Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Steroid Biochemistry & Molecular Biology 100 (2006) 117–128
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Effect of ormeloxifene on ovariectomy-induced bone resorption, osteoclast differentiation and apoptosis and TGF beta-3 expression

P.S. Narayana Murthy, S. Sengupta, S. Sharma, M.M. Singh *

Endocrinology Division, Central Drug Research Institute, Lucknow 226001, India

Received 27 October 2005; accepted 31 March 2006

Abstract

Effect of ormeloxifene, a multifunctional selective estrogen receptor modulator, on prevention of ovariectomy-induced bone resorption in retired breeder female rats, osteoclastogenesis using bone marrow cells from adult Balb/c mice cultured in presence of M-CSF and RANKL, osteoclast apoptosis using terminal deoxynucleotidyl transferase fragment end labeling and TGF beta-3 expression were investigated. Raloxifene, a benzothiophene reported to mimic effects of estrogen in bone, and estradiol were used for comparison. Ormeloxifene (10^{-6} and 10^{-8} M) significantly inhibited osteoclastogenesis ($P<0.001$ versus vehicle control) as evidenced by lower number of TRAP-positive osteoclasts in bone marrow cultures and caused apoptosis of osteoclasts. The effect was almost equivalent to that observed in presence of estradiol-17 beta, except that significant number of cells undergoing apoptosis was evident even at 10^{-9} M concentration of estradiol-17 beta ($P<0.001$). Raloxifene, though inhibited osteoclastogenesis at much lower concentrations (10^{-8} to 10^{-12} M; $P<0.001$), failed to cause apoptosis of osteoclasts at any of the concentrations used. While ormeloxifene, raloxifene and ethynylestradiol significantly prevented ovariectomy-induced bone loss in vivo in retired breeder female rats, prevention of ovariectomy-induced decrease in BMD and trabecular network of proximal tibia, calcium and phosphorus levels in femur and tibia and prevention of ovariectomy-induced down-regulation of TGF beta-3 expression in lumbar vertebrae was of lower order in raloxifene- than ormeloxifene- or ethynylestradiol-supplemented females. Both the SERMs, however, produced considerable estrogenic effects at the uterine level as evidenced by increase in weight, total and endometrial area and luminal epithelial cell height; the effect being generally greater in raloxifene- than ormeloxifene-treated rats. Findings demonstrate that inhibition of estrogen-deficiency osteoporosis by ormeloxifene, as in case of estradiol, was mediated via inhibition of osteoclastogenesis, apoptosis of osteoclasts and up-regulation of TGF beta-3 expression. Raloxifene, though effective in inhibiting osteoclastogenesis in vitro at much lower concentrations, was not only less potent in preventing ovariectomy-induced bone loss in retired breeder female rats in vivo but also appeared to have a different mechanism of action than ormeloxifene and estradiol.

Keywords: Ormeloxifene; Raloxifene; SERM; Ethynylestradiol; Antiresorptive; Bone mineral density; Bone mineral content; Osteoclastogenesis; Osteoclast apoptosis; TGF beta-3; Retired breeder rats

1. Introduction

Osteoporosis is a condition of low bone mass and microarchitectural disruption that results in fractures with minimal trauma. In post-menopausal women, it is associated with low estrogen levels [1]. The activity of osteoclasts that resorb bone, relative to bone forming osteoblasts, dictates the development of osteoporosis [2]. Several studies have shown that estrogen replacement therapy (ERT) maintains skeletal mass and reduces fracture risk in post-menopausal women [3]. The protective effect of estrogens on bone tissue is believed to be due primarily to their antiresorptive action [2]. However, long term ERT/HRT has been associated with increased risk of cancer in estrogen target tissues, including mammary gland and endometrium [4]. New synthetic, non-steroidal compounds, called the selective estrogen receptor (ER) modulators or SERMs, have recently been shown to possess ER agonistic or antagonistic selectivity depending on the target tissue [5]. Ormeloxifene is a member of type-1 class of non-steroidal SERMs in clinical use [6], which possesses

* Corresponding author. Tel.: +91 522 2613894 (O)/2310757 (R); fax: +91 522 2623405.
E-mail address: singhmm@rediffmail.com (M.M. Singh).

0960-0760/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2006.03.009

desirable bone-protective [7–10] and lipid lowing activities [11] of estrogen and reported to prevent advanced breast cancer in both women and men [6,12,13] without side effects associated with conventional HRT/ERT. The precise mechanism by which ormeloxifene and other such SERMs exert tissue-specific estrogen agonist/antagonistic effect remains enigmatic. Molecular mechanism by which estrogen exerts its bone protective effect is also not clearly understood. Recent evidence suggests that estradiol-17 beta and raloxifene decrease IL-6 mRNA levels and suppress osteoclast development in primary murine bone cell cultures [14]. Estrogen deficiency also affects availability of various other multifunctional cytokines such as IL-1, IL-6, TGF beta, etc., and affecting cells in the microenvironment. TGF betas play important role in bone formation, induction and repair. These are localized at bone fracture sites during healing and can regulate cell proliferation as well as phenotypic gene expression in the fracture callus and initiate osteogenesis and chondrogenesis [15]. It has been reported that ovariectomy significantly reduces mRNA expression level of TGF beta-3 isoform in bone, which was well maintained when supplemented with estrogen or raloxifene [16]. TGF beta-3 has also been reported to inhibit osteoclast differentiation, suggesting its role in estrogen-mediated bone maintenance [17]. The purpose of this study was to understand bone specific effects of ormeloxifene using osteoclast differentiation and apoptosis in osteoclast cultures derived from mouse bone marrow and ovariectomy-induced bone resorption and TGF beta-3 expression in retired breeder rats.

2. Materials and methods

2.1. Animals

Inbred Balb/c mice (6–8 week old) and retired breeder female Sprague–Dawley rats (age: 12–14 months; parity: ±3; body weight: 250–300 g), referred as retired after 3–6 litters due to age-related increased incidence of reproductive failure/litter size, of the Institute's breeding colony maintained under standard conditions (22 ± 1 °C) with alternate 12-h light:12-h dark periods and free access to regular pellet diet (Lipton India Ltd., Bangalore, India) and tap water were used in this study.

2.2. Chemicals

Ormeloxifene (International Nonproprietary Name for Centchroman) was synthesized [18] at this institute. Raloxifene was a generous gift from M/s. Cadila Healthcare Ltd., Ahmedabad, India. All other chemicals including macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL) were purchased from Sigma–Aldrich, St. Louis, MO, USA. Enhanced chemiluminescence (ECL-Plus) kit was purchased from Amersham Biosciences, Amersham, UK, and TdT-FragEL kit (QIA33) and anti-TGF beta-3 antibody (raised in mouse) (GL-16) from Oncogene, Boston, MA, USA.

2.3. Experimental design

2.3.1. Antiresorptive activity in vivo

All protocols used in this study were approved by the Institute's Animal Ethical Committee. Retired breeder female rats from the Institute's breeding colony were randomized into 5 groups of 10 each and kept in separate steel cages. Animals of four groups were bilaterally ovariectomized under light ether anesthesia and treated orally with ormeloxifene (1.25 mg/kg), raloxifene (3 mg/kg), ethynylestradiol (0.5 mg/kg) or the vehicle (gum acacia in distilled water) once daily on days 1–30 post-ovariectomy (day 1: day of ovariectomy). Dose of ormeloxifene used was chosen based on its contraceptive dosing [10] and that of raloxifene from earlier reports [19]. Dose of ethynylestradiol, though high, was aimed to delineate its beneficial effects on bone rather than its uterus stimulatory effects [20]. One group of females was sham-operated and treated similarly with the vehicle.

2.3.2. Autopsy and collection of tissues

Twenty-four hours after the last treatment, the animals were autopsied by excessive ether inhalation and femur and tibia bones and uterus of each rat were dissected free of adhering tissues, fixed in 70% ethanol and stored at −20 °C until BMD measurements. After BMD analysis, the isolated femur and tibia bones were kept in desiccators containing fused $CaCl_2$ until further analysis. Lumbar vertebrae (nos. 2–4) were excised, thoroughly cleaned of adhering tissues and stored at −20 °C for TGF beta-3 expression studies. The uterus of each rat was weighed and fixed in 4% formaldehyde in phosphate buffer (pH 7.4) for histomorphometry.

2.3.3. Bone mineral density (BMD) measurement

BMD measurement of isolated femur and tibia bones was performed using DEXA Fan Beam Densitometer (Model: QDR-4500A, Hologic, USA) calibrated daily with hydroxyapatite anthropomorphic spine phantom (Hologic #3256) using manufacturer provided high-resolution software for small animals. Scans of isolated bones were performed using identical regions of interest (femur: global and neck; tibia: global and proximal region) at a scan speed of 1 mm/s or 4 lines/mm [10].

2.3.4. Drying and ashing of bones

Each dried bone kept in the desiccator was weighed and placed in a pre-weighed clean silica crucible, soaked with 500 μl of concentrated HCl and kept in a Muffle furnace set at a constant temperature of 500 °C for 3–4 h, allowed to cool to room temperature and then weighed to determine the weight of bone ash.