Effects of Vitamin E on the Osteoblast Differentiation

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ABSTRACT. Vitamin E is thought to affect bone formation and bone remodeling. In this study, we investigated the effects of vitamin E (α-tocopherol and δ-tocopherol) on the osteoblasts isolated from rat calvariae. At 4 and 7 days (Day 4 and 7) after induction of osteoblastic differentiation, treatment of α-tocopherol (100 and 200 μM) and δ-tocopherol (2 and 20 μM) for 3 days significantly decreased alkaline phosphatase activity of the cultured osteoblasts. At Day 14, however, no significant change was detected in ALP activity and expression of bone sialoprotein mRNA in the osteoblasts treated with α-tocopherol or δ-tocopherol for 3 days. Expression of osteocalcin mRNA was decreased by treatment of α-tocopherol (100 and 200 μM) and δ-tocopherol (2 and 20 μM) at Day 4 and 7. At Day 14, expression of osteocalcin mRNA was decreased only with treatment of 200 μM α-tocopherol. In addition, the noncalcified nodules were decreased by treatment of α-tocopherol (200 μM) and δ-tocopherol (20 μM) at Day 7. However, treatment of α-tocopherol and δ-tocopherol showed no significant change of formation of calcified nodules at Day 14. These results indicate that vitamin E inhibits differentiation of osteoblasts especially from early stage to osteoid-producing stage.

KEY WORDS: alkaline phosphatase, cell culture, osteoblast, osteocalcin, tocopherol.

Vitamin E (tocopherols and toctrienols) is an important cellular antioxidant that participates in several reactions to prevent peroxidation of membrane-bound lipids. Recently, many non-antioxidant functions of α-tocopherol, a major vitamin E isomer, were described at cellular level, such as inhibition of protein kinase C, inhibition of the smooth muscle cell proliferation and the activation of diacylglycerol kinase [3, 4, 7–9]. In the skeletal system, several reports demonstrated effects of vitamin E on bone and cartilage tissues [1, 20, 28, 32]. Ebina et al. reported that Fe-induced impairment of bone formation was prevented by dietary vitamin E supplementation in rats [13]. In addition, vitamin E stimulated trabecular bone formation in chicks [25]. Therefore, vitamin E is thought to affect bone formation and bone remodeling. However, direct effects of vitamin E on bone tissue at cellular level are still remained unclear. Among tocopherol isoforms, α-tocopherol shows the most biologically active and is selectively retained in the body [2, 15], and δ-tocopherol exhibits the most inhibitory effect to cell proliferation in several cell-types [23, 24]. In this study, to test the influence of vitamin E on differentiation of osteoblasts at several stages of osteogenesis, we investigated change of alkaline phosphatase activity (ALP), and expression of osteocalcin and bone sialoprotein (BSP) as a marker of maturation of osteoblasts [17, 27, 31] in cultured osteoblasts by treatment with α and δ-tocopherol.

Osteoblasts were isolated from calvariae of newborn rats by collagenase digestion according to the method described by Spector et al. [29]. The isolated osteoblasts were suspended in DMEM/F12 medium containing 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 μg/ml streptomycin (growth medium), and seeded at a density of 1 × 10⁵/3 cm-diameter dish. After the cultured osteoblasts became confluent (Day 1), the cells were maintained in the complete medium (DMEM/F12 medium containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin containing 10 mM β-glycerophosphate and 100 μg/ml ascorbic acid) to induce maturation and mineralization of the osteoblasts. To dissolve highly lipophilic vitamin E compounds in aqueous culture media, these compounds were conjugated to bovine serum albumin (BSA). Alpha-tocopherol and δ-tocopherol (Sigma, St. Louis, MO, U.S.A.) dissolved in 100% ethanol at various concentrations [14, 15] were added to 9 volumes of 10% BSA and incubated at 37°C over night to conjugate tocopherols to BSA. These tocopherol solutions were added to osteoblasts at concentration of 100 and 200 μM (α-tocopherol) or 2 and 20 μM (δ-tocopherol) from Day 1 to 4, Day 4 to 7 or Day 11 to 14. These doses and treatment period of α and δ-tocopherol were determined according to our preliminary examinations based on several investigations on the effects of tocopherols using cultured cells [7, 8, 14, 15, 18]. The control cultures were received an equivalent volume of 10% ethanol in 10% BSA. The osteoblasts were cultured with a change of the medium every 2 days and at 24 hr before the harvest of the osteoblasts. After the treatment for 96 hr, the osteoblasts were underwent lysis in 0.9% NaCl and 0.2% Triton × 100 for the determination of ALP activity and protein content. ALP activity was determined in the cell-lysate using p-nitrophenyl phosphate as a substrate according to the method of Pacifici et al. [26]. One unit of
ALP activity was defined as the ALP activity that liberated 1 μmol p-nitrophenol per mg protein determined by Lowry’s method. Total cellular RNA was extracted from cultured osteoblasts according to acid-guanidine-phenol-chloroform method. Expression of osteocalcin and BSP mRNA were detected with reverse transcription polymerase chain reaction (RT-PCR) using 1 μg of total RNA with GeneAmp RNA PCR kit (Applied Biosystems, CA, U.S.A.) according to the manufacture’s protocol. Aliquots of PCR mixes were removed at multiple points in the cycles, applied on an agarose gel, and DNA bands were visualized by ethidium bromide. Band intensities were compared to each other at the lowest number of cycles at which products started to be visible. Detection of glyceraldehydes-3-phosohate dehydrogenase (GAPDH, housekeeping gene) mRNA was also performed as an internal control. The following primers were used; rat osteocalcin (GneBank accession Nr. NM013414): forward 5’ CAT GAG GAC CCT CTC TCT GC 3’, reverse 5’ CCT AAA CGG TGG TGC CAT AG 3’, rat BSP (J04215): forward 5’ CAC TCA CTT GCT CTC TCC AG 3’, reverse 5’ CTG AGA GTG TGG CGT TGT GT 3’, rat GAPDH (NM017008): forward 5’ TCC ACC ACC CTG TGG CAT GCC ATC AC 3’, reverse 5’ ACC ACA GTC CAT GCC ATC AC 3’. Semi-quantitative analysis of RT-PCR products was performed by volume densitometry using NIH image J software after scanning of the gel photographs. Data from separate RNA isolated from 5-8 different experiments were presented. Levels of all amplification products were normalized to values obtained by that of GAPDH expression, and were expressed as percentage of levels of the control of same experiments. The osteoblasts in some dishes were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer to detecte calcium deposition in the cultured osteoblasts histologically by von Kossa’s staining.

In the control osteoblasts, ALP activity was detected at Day 4 and increased during the remainder of the culture period in the control osteoblasts (Fig. 1). At Day 4, ALP activity significantly decreased in α-tocopherol treated osteoblasts at doses of 100 and 200 μM, and in δ-tocopherol treated osteoblasts at doses of 20 μM (Fig. 2). Similarly, ALP activity significantly decreased in α-tocopherol treated osteoblasts (100 and 200 μM) and in δ-tocopherol treated osteoblasts (20 μM) (Fig. 3). At Day 14, however, ALP activities of α and δ-tocopherol treated osteoblasts showed no significant change compared with the control osteoblasts (Fig. 4).

Expression of osteocalcin mRNA was detected in the control osteoblasts at Day 4, 7 and 14 (Fig. 5). The levels of osteocalcin mRNA were gradually increased from Day 4 to Day 14. In 100 and 200 μM α-tocopherol and 2 and 20 μM δ-tocopherol treated osteoblasts, osteocalcin mRNA was lower than that of the control osteoblasts at Day 4 (Fig. 6) and Day 7. At Day 14, however, only 200 μM α-tocopherol treated osteoblasts showed lower levels of osteocalcin mRNA than that of the control osteoblasts (Fig. 6).

Expression of BSP mRNA was detected in the control osteoblasts at Day 14, but not at Day 4 and 7 (Fig. 5). No significant change of the level of BSP mRNA was detected in α and δ-tocopherol treated osteoblasts from Day 4 to 14 (Fig. 6).

In the control osteoblasts, no calcium deposition was detected in the osteoblasts at Day 4 and 7 in von Kossa staining. However, most of osteoblasts became small polygonal cells forming noncalcified nodules at Day 7. At Day 14, small calcified nodules were scattered throughout the cultured osteoblasts. In 200 μM α-tocopherol and 20 μM δ-tocopherol treated osteoblasts, the noncalcified nod-
ules were smaller than those of the control osteoblasts at Day 7 (Fig. 7). At Day 14, however, the numbers and areas of the calcified nodules showed no significant change in \( \alpha \) or \( \delta \)-tocopherol treated osteoblasts (Fig. 8).

This is the first report that demonstrates the effects of vitamin E on osteoblasts. In the present study, \( \alpha \) and \( \delta \)-tocopherol decreased ALP activity and expression of osteocalcin mRNA in cultured osteoblasts at Day 4 and 7. However, \( \alpha \) and \( \delta \)-tocopherol treatment showed no significant effect at Day 14, except for the treatment of 200 \( \mu M \) \( \alpha \)-tocopherol which showed inhibition of expression of osteocalcin mRNA. The culture system of osteoblasts isolated from the calvariae of newborn rats is a suitable model to investigate the process of osteoblast differentiation from their progenitor to mature cells producing calcified bone matrix [29, 31]. Igarashi et al. demonstrated that these cultured osteoblasts increased the expression of several molecules expressed by mature osteoblasts, such as ALP, osteocalcin, BSP and PTH/PTHrP receptor, during culture period [17]. In the present study, ALP activity and expression of osteocalcin mRNA were gradually increased in the control osteoblasts according to days in culture. In addition, the control osteoblasts showed many non-calcified nodules containing small polygonal osteoblasts at Day 7, and the calcified nodules and the expression of BSP mRNA at Day 14. The non-calcified nodules are formed prior to the calcium deposition in the cultured osteoblasts, and revealed to be composed mainly of type I collagen, and resemble osteoid [5, 6, 19, 31]. The small polygonal cells in the non-calcified nodules were considered to be osteoblasts producing osteoid [19, 31]. These lines of evidence indicate that the cultured osteoblasts used in this study gradually differentiated from the osteoblasts at relatively early stage to those producing osteoid, and then to those producing the calcified bone matrix during the culture period. Therefore, the results of the present study suggest that \( \alpha \) and \( \delta \)-tocopherol inhibit the differentiation of osteoblasts from early stage to osteoid.

Fig. 3. Changes of ALP activity in cultured osteoblasts at Day 7 by treatment of \( \alpha \) and \( \delta \)-tocopherol for 3 days. ALP activity significantly decreased in osteoblasts treated at dose of 100 and 200 \( \mu M \) \( \alpha \)-tocopherol, and 20 \( \mu M \) \( \delta \)-tocopherol compared with control osteoblasts. Control: n=10, \( \alpha \)-tocopherol: n=12, \( \delta \)-tocopherol: n=6, *: p<0.001.

Fig. 4. Changes of ALP activity in cultured osteoblasts at Day 14 by treatment of \( \alpha \) and \( \delta \)-tocopherol for 3 days. No significant changes of ALP activity was detected in the osteoblasts treated with \( \alpha \)-tocopherol or \( \delta \)-tocopherol compared with control osteoblasts, n=6.

Fig. 5. Expression of osteocalcin, BSP and GAPDH mRNA in cultured osteoblasts at Day 4, 7 and 14. Osteocalcin mRNA gradually increased according to days in culture. BSP mRNA was detected only at Day 14. M: 100 bp ladder size marker.
producing stage, but not the differentiation to the osteoblasts producing the calcified bone matrix. In addition, 20 μM δ-tocopherol treated osteoblasts at Day 7 showed lower ALP activity than those at Day 4, indicating that δ-tocopherol inhibit the differentiation to osteoblasts producing osteoid more effectively than those at early stage.

Both α and δ-tocopherol inhibited formation of the non-calcified nodules at Day 7. At Day 14, however, α and δ-tocopherol showed no effect on formation of the calcified nodules. These findings also indicate the inhibition of the differentiation to the osteoblasts producing osteoid, but not of the differentiation to the osteoblasts producing the calcified nodules.
fied bone matrix, by α and δ-tocopherol. Murphy et al. observed that excessive vitamin E supplementation reduced calcium and phosphorus in bone in chick [25]. From our result, it seems to be possible that the decrease of the amount of calcified bone matrix by vitamin E supplementation in chick was caused by inhibition of osteoid formation.

It was unclear whether the effects of α and δ-tocopherol on differentiation of osteoblasts are caused by their antioxidant property or non-antioxidant functions in this study. Many reports demonstrated that several antioxidants increased ALP activity in cultured osteoblasts [10, 11, 12, 21, 22], indicating that the decrease of ALP activity induced by α and δ-tocopherol in cultured osteoblasts is not caused by their antioxidant effects. Tocopherol shows several functions at cellular level independent of its antioxidant properties. One of the most important non-antioxidant func-
tions of α and δ-tocopherol is inhibition of protein kinase C activity by producing dephosphorylation of the enzyme, resulting in inhibition of cell proliferation [7, 8, 15, 18]. In addition, tocopherol functions as a transcriptional regulator with binding to tocopherol-associated protein [33]. Such non-antioxidant functions of tocopherol may affect the expression of several factors associated with differentiation of the osteoblasts.

In conclusion, our results have indicated that α and δ-tocopherol inhibited the differentiation of osteoblasts especially from early stage to osteoid-producing stage. Therefore, vitamin E was thought to have an essential role in regulation of osteoblastic differentiation and producing bone matrix.

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REFERENCES


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