

# Effects of estriol on cell viability and 1,25-dihydroxyvitamin D<sub>3</sub> receptor mRNA expression in cultured human osteoblast-like cells

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## ABSTRACT

It is clinically evident that administration of estriol (E<sub>3</sub>) increases the bone mass density of the lumbar vertebrae in postmenopausal women, and that combined treatment with estrogen and 1,25-dihydroxyvitamin D<sub>3</sub> (VD<sub>3</sub>) increases femoral neck bone mass density compared with treatment with estrogen alone in postmenopausal osteoporotic women. However, the molecular mechanism whereby treatment with E<sub>3</sub> affects osteoblast cell function is still unknown. This study was conducted first to examine the comparative effects of E<sub>3</sub> and VD<sub>3</sub> on the cell viability of cultured human osteoblast-like cells (HOS) and second to determine whether E<sub>3</sub> affects VD<sub>3</sub> receptor mRNA expression in HOS. The cell viability and VD<sub>3</sub> receptor mRNA expression of cultured HOS were assessed by MTT assay and semi-quantitative reverse transcriptase-polymerase chain reaction with Southern blot analysis, respectively. The treatment with E<sub>3</sub> increased the cell viability of cultured HOS compared with untreated control cultures. The increase in cell viability caused by the treatment with E<sub>3</sub> was further augmented by the combined treatment with VD<sub>3</sub>. The addition of either E<sub>3</sub> ( $3.52 \times 10^{-8}$  mol/l) or E<sub>3</sub> ( $3.52 \times 10^{-7}$  mol/l) to cultured HOS for 24 h resulted in a fourfold and eightfold increase, respectively, in VD<sub>3</sub>

receptor mRNA expression in HOS, compared with that in untreated control cultures. These results suggest that E<sub>3</sub> may up-regulate the cell viability of osteoblast cells, and that the concomitant treatment with E<sub>3</sub> and VD<sub>3</sub> further augments the cell viability being associated with an E<sub>3</sub>-induced increase in VD<sub>3</sub> receptor mRNA expression in those cells.

## INTRODUCTION

The loss of bone mass that occurs in postmenopausal women can be attributed to lack of estrogen. This is confirmed by the clinical observation that postmenopausal osteoporosis can be successfully prevented by estrogen replacement therapy. 17 $\beta$ -Estradiol (E<sub>2</sub>) has an important role in the prevention of postmenopausal osteoporosis<sup>1,2</sup>. E<sub>2</sub> inhibits bone resorption and stimulates bone formation in the tibia and femur in ovariectomized rats by promoting calcium absorption in the intestine<sup>3-5</sup>. E<sub>2</sub> also exerts an inhibitory effect on bone resorption via the estrogen receptor detected in rat osteoblast-like cells and normal human osteoblasts<sup>6-9</sup>. The suppression of bone resorption is attributed to inhibition of

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bone-resorbing cytokine action on bone-marrow stromal cells and osteoblasts<sup>9</sup>. The E<sub>2</sub>-induced increase in cell proliferation of osteoblasts might be mediated by insulin-like growth factor I (IGF-I), induced by E<sub>2</sub><sup>6,10</sup>.

Recently, Tuppurainen *et al.*<sup>11</sup> showed that combined treatment with E<sub>2</sub> and 1,25-dihydroxyvitamin D<sub>3</sub> (VD<sub>3</sub>) further increases femoral neck bone mass density in osteoporotic women compared with treatment with E<sub>2</sub> alone. Several reports have shown that E<sub>2</sub> increases the number of VD<sub>3</sub> receptors in the rat uterus, liver and kidney, and in human breast cancer cells<sup>12–18</sup>. It has also been reported that E<sub>2</sub> increases the number of VD<sub>3</sub> receptors in the ROS 17/2.8 cell line and the VD<sub>3</sub> receptor mRNA levels in human osteosarcoma cells (MG-63)<sup>19,20</sup>.

Estriol (E<sub>3</sub>) is an estrogen with fewer adverse effects on endometrial proliferation, and it requires no combined use of progestin when used for hormone replacement therapy. For hormone replacement therapy, E<sub>3</sub> is an alternative agent to E<sub>2</sub><sup>21</sup>. However, the molecular mechanism whereby E<sub>3</sub> affects osteoblast cell function is still unknown. The present study was conducted in order to examine the effects of E<sub>3</sub> on the cell viability of cultured human osteoblast-like cells (HOS), and to determine whether E<sub>3</sub> affects the VD<sub>3</sub> receptor mRNA expression in HOS.

## MATERIALS AND METHODS

### Materials

VD<sub>3</sub> was purchased from Chugai Pharmaceutical Co., Ltd (Tokyo, Japan). E<sub>2</sub> and E<sub>3</sub> were obtained from Sigma (St Louis, MO, USA) and Mochida Pharmaceutical Co., Ltd (Tokyo, Japan), respectively. The sources of other materials used were as follows. Fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Life Technologies, Inc. (Grand Island, NY, USA) and Sigma (St Louis, MO, USA), respectively. Penicillin and streptomycin were obtained from Invitrogen Corp. (Carlsbad, CA, USA). HOS was purchased from Dainippon Pharmaceutical Co., Ltd (Tokyo, Japan).

### Cell culture

HOS was subcultured in 1.0 × 10<sup>5</sup>-mm<sup>3</sup> plastic culture flasks at 37°C in a 5% CO<sub>2</sub> humidified atmosphere in phenol-red-free DMEM supplemented with

10% dextran-coated charcoal-stripped FBS, penicillin (100 IU/ml) and streptomycin (100 µg/ml). At approximately 60–70% confluence, the cultured medium was stepped down to serum-free conditions by incubating the cells in serum-free DMEM in the absence or presence of E<sub>2</sub> (3.67 × 10<sup>-8</sup> mol/l), E<sub>3</sub> (3.52 × 10<sup>-8</sup> or 3.52 × 10<sup>-7</sup> mol/l) and VD<sub>3</sub> (10<sup>-9</sup> mol/l) for indicated periods of time (24 h to 72 h).

### MTT assay for cell viability

HOS cell number and viability were evaluated by means of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium biomet) assay using the Mosmann method<sup>22</sup>. This assay was based on the cleavage of the tetrazolium salt MTT to a blue formazan product by mitochondrial dehydrogenase in viable cells. Briefly, after being treated in the absence or presence of E<sub>2</sub> (3.67 × 10<sup>-8</sup> mol/l), E<sub>3</sub> (3.52 × 10<sup>-8</sup> or 3.52 × 10<sup>-7</sup> mol/l) and VD<sub>3</sub> (10<sup>-9</sup> mol/l) in serum-free DMEM for 24–72 h in 96-well tissue-culture plates, 10 µl of MTT (Sigma, St Louis, MO, USA) solution was added to each well, and cultured cells were incubated at 37°C for another 4 h. Then 100 µl of isopropanol/HCl solution was added to each well and mixed thoroughly with an EM-36N microtube mixer (Taitec, Tokyo, Japan). The absorbance was measured by an MTP-120 ELISA plate reader (Corona Electric Co., Osaka, Japan) with a test wavelength of 570 nm and a reference wavelength of 630 nm. These experiments with HOS were repeated at least three times with similar results.

### Semi-quantitative RT-PCR with Southern blot analysis

The cells were seeded on to 1.0 × 10<sup>5</sup>-mm<sup>3</sup> culture plates and cultured into subconfluency (approximately 8 × 10<sup>4</sup> cells/cm<sup>2</sup>). Thereafter, the cells were stepped down to serum-free conditions by being incubated in serum-free DMEM in the absence or presence of E<sub>2</sub> (3.67 × 10<sup>-8</sup> mol/l), or E<sub>3</sub> (3.52 × 10<sup>-8</sup> or 3.52 × 10<sup>-7</sup> mol/l) for 24–48 h. Total RNA was isolated from HOS cultured by the guanidinium thiocyanate and phenol/chloroform method. First-strand complementary DNA (cDNA) was synthesized from 4 µg of total RNA using a cDNA synthesis kit (QIAGEN GmbH, Hilden, Germany).



The polymerase chain reaction (PCR) was performed using 1  $\mu$ l of cDNA as template, 6.25 pmol/l of each primer, 2.5 mmol/l dNTPs, 0.125 U Taq DNA polymerase (Roche-Diagnostics GmbH, Mannheim, Germany), 1X reaction buffer containing 10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l of KCl, 1.5 mmol/l of  $MgCl_2$  and 0.01% gelatin in a reaction volume of 25  $\mu$ l. The primers that were used to amplify the  $VD_3$  receptor cDNA were 5'-CAACAAAGACTACAAGTACCGCGTCAGTGA-3' (sense) and 5'-GTGAGGAGGGCTGCTGAGTAG-3' (antisense)<sup>23</sup>. The sense primer annealed to 982–1011, while antisense corresponded to bases 1471–1491 of the  $VD_3$  receptor's cDNA. The length of the expected PCR product was 510 base pairs (bp). PCR amplification was performed using a Gene Amp PCR System 9600-R (Perkin Elmer Corp., Norwalk, CT, USA). The amplification procedure included an initial denaturation step at 94°C for 5 min and 30 cycles, as follows: denaturation step at 94°C for 30 s, annealing step at 55°C for 30 s and extension step at 72°C for 30 s. The sequence of the primer used to amplify the housekeeping  $\beta$ -actin gene was 5'-CTTCTACAATGAGCTGCGTG-3' (sense) and 5'-TCATGAGGTAGTCAGTCAGG-3' (antisense). The sense primer annealed to bases 308–327, while the antisense primer corresponded to bases 593–612 of the  $\beta$ -actin gene. The length of the expected PCR product was 305 bp. The amplification procedure included an initial denaturation step at 94°C for 5 min and 15 cycles, as follows: denaturation step at 94°C for 30 s, annealing step at 55°C for 30 s, extension step at 72°C for 30 s.

To allow the quantification of the  $VD_3$  receptor mRNA expression, Southern blot analysis was used. The amplified products were electrophoresed on 3% agarose gel, and after denaturation with alkaline solution they were transferred to a nylon membrane filter for Southern blotting. After transfer, the DNA was fixed by UV irradiation, and hybridization was performed using the 5'-<sup>32</sup>P-end-labeled oligonucleotide probe. The conditions for hybridization were prehybridization at 65°C for 2 h and hybridization at 65°C for 18 h. The probe specific for  $\beta$ -actin was prepared with a full-length cDNA of  $\beta$ -actin labeled with <sup>32</sup>P. The washing conditions were as follows: saline sodium citrate (SSC = 300 mmol/l NaCl and 30 mmol/l trisodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS) at room temperature for 20 min, performed twice, and SSC (30 mmol/l

NaCl and 3 mmol/l trisodium citrate, pH 7.0) and 0.1% SDS at 60°C for 5 min.

After washing, the blot was exposed to X-ray film for 3 h. The film was scanned on ScanJet 3C/ADF (Hewlett Packard, Miami, FL, USA), and the strength of the signal was measured by NIH Image version 1.58. The amount of  $VD_3$  mRNA was expressed relative to the amounts of  $\beta$ -actin mRNA present in each specimen.

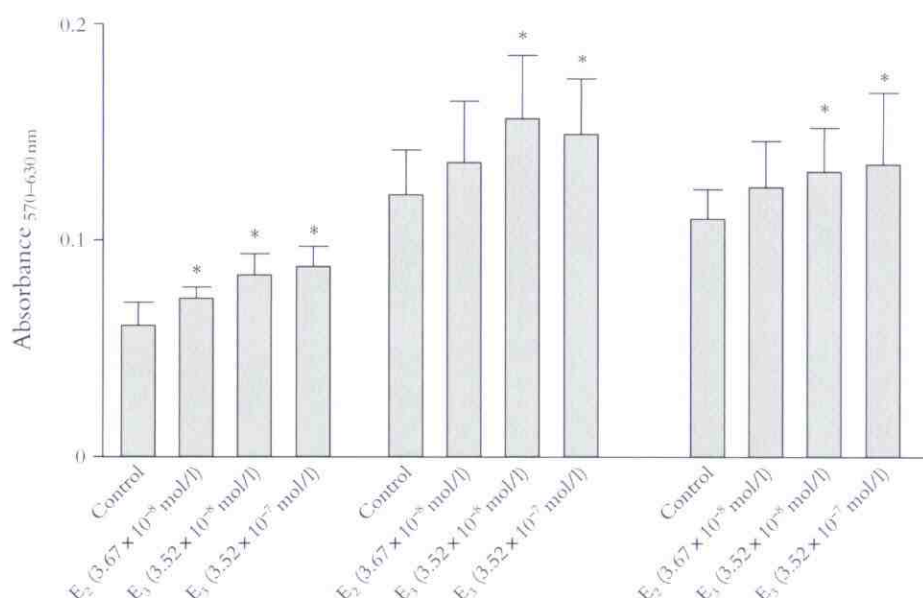
## Statistical analysis

The data collected are shown as mean values  $\pm$  SD from at least three independent experiments. Statistical significance was evaluated using two-way analysis of variance (ANOVA). Differences with a *p*-value of  $< 0.05$  were considered to be statistically significant.

## RESULTS

### Effects of $E_2$ , $E_3$ and $VD_3$ on cell viability

The treatment with  $E_2$  ( $3.67 \times 10^{-8}$  mol/l) resulted in a significant increase in the cell viability of cultured HOS compared with untreated control cultures at 24 h exposure, but did not do so at 48 h or 72 h exposure. The treatment with either  $E_3$  ( $3.52 \times 10^{-8}$  mol/l) or  $E_3$  ( $3.52 \times 10^{-7}$  mol/l) also significantly increased the cell viability of cultured HOS compared with untreated control cultures throughout 24–72 h exposure (Figure 1). The treatment with  $VD_3$  ( $10^{-9}$  mol/l) exerted similar stimulatory effects on cell viability to those observed with  $E_3$  ( $3.52 \times 10^{-8}$  mol/l). The combined treatment with  $E_2$  ( $3.67 \times 10^{-8}$  mol/l) and  $VD_3$  ( $10^{-9}$  mol/l) further increased the cell viability of cultured HOS compared with the treatment with  $E_2$  ( $3.67 \times 10^{-8}$  mol/l) alone or  $VD_3$  ( $10^{-9}$  mol/l) alone throughout 24–72 h exposure. The increase in cell viability of cultured HOS caused by the treatment with  $E_3$  ( $3.52 \times 10^{-8}$  mol/l) alone was also further augmented in response to the concomitant treatment with  $VD_3$  ( $10^{-9}$  mol/l) at 72 h exposure. Compared with the combined treatment with  $E_3$  ( $3.52 \times 10^{-8}$  mol/l) and  $VD_3$  ( $10^{-9}$  mol/l), the combined treatment with  $E_2$  ( $3.67 \times 10^{-8}$  mol/l) and  $VD_3$  ( $10^{-9}$  mol/l) had a higher cell viability of cultured HOS at 24 h exposure, but did not do so at 48 h and 72 h exposure (Table 1).



**Figure 1** Effects of 17 $\beta$ -estradiol (E<sub>2</sub>) and estriol (E<sub>3</sub>) on the cell viability of cultured human osteoblast-like cells (HOS) assessed by MTT assay. Treatment with E<sub>2</sub> ( $3.67 \times 10^{-8}$  mol/l) or E<sub>3</sub> ( $3.52 \times 10^{-8}$  or  $3.52 \times 10^{-7}$  mol/l) alone was carried out for 24–72 h in serum-free conditions. Values are presented as the mean values  $\pm$  SD from three independent experiments. \* $p < 0.05$  vs. control cultures

**Table 1** Effects of 17 $\beta$ -estradiol (E<sub>2</sub>), estriol (E<sub>3</sub>) and 1,25-dihydroxyvitamin D<sub>3</sub> (VD<sub>3</sub>) on cell viability of cultured human osteoblast-like cells (HOS) assessed by MTT assay

	Exposure time		
	24 h	48 h	72 h
Control	0.059 $\pm$ 0.011	0.120 $\pm$ 0.019	0.108 $\pm$ 0.011
VD <sub>3</sub> ( $10^{-9}$ mol/l)	0.088 $\pm$ 0.010*	0.144 $\pm$ 0.017*	0.129 $\pm$ 0.018*
E <sub>2</sub> ( $3.52 \times 10^{-8}$ mol/l)	0.072 $\pm$ 0.008*	0.134 $\pm$ 0.025	0.123 $\pm$ 0.019
E <sub>3</sub> ( $3.67 \times 10^{-8}$ mol/l)	0.084 $\pm$ 0.010*	0.155 $\pm$ 0.015*	0.128 $\pm$ 0.014*
E <sub>2</sub> ( $3.52 \times 10^{-8}$ mol/l) + VD <sub>3</sub> ( $10^{-9}$ mol/l)	0.121 $\pm$ 0.030 <sup>†††</sup>	0.179 $\pm$ 0.019 <sup>††</sup>	0.147 $\pm$ 0.014 <sup>††</sup>
E <sub>3</sub> ( $3.67 \times 10^{-8}$ mol/l) + VD <sub>3</sub> ( $10^{-9}$ mol/l)	0.089 $\pm$ 0.011	0.160 $\pm$ 0.021	0.147 $\pm$ 0.018 <sup>†**</sup>

Values are presented as the mean values  $\pm$  SD from three independent experiments. \* $p < 0.05$  vs. control; <sup>†</sup> $p < 0.05$  vs. VD<sub>3</sub> alone; <sup>††</sup> $p < 0.05$  vs. E<sub>2</sub> alone; <sup>\*\*</sup> $p < 0.05$  vs. E<sub>3</sub> alone; <sup>†††</sup> $p < 0.05$  vs. combined treatment with E<sub>3</sub> and VD<sub>3</sub>

### Effect of E<sub>2</sub> and E<sub>3</sub> on VD<sub>3</sub> receptor mRNA expression

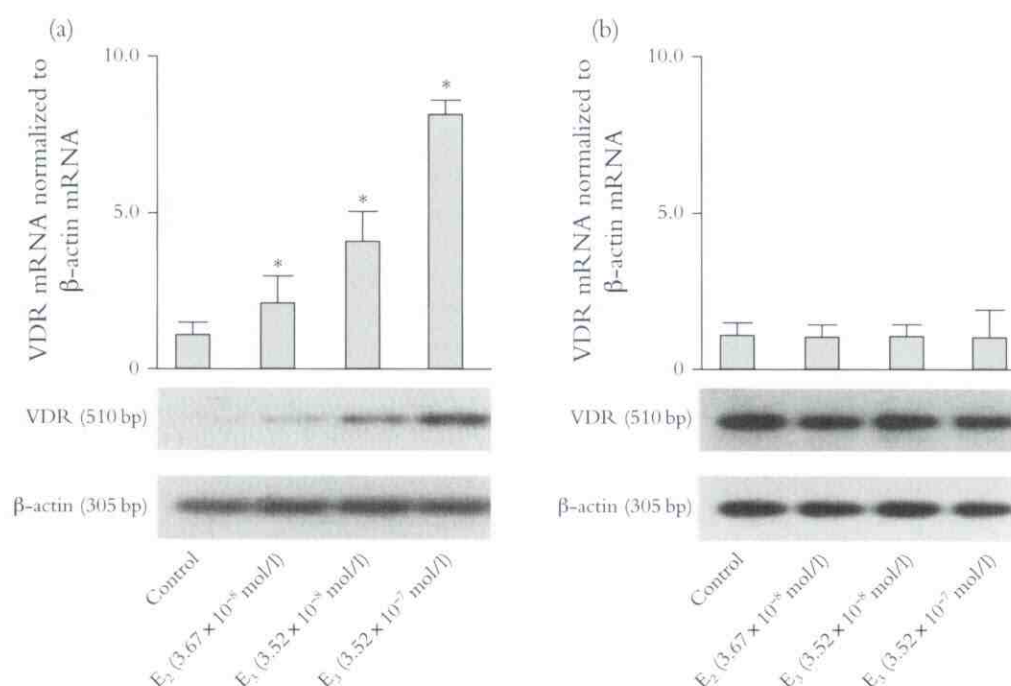
Densitometric quantification of VD<sub>3</sub> receptor mRNA with a molecular basis of 510 bp produced by semi-quantitative reverse transcriptase (RT)-PCR with Southern blot analysis revealed that the treatment with either E<sub>2</sub> ( $3.67 \times 10^{-8}$  mol/l) alone or E<sub>3</sub> ( $3.52 \times 10^{-8}$  or  $3.52 \times 10^{-7}$  mol/l) alone resulted in a twofold, fourfold and eightfold increase, respectively, in VD<sub>3</sub> receptor mRNA expression in cultured HOS at 24 h exposure compared with untreated control

cultures (Figure 2a). By contrast, at 48 h exposure, no such increases in VD<sub>3</sub> receptor mRNA expression in cultured HOS were noted with the treatment with either E<sub>2</sub> alone or E<sub>3</sub> alone (Figure 2b).

### DISCUSSION

This study has demonstrated that the treatment with E<sub>3</sub> increases the cell viability of cultured HOS compared with untreated control cultures, that combined treatment with E<sub>3</sub> and VD<sub>3</sub> increases the





**Figure 2** Effects of 17- $\beta$ -estradiol (E<sub>2</sub>) or estriol (E<sub>3</sub>) on VD<sub>3</sub> receptor (VDR) mRNA expression in cultured human osteoblast-like cells (HOS) analyzed by semi-quantitative RT-PCR and Southern blot. HOS were exposed to either E<sub>2</sub> ( $3.67 \times 10^{-8}$  mol/l) or E<sub>3</sub> ( $3.52 \times 10^{-8}$  or  $3.52 \times 10^{-7}$  mol/l) for (a) 24 h and (b) 48 h. The amount of mRNA was expressed relative to the abundance of  $\beta$ -actin mRNA. Data are presented as the fold increase over the control value and as the mean values  $\pm$  SD from three independent experiments. \* $p < 0.05$  vs. control cultures

cell viability relative to the treatment with E<sub>3</sub> alone, and that treatment with E<sub>3</sub> substantially increases VD<sub>3</sub> receptor mRNA expression in those cells. These findings suggest that E<sub>3</sub> exerts direct effects on osteoblast function, and that an interaction between E<sub>3</sub> and VD<sub>3</sub> receptors occurs in cultured HOS.

Nishibe *et al.*<sup>21</sup> showed that the administration of E<sub>3</sub> increases bone mass density of the lumbar vertebrae in postmenopausal women. E<sub>3</sub> has been considered to be an estrogen with a much weaker stimulatory effect on endometrial proliferation. Accordingly, E<sub>3</sub> therapy is associated with less frequent genital bleeding and requires no concomitant use of progestin. Nevertheless, Weiderpass *et al.*<sup>24</sup> have recently reported that oral use of E<sub>3</sub> 1–2 mg daily increases the relative risk of endometrial cancer and endometrial hyperplasia. Thus it is necessary to monitor the endometrium during such treatment with E<sub>3</sub>. The latter is also referred to as a terminal metabolite or as an impending estrogen, and is known to play a role in the metabolic responses in the target tissues that express the estrogen receptor<sup>25</sup>. E<sub>3</sub> has a sevenfold and a fivefold lower affinity relative to E<sub>2</sub> for the estrogen receptor  $\alpha$  and the estrogen receptor

$\beta$ , respectively<sup>26</sup>. The relative association constant of E<sub>3</sub> with the estrogen receptor has been reported to be 12.5% relative to that of E<sub>2</sub>, and the potency of E<sub>3</sub> in inducing positive co-operativity in the estrogen receptor is 50% that of E<sub>2</sub><sup>27</sup>. Onoe *et al.*<sup>28</sup> reported that higher levels of estrogen receptor  $\beta$  mRNA compared with estrogen receptor  $\alpha$  mRNA are expressed in rat calvaria cells and ROS 17/2.8. E<sub>3</sub> has a higher affinity for estrogen receptor  $\beta$  than for estrogen receptor  $\alpha$ <sup>26</sup>. Interestingly, in the present study the treatment with E<sub>3</sub> increased the cell viability of cultured HOS in a similar manner to the treatment with E<sub>2</sub>. Furthermore, it is worth noting that E<sub>3</sub> treatment produced a greater increase in VD<sub>3</sub> receptor mRNA expression in cultured HOS compared with E<sub>2</sub> treatment. We have also noted that E<sub>3</sub> treatment augments the proliferative activity of cultured HOS assessed by bromodeoxyuridine uptake as much as E<sub>2</sub> treatment (unpublished data). It is therefore likely that E<sub>3</sub> may be an effective alternative to E<sub>2</sub> for preventing loss of bone mineral density in postmenopausal women.

It has become evident that not only VD<sub>3</sub> but also E<sub>2</sub> regulates the number of VD<sub>3</sub> receptors through its

own specific receptor<sup>29-31</sup>. Several investigators have reported that E<sub>2</sub> increases the amount of both VD<sub>3</sub> receptor mRNA and protein in human and rat bone cells. Liel *et al.*<sup>19</sup> showed that E<sub>2</sub> treatment results in a twofold increase in the number of VD<sub>3</sub> receptors in rat osteosarcoma-derived osteoblast-like cells, ROS 17/2.8. Mahonen *et al.*<sup>20</sup> reported that E<sub>2</sub> stimulates VD<sub>3</sub> receptor mRNA expression in human osteosarcoma cells. In their studies, the addition of E<sub>2</sub> at a concentration of 10<sup>-8</sup> mol/l increases VD<sub>3</sub> receptor expression in various cells. The present study has demonstrated that treatment with either E<sub>2</sub> or E<sub>3</sub> increases VD<sub>3</sub> receptor mRNA expression in cultured HOS compared with that in untreated control cultures. It is likely that the effects of E<sub>3</sub> on bone may be mediated at least in part through its action on VD<sub>3</sub> receptor mRNA expression, followed by an increase in the sensitivity of osteoblasts to VD<sub>3</sub>. Understanding the mechanism of E<sub>3</sub> action on the bone should encourage much wider investigation of its potential clinical use in populations of different ethnic origin, although most of the studies on E<sub>3</sub> action were carried out in Japanese women.

Steroid and nuclear receptor coactivators have been implicated in the regulation of nuclear receptor function by enhancing ligand-dependent transcriptional activation of target gene expression<sup>32</sup>. A number of coactivator molecules of the steroid receptor coactivator/nuclear receptor coactivator family interact with activation functions within nuclear receptors through a conserved region containing helical domains of a core LXXLL sequence, and thus participate in transcriptional regulation<sup>33</sup>. A recent study has shown that the VD<sub>3</sub> receptor and estrogen receptor  $\beta$  interact with different  $\alpha$ -helical LXXLL motifs of receptor-associated

coactivator 3<sup>32</sup>. Thus it is possible to speculate that E<sub>3</sub>-bound nuclear receptors recruit coactivators, including receptor-associated coactivator 3, and enhance the ligand-dependent transcriptional activation of VD<sub>3</sub> receptor mRNA expression. It seems likely that the combined treatment with E<sub>3</sub> and VD<sub>3</sub> augments the cell viability of cultured HOS as much as the combined treatment with E<sub>2</sub> and VD<sub>3</sub>. However, the molecular mechanism whereby E<sub>3</sub> affects VD<sub>3</sub> receptor mRNA expression in cultured HOS is still unknown.

In conclusion, we have demonstrated that E<sub>3</sub> treatment increases the cell viability of cultured HOS compared with untreated control cultures. Combined treatment with E<sub>3</sub> and VD<sub>3</sub> further increases the cell viability of cultured HOS compared with treatment with E<sub>3</sub> alone, and E<sub>3</sub> treatment also results in a substantial increase in VD<sub>3</sub> receptor mRNA expression in cultured HOS compared with untreated control cultures. Although the biological role of E<sub>3</sub> in bone cells has yet to be explored, the present study moves toward a better understanding of the molecular mechanisms underlying the clinical effects of E<sub>3</sub> alone or combined therapy with E<sub>3</sub> and VD<sub>3</sub> in the maintenance of bone density in postmenopausal women.

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