverse effects on bone tissue both in vivo and in vitro, but the mechanism(s) by which EDs influence osteoblasts and osteoclasts are still largely unsettled.

Progress in tissue and cell culture has provided new and powerful tools for the study of differentiation and function of the major types of bone cells [10,11]. Primary cultures or cell lines established from osteoblasts, osteoclasts and chondrocytes enable the recognition of factors involved in bone formation and resorption, and allow detailed studies on factors interfering with these processes. One of the in vitro models for investigating specific gene events associated with osteoblast proliferation and differentiation, as well as mineralization of the extracellular matrix, is primary culture of rat calvarial osteoblast-like (ROB) cells [12–17]. In fact, ROB cells in primary culture spontaneously differentiate into osteoblasts, and this process is associated with increased expression of both collagen and non-collagenous bone proteins, including osteocalcin, osteopontin and osteonectin [12,18,19].

Some EDs are known to interfere with osteoblast differentiation and function [17,20]. Earlier in vitro data demonstrated adverse effects of dioxin derivatives that are estrogen antagonists, on rat osteoblasts and UMR-osteosarcoma cell line: inhibition of osteoblast differentiation in primary culture or impairment of collagenase-3 secretion from osteosarcoma cell line [21–23]. However, only scarce data are available on the effects of other EDs, like diphenylolpropane (bisphenol-A; BSP), benzophenone-3 (Eusolex-4360; BP3), resveratrol and silymarin.

BSP is an important chemical in the production of epoxy resins and polycarbonate plastics, and is known to act as a weak estrogen [24,25]. BP3, a UV-absorber used in cosmetics and plastics to improve product stability and durability, also exert estrogenic activity [26]. Resveratrol, a phytoalexin found in the red wine, belongs to phytoestrogens and exhibits SERM activity, thereby modulating numerous biological activities [27,28]. Silymarin, a plant flavonoid exerting preventive effects against carcinogenesis and cell damage in various animal models, in some organs displays estrogenic effects [29,30]. It, therefore, seemed worthwhile to investigate the expression of marker genes in ROB cells in relation to the duration of their in vitro culture, and to study the effects of EDs on differentiation and functions of ROB cells at the nodule-forming stage [8,31].

2. Materials and methods

2.1. Animals and reagents

Two-day-old Wistar rats, born in our laboratory facilities, were used, and the study protocol was approved by the local Ethics Commitee for Animal Studies. BSP and BP3 were purchased from Merck & Co. (Whitehouse Station, NJ). Resveratrol was provided by Nabio Biotech. Co. (Shanghai, China). Silymarin, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and all other reagents were obtained from Sigma–Aldrich Corp. (St. Louis, MO).

2.2. ROB cell culture

The technique was that described by Boden et al. [14] with few modifications. Briefly, calvarias of eight rats were placed in DMEM, and the connective tissue was removed. Calvarias were then cut into small fragments, which were dissociated to cell suspensions by enzymatic digestion with 0.1% collagenase-I for 30 min at 37 °C. ROB cells were harvested by centrifugation and resuspended in DMEM supplemented with $NaHCO_3$, 6% FCS and antibiotic-antimycotic solution. Cells were then plated in culture dishes (10^4 cells/dish), and cultured for up 30 days at 37 °C in a humidified atmosphere of 95% air–5% CO_2, medium being changed every 24 h. Conventional PCR was performed on ROB cells cultured for 10, 20 and 30 days, and semiquantitative real time-PCR on cells at days 2, 7 and 21 of culture.

2.3. RT-PCR

ROB cells were harvested, and total RNA was extracted, as previously detailed [32]. Contaminating DNA was eliminated by DNase-I treatment (RNase-Free