Effect of Coumestrol on Bone Metabolism in Organ Culture

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Coumestrol, a representative phytoestrogen, inhibited bone resorption-stimulating agent-induced bone resorption, whereas it did not inhibit basal bone resorption of cultured fetal rat limb bone. Coumestrol increased the calcium content of 9-d-old chick embryonic femurs in organ culture, indicating that this phytoestrogen stimulated bone-mineralizing activity. Therefore, coumestrol is a unique substance in that it inhibits bone resorption and, at the same time, stimulates bone mineralization. Coumestrol exhibited less estrogenic activity than 17β -estradiol as estimated by the increase in uterine weight of ovariectomized rats. As coumestrol has less estrogenic activity than 17β -estradiol, it may prove to be a more potent drug against bone diseases including osteoporosis.

Key words coumestrol; bone resorption; bone mineralization; phytoestrogen; 17β -estradiol

Deficiency of 17β -estradiol has long been recognized as a cause of post-menopausal osteoporosis, and estrogen replacement therapy is an effective treatment for the prevention of bone loss.^{1–3)} Many studies have shown that estrogen deficiency is associated with the accelerated resorption of bone.^{4,5)} However, the mechanism for the protective effect of estrogen from bone resorption is yet unclear. The discovery of estrogen receptors in osteoblasts^{6,7)} suggested that estrogen affects the osteoblast first to synthesize or secrete some substance to regulate the activity of osteoclasts. In fact, 17β -estradiol inhibited interleukin-6 (one of the bone resorption-stimulating substances) production from bone marrow-derived stromal cells⁸⁾ and stimulated the synthesis of cell/matrix-associated inhibitor⁹⁾ of bone resorption-stimulating activity¹⁰⁾ in osteosarcoma cells induced by various stimulators of bone resorption. However, these effects of estrogen were obtained with 17β -estradiol, the naturally occurring active estrogen in animals and few estrogens exist in plants. Coumestrol (Fig. 1) has been found in forage crops and exhibited estrogenic activity as shown by the increase in uterine weight of ovariectomized mice.^{11–13)} In this present study, to investigate whether phytoestrogen also has bone-metabolizing activity or not, I examined the effect of coumestrol, a representative phytoestrogen, on bone-resorbing and -mineralizing activity in cultures of fetal rat and embryonic chick limb bones.

MATERIALS AND METHODS

Chemicals Agents used and their sources were as follows: coumestrol, Eastman Kodak, Rochester, NY, U.S.A.; 17β -estradiol and bovine serum albumin (BSA), cell culture grade, Sigma Chemical Co., St. Louis, MO, U.S.A.; synthetic human parathyroid hormone (fragment 1–34) (PTH), Peptide Institute, Inc., Minoh; prostaglandin E_2 (PGE_2), Funakoshi, Tokyo; $1\alpha,25$ -dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$], Calcium C-test WakoTM, and dimethyl sulfoxide (DMSO), Wako Pure Chemical Co., Osaka; Dulbecco's phosphate-buffered saline (Ca^{2+} , Mg^{2+} free) [PBS(–)] and BGJb-HW2 (phenol red free), Nissui Pharmaceutical Co., Tokyo; and $^{45}CaCl_2$, Du Pont/NEN Research Products, Boston, MA, U.S.A.

BGJb-HW2 medium containing 0.01% BSA¹⁴⁾ was sterilized by passage through a 0.22- μ m filter.

Determination of Bone-Resorbing Activity Bone-resorbing activity was determined by the method of Raisz.¹⁵⁾ Briefly, ^{45}Ca (1.85 MBq/rat) was subcutaneously injected into pregnant Sprague-Dawley rats (Japan SLC, Inc., Hamamatsu) on day 18 of pregnancy. They were sacrificed by decapitation under ether anesthesia two days after the ^{45}Ca -injection. The isotope-labeled limb bones were isolated from fetuses aseptically, and the adhering soft connective tissue was completely removed while the bones were being rotated on dry paper. The shaft, a calcified portion of the ^{45}Ca -labeled limb bone, was chase-cultured in BGJb-HW2 medium. After incubation for a day to remove the contaminating ^{45}Ca , the test compound was added to fresh chase-medium, and incubation was continued for 5 d with a change of medium every day. The same part of shaft from each fetal sibling was used for a given experiment. Before the medium was exchanged, an aliquot of the medium was withdrawn for liquid scintillation counting to measure the radioactivity of released ^{45}Ca . After the incubation, ^{45}Ca remaining in the bones was liberated by hydrolysis in 6N HCl at 110°C and the radioactivity was measured in a liquid scintillation counter.

Bone-resorbing activity was expressed as a percentage of released ^{45}Ca : ^{45}Ca release (%) = $100 \times (^{45}Ca \text{ released into the medium}) / [(^{45}Ca \text{ released into the medium}) + (^{45}Ca \text{ remaining in the bone})]$.

A concentrated stock solution of the test compound was prepared in DMSO and a precise amount of this solution was added to the medium to obtain the desired concentration. The final concentration of DMSO was restricted to 0.1%. The same amount of DMSO was added

Fig. 1. Chemical Structures of Coumestrol and 17β -Estradiol

to the control cultures.

Determination of Bone-Mineralizing Activity Fertilized eggs from White Leghorns (Komatsu Poultry Farm) were incubated for 9 d at 37.6°C. The femurs isolated from 9-d-old chick embryos as described above were cultured by the roller tube method^{14,16} for 6 d in the presence or absence of the test compound, while changing the medium every other day. Next the femurs were rinsed with PBS(–) and then hydrolyzed in 1 N HCl at 110°C for 24 h, after which the calcium content in the hydrolysate was determined using a commercial kit, Calcium C-test Wako™. For control of individual variations, pair-mate cultures were employed, with one bone taken for the control and the contralateral bone from the same embryo used as the test sample.

Ovariectomized Rats Female Wistar rats (Japan SLC Inc.) 8 weeks old were housed in a room with a 12 h/12 h light–dark cycle at a constant temperature (23 ± 1°C). They were ovariectomized or sham-operated under ether anesthesia and used for experiments 7 d later. The rats were randomized into groups and the test compound dissolved or suspended in ethanol, or vehicle (ethanol) alone, was subcutaneously administered for 3 d and then,

on the fourth day, they were sacrificed under ether anesthesia. Their uteri were dissected free of adhering fat, and then weighed.

Statistical Analyses Data were expressed as means ± S.E.M. Statistical significance was determined by Student's *t*-test for the comparison between two groups or by one-way analysis of variance followed by Dunnett's test for multiple group comparisons.

RESULTS

Effects of Coumestrol and 17β-Estradiol on Bone Resorption in Fetal Rat Bone The maximal effective dose obtained from our preliminary experiments was used for each bone resorption-stimulating agent. Coumestrol, whose structure is shown in Fig. 1, inhibited PTH (1.8 × 10^{−8} M)-, 1α,25(OH)₂D₃ (10^{−9} M)- and PGE₂ (10^{−6} M)-stimulated bone resorption in a concentration-dependent manner (Fig. 2B–D). However, it did not influence basal resorption up to 10^{−4} M (Fig. 2A).

17β-Estradiol inhibited PTH- and PGE₂-induced bone resorption of fetal rat bone in a concentration-dependent

Fig. 2. Effects of Coumestrol on Basal, PTH-, 1α,25(OH)₂D₃- and PGE₂-Induced Bone Resorption in Fetal Rat Bone

Humeral (A and B), radial (C) and femoral (D) shafts obtained from fetal rats labeled with ⁴⁵Ca given by subcutaneous injection of the isotope into pregnant rats were chase-cultured for 5 d with various concentrations of coumestrol (vertically striped column) or without coumestrol (control, vehicle, closed column) in the absence (A) or presence of PTH (B, 1.8 × 10^{−8} M), 1α,25(OH)₂D₃ (C, 10^{−9} M) or PGE₂ (D, 10^{−6} M). Bone-resorbing activity is expressed as % release of ⁴⁵Ca. Open columns in B, C and D represent basal bone resorption. Values represent means ± S.E.M. (n = 5). *, *p* < 0.05 and **, *p* < 0.01 vs. the control value.

Fig. 3. Effects of 17β-Estradiol on Basal, PTH-, 1α,25(OH)₂D₃- and PGE₂-Induced Bone Resorption in Fetal Rat Bone

Humeral (A), femoral (B and D) and ulnar (C) shafts obtained from fetal rats labeled with ⁴⁵Ca given by subcutaneous injection of the isotope into pregnant rats were chase-cultured for 5 d with various concentrations of 17β-estradiol (horizontally striped column) or without 17β-estradiol (control, vehicle, closed column) in the absence (A) or presence of PTH (B, 1.8 × 10^{−8} M), 1α,25(OH)₂D₃ (C, 10^{−9} M) or PGE₂ (D, 10^{−6} M). Bone-resorbing activity is expressed as % release of ⁴⁵Ca. Open columns in B, C and D represent basal bone resorption. Values represent means ± S.E.M. (n = 5). *, *p* < 0.05 and **, *p* < 0.01 vs. the control value.

Fig. 4. Effects of Coumestrol and 17 β -Estradiol on the Calcium Content of Cultured Embryonic Chick Femurs

Femurs from 9-d chick embryos were cultured for 6 d in the absence (vehicle, paired control, open column) or presence of various concentrations of coumestrol (A, vertically striped column) or 17 β -estradiol (B, horizontally striped column), and then their calcium contents were determined. Values are means $\pm$ S.E.M. ($n=5$). *, $p < 0.05$ vs. the paired control value.

Fig. 5. Effects of Coumestrol and 17 β -Estradiol on Uterine Weight of Ovariectomized Rats

Female Wistar rats 8 weeks old were ovariectomized or sham-operated under ether anesthesia. The test compounds dissolved or suspended in ethanol, or vehicle (ethanol) alone were subcutaneously administered for 3 d and then on the fourth day, the animals were sacrificed. Their uteri were dissected free of adhering fat and weighed. Dotted area, control (ovariectomized, vehicle); checkered area, sham-operated (vehicle); $\circ$, 17 β -estradiol; $\bullet$, coumestrol. Values are means $\pm$ S.E.M. ($n=5-6$). **, $p < 0.01$ vs. the control value.

manner (Fig. 3B and D). It also inhibited 1 α ,25(OH) $_2$ D $_3$ -induced bone resorption, but not significantly (Fig. 3C). It did not influence basal resorption up to 10^{-4} M (Fig. 3A).

Effects of Coumestrol and 17 β -Estradiol on Mineral Deposition in 9-d Chick Embryonic Femurs Coumestrol, at a concentration of 10^{-4} M, increased the calcium content of 9-d chick embryonic femurs cultured for 6 d in chemically defined BGJb-HW2 medium (Fig. 4A). However, 17 β -estradiol (10^{-6} and 10^{-5} M) had no effect on the calcium content (Fig. 4B).

Effects of Coumestrol and 17 β -Estradiol on the Uterine Weight of Ovariectomized Rats Coumestrol and 17 β -estradiol significantly increased the uterine weight of ovariectomized rats (Fig. 5). The uterotrophic activity of 17 β -estradiol was about 10000 times more potent than that of coumestrol.

DISCUSSION

Coumestrol, a representative phytoestrogen, inhibited PTH-, 1 α ,25(OH) $_2$ D $_3$ -, and PGE $_2$ -induced bone resorption without affecting basal bone resorption in fetal rat bone, indicating that phytoestrogens also affect bone metabolism. Since coumestrol did not affect basal bone resorption even at a concentration of 10^{-4} M, its inhibitory effect on bone resorption induced by the various agents was not caused by any toxic action. The culture of 9-d-old embryonic chick femurs is a good tool for determining bone mineralizing activity¹⁶⁾ because bone-mineralizing activity largely exceeds bone-resorbing activity in the early developmental stage. In addition to its inhibitory effect on bone resorption, coumestrol also stimulated the bone mineralization as estimated by the increase in the calcium content of 9-d-old embryonic chick femurs cultured *in vitro*. These results show that coumestrol is a unique substance that inhibits bone resorption and, at the same time, stimulates bone mineralization, because the effective concentrations of coumestrol for inhibiting bone resorption and stimulating bone mineralization were about the same.

17 β -Estradiol also inhibited the bone resorption-stimulating agent-induced resorption of fetal rat bone at concentrations of 10^{-6} M and above but had no effect on bone mineralization in organ cultures of chick embryonic bone. The effective doses of 17 β -estradiol and coumestrol required for the inhibition of stimulus-induced bone resorption of fetal rat bone were almost the same. However, the estrogenic activity of coumestrol, as estimated by the increase in uterine weight of ovariectomized rats, was 10000 times lower than that of 17 β -estradiol. Therefore, the degree of the inhibitory effect of coumestrol and 17 β -estradiol on bone resorption does not correlate with the estrogenic activity.

The structure of coumestrol is slightly different from that of 17 β -estradiol. It is possible that they share a common functional structure for inhibitory action on bone resorption which does not mediate estrogenic action. It is important to understand such functional effects because a decreased estrogenic activity is desirable for the treatment of osteoporosis to avoid any severe side-effects.^{17,18)} Such a series of studies may provide new substances that inhibit bone resorption but are free from any estrogenic activity.

Considering its weaker estrogenic activity and more potent mineralizing activity, coumestrol may prove to be a more useful therapeutic agent than 17 β -estradiol.

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REFERENCES

- 1) Lindsay R., Hart D. M., Aitken J. M., MacDonald E. B., Anderson J. B., Clarke A. C., *Lancet*, **1**, 1038—1041 (1976).
- 2) Lindsay R., *Am. J. Obstet. Gynecol.*, **156**, 1347—1351 (1989).
- 3) Ettinger B., *Obstet. Gynecol.*, **72**, 12S (1988).
- 4) Heaney R. P., Recker R. R., Saville P. D., *J. Lab. Clin. Med.*, **92**, 964—970 (1978).
- 5) Selby P. L., Peacock M., Barkworth S. A., Brown W. B., Taylor G. A., *Clin. Sci.*, **69**, 265—271 (1985).
- 6) Komm B. S., Terpening C. M., Benz D. J., Graeme K. A., Gallegos A., Korc M., Greene G. L., O'Malley B. W., Haussler M. R., *Science*, **241**, 81—83 (1988).
- 7) Eriksen E. F., Colvard D. S., Berg N. J., Graham M. L., Mann K. G., Spelsberg T. C., Riggs B. L., *Science*, **241**, 84—86 (1988).
- 8) Girasole G., Jilka R. L., Passeri G., Boswell S., Boder G., Williams D. C., Manolagas S. C., *J. Clin. Invest.*, **89**, 883—891 (1992).
- 9) Ishii T., Saito T., Morimoto K., Takeuchi Y., Asano S., Kumegawa M., Ogata E., Matsumoto T., *Biochem. Biophys. Res. Commun.*, **191**, 495—502 (1993).
- 10) Fuller K., Gallagher A. C., Chambers T. J., *Biochem. Biophys. Res. Commun.*, **181**, 67—73 (1991).
- 11) Bickoff E. M., Booth A. N., Lyman R. L., Livingston A. L., Thompson C. R., DeEds F., *Science*, **126**, 969—970 (1957).
- 12) Bickoff E. M., Booth A. N., Lyman R. L., Livingston A. L., Thompson C. R., Kohler G. O., *J. Agric. Food Chem.*, **6**, 536—539 (1958).
- 13) Bickoff E. M., Livingston A. L., Hendrickson A. P., Booth A. N., *J. Agric. Food Chem.*, **10**, 410—412 (1962).
- 14) Tsutsumi N., Kawashima K., Arai N., Nagata H., Kojima M., Ujiiie A., Endo H., *Bone Miner.*, **24**, 201—209 (1994).
- 15) Raisz L. G., *J. Clin. Invest.*, **44**, 103—116 (1965).
- 16) Endo H., Kiyoki M., Kawashima K., Naruchi T., Hashimoto Y., *Nature* (London), **286**, 262—264 (1980).
- 17) Peck W. A., Riggs B. L., Bell N. H., Wallace R. B., Johnston C. C., Jr., Gordon S. L., Shulman L. E., *Am. J. Med.*, **84**, 275—282 (1988).
- 18) Vessey M. P., *J. Roy. Soc. Med.*, **77**, 542—549 (1984).