Effects of 17β-estradiol, tamoxifen and raloxifene on the protein and mRNA expression of interleukin-6, transforming growth factor-β1 and insulin-like growth factor-1 in primary human osteoblast cultures

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ABSTRACT. We investigated the effects of 17β-estradiol and two selective estrogen receptor modulators, tamoxifen and raloxifene, on the expression and release of constitutive and interleukin-1-stimulated interleukin (IL)-6, transforming growth factor-β1 (TGF-β1) and insulin-like growth factor-1 by osteoblasts in primary culture from trabecular bone of healthy post-menopausal women. After 24 h incubation with 10⁻⁸ M concentration of these compounds, there was no decrease in: a) the constitutive or IL-1β-induced levels of IL-6 protein released to culture medium; b) the constitutive IL-6 mRNA expression after incubation of osteoblasts with 10⁻⁸ M 17β-estradiol or 10⁻⁸ M tamoxifen for 1, 3, 6, 24 or 30 h. Although a decrease after 30 h of treatment with 10⁻⁸ M, raloxifene was found in mRNA IL-6 expression, and this fact was not reflected by a decrease in the release of IL-6 protein to the culture medium after 48 h of incubation with 10⁻⁸ M or 10⁻⁷ M raloxifene. Tumoral growth factor TGF-β1 expression was not influenced by incubation with these compounds. Gene expression of IGF-I increased following 24 or 30 h incubation with 10⁻⁸ M 17β-estradiol and 30 h incubation with raloxifene. Tamoxifen did not affect IGF-I expression. In conclusion, the effects of estradiol or tamoxifen on bone metabolism do not appear to be mediated through the regulation of osteoblast IL-6 release or synthesis, but raloxifene produces a decrease in mRNA IL-6 expression. The actions of estradiol, tamoxifen and raloxifene do not appear to be mediated by tumoral growth factor TGF-β1. On the other hand, an increase in IGF-I synthesis induced by raloxifene and estradiol could mediate, in part, the effects of these compounds on bone.

INTRODUCTION

In the adult skeleton, bone mass remains relatively constant throughout the continuous turnover of bone. During this remodelling process, the resorptive activity of osteoclasts is tightly coupled to and kept in balance with the synthetic activity of osteoblasts (1). In post-menopausal osteoporosis, this remodelling process is disrupted with uncoupling between osteoblast and osteoclast activity, and the net result is bone loss. Although a number of factors have been implicated in the etiology of post-menopausal osteoporosis, the lack of estradiol – a systemic regulator of bone remodelling – appears to play a significant role (2). However, the physiological bases for the action of estrogens on bone are not fully understood. Estrogens exert direct inhibitory effects on osteoclasts and their precursors, as well as direct but variable effects on mature osteoblasts. Moreover, estrogens regulate the osteoblastic production of cytokines and growth factors that, in turn, regulate the osteoclastic function in a paracrine fashion (3). Human osteoblast production of a wide range of growth factors and cytokines, playing a crucial role in bone metabolism, has been demonstrated (4): interleukin-6 (IL-6), transforming growth factor-β1 (TGF-β1) and IGF-I are among the most important (5, 6). Due to its pro-osteoclastogenic effect, IL-6 has received considerable interest as a possible intermediate in the protective action of estradiol on bone. Although the suppression of IL-6 production by osteoblasts has been reported as one mechanism contributing to es-
trogen-dependent inhibition of osteoclast activity (7, 8), conflicting data have been accumulated (9-12). TGF-β is also secreted from osteoblasts and incorporated in, and released from, the bone matrix during bone formation and resorption, respectively. It is considered to be one of the most important “coupling factors” between osteoblasts and osteoclasts (13). TGF-β may also act as an anabolic factor through its influence on osteoblast proliferation and differentiation, and on the bone matrix synthesis (14-16). Several reports have suggested that TGF-β is involved in both the direct inhibition of osteoclast resorption (17, 18) and inhibition of osteoclast precursor recruitment (19, 20). TGF-β also influences osteoblast and osteoclast apoptosis (21).

IGF-I acts as an anabolic factor through its positive influence on osteoblast proliferation and differentiation and on collagen synthesis (22, 23). In studying the effects of estradiol on osteoblasts, an important consideration regards the experimental model used: at the species level, there are potentially important differences between murine and human osteoblasts, which may create difficulties in accurate data interpretation (24). To avoid this, “in vitro in situ” experiments using human cells of osteoblastic phenotype may be preferable. Transformed osteosarcoma cell lines (MG-63, Saos-2, TE-85-H0S, FM-30/2) and, genetically-manipulated and immortalised osteoblastic, human cell lines (HOBIT, hFOB 1.19, hFOB/ER9, BOP 37) are often used, but is not possible to assume that the response of these cell types to different agents is the same as would occur in non-transformed osteoblasts from healthy subjects. The estrogen receptor isoforms present and the stage of cell differentiation also appear to influence the response of osteoblast cells to estradiol (25). It is important that the conditions employed mimic physiological conditions as far as possible. Although the production and secretion of TGF-β and IGF-I have been shown to be modulated by estrogen in several reports (26), in the majority of these studies transformed cell lines were used. Like estrogens, selective estrogen receptor modulators (SERMs), through their binding to estrogen receptors, produce estrogen-like effects in some tissues, including bone. However, they antagonise estrogen actions in other tissues, thus limiting collateral undesirable effects produced by estrogen treatment (27). Tamoxifen and raloxifene are SERMs widely used in the breast cancer treatment and post-menopausal osteoporosis, respectively. Their effect on the synthesis and release of local factors by osteoblasts has not been established. This study aimed to determine the effect of estradiol, tamoxifen and raloxifene on the expression and release of IL-6, TGF-β1 and IGF-I by osteoblasts.

It was performed in a primary culture of human mature osteoblasts from trabecular bone of healthy post-menopausal women.

**MATERIALS AND METHODS**

**Osteoblast culture**

Human osteoblasts (HOB) were cultured using the method of Marie et al. (28) with some modifications (29). Briefly, the trabecular bone femur specimens were obtained from 7 post-menopausal women, aged 55-75 yr, who were undergoing corrective surgery after accidental injury and not exhibiting metabolic bone disease. None of the women were taking drugs known to affect bone metabolism. The Local Ethics Committee approved this study and patients’ consent was obtained. The trabecular bone was fragmented and placed on an 80 μm pore size nylon mesh in a Petri dish (Corning Incorporated, NY, USA), to further eliminate the remaining small marrow cells and to allow the cells lining the trabecular bone surfaces to migrate onto the mesh. The bone explants were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 20% foetal calf serum (FCS) and 1% P/S (penicillin-streptomycin; Invitrogen-Life Technologies, Grand Island, NY, USA) and incubated at 37 °C in a humidified atmosphere with 5% CO₂. Cells outgrowing from the bone surface were collected by trypsinisation at preconfluence (about 45 days from the beginning of culture) and plated in F-75 flasks (Corning Incorporated, NY, USA). Experiments were performed between the second and the fourth passages. Osteoblasts were characterized by their alkaline phosphatase activity and by osteocalcin production in response to 1,25 (OH)₂ D₃ (28, 29).

**Compounds**

17β-estradiol was obtained from Sigma, St Louis, MO. This compound was dissolved in 100% ethanol to prepare a stock solution (10⁻³ M) which was stored at –20 °C. On the day of the experiment, a 10⁻⁵ M solution was prepared in culture medium and an aliquot added to the osteoblast culture medium in order to obtain a final concentration of 10⁻⁸ M estradiol.

**Tamoxifen**

This compound was kindly supplied by Astra-Zeneca Pharmaceuticals (Macclesfield, UK). It was dissolved in 100% ethanol to prepare a stock solution (10⁻³ M) which was stored at –20 °C. On the day of the experiment, a 10⁻⁵ M solution was prepared in culture medium and an aliquot was added to the osteoblast culture medium in order to obtain a final concentration of 10⁻⁸ M tamoxifen.

**Raloxifene analog (LY117018)**

This compound was kindly supplied by Lilly Research Laboratories (Indianapolis, IN). It was dissolved in 100% ethanol to prepare a stock solution (10⁻³ M) which was stored –20°C. On the day of the experiment, a 10⁻⁵ M solution was prepared in culture medium and an aliquot added to the osteoblast culture medium in order to obtain a final concentration of 10⁻⁸ M or 10⁻⁷ M raloxifene analog. After these compound additions to culture media, the ethanol concentration was lower than 0.005% (v/v) in all cases.

**Human IL-1β**

(hIL-1β; Boehringer Mannheim Biochemical, Basel, Switzerland; 1: 10 and 1:100 dilutions of hIL-1β were made in phosphate buffered