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The effect of adsorbed vitamin D and K to hydroxyapatite on ALP activity of MC3T3-E1 cell

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Abstract This study describes the adsorptive property of vitamins on HA and the effect of the HA adsorbed vitamin on the alkaline phosphatase (ALP) activity for effective use as a bone graft substitute. The vitamins used were calciferol (D_3), menaquirone (K_2) and 25-hydroxycholecalciferol ($25(OH)D_3$). These vitamins were adsorbed on HA at 4, 10, 20, 37 and 50 °C. The adsorption amount was constant below 20 °C, and decreased as the incubation temperature increased over 20 °C. The order of the adsorption amount was: $25(OH)D_3 > K_2 > D_3$. The HA adsorbed vitamins (HA/D_3, $HA/25(OH)D_3$ and HA/K_2) were suspended in physiological saline for 48 h for the release test. The release ratio of all vitamins increased with incubation time. The order of the release ratio was: $25(OH)D_3 > K_2 > D_3$, which was proportional to that of the adsorption amount. The ALP activity of MC3T3-E1 osteoblast-like cells on HA, HA/D_3, $HA/25(OH)D_3$ and HA/K_2 was also investigated. The ALP activity was higher on $HA/25(OH)D_3$ than on any other samples. However, HA/K_2 and HA/D_3 showed similar ALP activity to HA.

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1 Introduction

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HA) has been known to have excellent absorption property as well as osteocompatibility. HA has been used extensively as a bone graft substitute and has the ability to pack proteins or enzymes due to its multiple site binding characteristics for proteins and much larger specific surface area compared to other calcium phosphates [1–4]. Zittle first reported protein adsorption onto calcium phosphates in 1951 [5]. Since then, extensive studies have been conducted and the adsorption of various organic compounds such as albumin, amino acid, insulin, growth factor, antitumor agents, and immunogamma globulin to HA has been tested [6–10]. However, there have been few reports about the adsorption of vitamins to HA. Vitamin D and K have been known to be important in bone metabolism [11–14]. Vitamin K_2 directly stimulates osteocalcin production in osteoblastic cells [15]. Vitamin D_3 is hydroxylated in the liver to form 25-hydroxycholecalciferol ($25(OH)D_3$) and subsequently to 1,25-hydroxycholecalciferol ($1,25(OH)_2D_3$) in the kidney. The $1,25(OH)_2D_3$ is biologically the most active metabolite of vitamin D_3, and has the highest stimulation of osteocalcin and alkaline phosphatase (ALP) production in osteoblastic cells [16–17]. Vitamin D_3 still has stimulation ability of type I collagen and ALP production in osteoblastic cells [18]. $25(OH)D_3$ decreases calcium uptake from bone cells, which relates to suppressing the bone resorption [19].

In the present study, the adsorptive property of HA to vitamin D_3, $25(OH)D_3$ and K_2 was investigated using high performance liquid chromatography (HPLC). The ALP activity of osteoblastic cells (MC3T3-E1) on the HA adsorbed vitamin were examined as a differentiation maker for a bone substitute use.

2 Materials and methods

2.1 Materials

Hydroxyapatite powder was supplied by Taihei Chemical Industrial Co., Ltd. (Ohsaka, Japan). The mean particle size and specific surface area of the HA powder was 20.2 μm and 50–60 m^2/g, respectively. The average Ca/P ratio was 1.67.

Calciferol (D_3), menaquirone (K_2) and 25-hydroxycholecalciferol ($25(OH)D_3$) were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan).

2.2 Methods

2.2.1 Adsorption of vitamins on HA

D_3 and $25(OH)D_3$ were dissolved in ethanol, and adjusted to a concentration of 1.0 mg/mL by rapid mixing in a vortex apparatus for 5 min just prior to use. Due to insolubility in ethanol, K_2 was dissolved in acetone and the concentration was adjusted with 1.0 mg/mL. Samples of HA powder (0.1 g) were dispersed into 1.0 mL of each dissolved vitamin in a conical polypropylene microtube (1.5 mL capacity) by vortexing for 15 s. The suspension was incubated without stirring in 4, 10, 20, 37, and 50 °C for 15 min and then centrifuged for 1 min at 6,500 rpm. The vitamin concentration of the supernatants were analyzed by HPLC (HITACHI Lachrom Elite with L-2420 UV–VIS detector, HITACHI, Japan). The amount of vitamin adsorbed was then calculated from the material balance. Blanks containing only each vitamin, prepared and incubated in solvents corresponding to the adsorption tests but without HA powder, were used as controls. Each final result was obtained from an average of three samples.

2.2.2 Vitamin release from HA

The HA adsorbed vitamins were prepared as described previously at 20 °C, and the initial adsorbed vitamins were determined. 0.2 g of the each HA adsorbed vitamin was resuspended in 0.2 mL of physiological saline in a microtube, and stored at 37 °C in a humidified atmosphere to prevent evaporation of the saline. After 24 and 48 h incubation, the supernatants were sampled and acetnitrile was added at 5.0% to dissolve the released vitamins in the supernatants. The released vitamin concentration of the supernatants was analyzed by HPLC. The release ratio was determined by dividing the released vitamin by the initial adsorbed vitamin. The final result was obtained by averaging five samples.

2.2.3 Cell culture

Osteoblast-like MC3T3-E1 cells (Riken BioResource Center, Japan) were cultured in a plastic cell culture flask (BD Bioscience, USA) in Eagle's MEM (Gibco BRL, USA) supplemented with 10% fetal bovine serum (Gibco BRL, USA), 0.1 mM of non-essential amino acids (L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine, and glycine), $NaHCO_3$ (2.2 g/L), glucose (4.0 g/L), ascorbic acid (5 mg/L), $Fe(NO_3)_3$ (0.1 mg/mL), and kanamycin (20 μg/mL). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2, and subcultured every 7 days with trypsin–EDTA (0.25% trypsin with 1 mM EDTA). The cells were seeded at a concentration of 1×10^5 cells/dish on each 35 mm dish. After 24 h, the medium was changed and included each of the prepared HA adsorbed vitamin (D_3/HA, $25(OH)D_3$/HA, K_2/HA) and HA powder alone at a concentration of 0.1 mg/mL. The adsorption of D_3/HA, $25(OH)D_3$/HA and K_2/HA was adjusted at 0.25 wt.%. The concentration of each vitamin can be estimated at 0.25 μg/mL in each medium including the HA adsorbed vitamin. The medium was also changed and included D_3, $25(OH)D_3$, K_2 at a concentration of 0.25 μg/mL as a comparison.

After 48 h, the cells were washed three times with the PBS(–) solution and were scraped using a cell scraper (Sumitomo Corp., Japan) in 0.5 mL of 0.2% Igepal CO-630 (Sigma-Aldrich Co., USA), 10 mM tris–HCl and 1 mM $MgCl_2$ at pH 8, and homogenized. The ALP activity was measured using a Wako LabAssay kit 291-58601 (Wako Pure Chemical Industries Ltd., Japan). *p*-Nitrophenol phosphate was used as a substrate for the ALP. The reaction product, *p*-nitrophenol, manifests itself as a yellow solution in alkaline solutions at pH 9.8 and was quantified at $\lambda = 405$ nm. The ALP activity was expressed as nmol/min/mg of protein. The protein content was determined using the Bradford Assay, employing bovine serum albumin as a standard [20]. Each experimental value was obtained using the average value of three wells.

3 Results and discussion

3.1 Adsorption of vitamins on HA

Figure 1 shows the adsorption amount of vitamins on HA powder incubated at each temperature. In all vitamins, the adsorption amount was constant below 20 °C, and decreased as the incubation temperature increased over 20 °C. All vitamins showed lower adsorption amounts over 37 °C, which might be caused by degradation of vitamins by heat. D_3, $25(OH)D_3$, and K_2 should be stored in a cool ambient temperatures below 20 °C because they are

susceptible to heat and light energy. The order of the adsorption amount was: $25(OH)D_3 > K_2 > D_3$. $25(OH)D_3$ showed higher adsorption on HA than D_3 and K_2. The affinity of water may be involved in the comparison between D_3 and $25(OH)D_3$. $25(OH)D_3$ has a greater affinity to water than D_3 because the $25(OH)D_3$ has one more O–H groups in the molecular structure than D_3. HA has a high affinity to water, and has a hydrated layer around the HA particle surface. $25(OH)D_3$ is considered to be easily adsorbed on HA through the hydrated layer. K_2 also showed a higher adsorption on HA than D_3. In this case, many factors might be related to this result, such as the difference in the solvents used (ethanol and acetone), as well as the difference in molecular structures.

Hydroxyapatite is known for its excellent adsorptive property of amino acid proteins. Matsumoto et al. reported its adsorptive property of cytochrome c, which is a growth factor protein. In a previous study, the maximum protein adsorption on HA was around 2% [21]. In this study, the adsorption amount of $25(OH)D_3$ reached 0.62%. The adsorption of the protein on HA occurs through the electrostatic interaction in water. However, the fat-soluble vitamins were adsorbed on HA in ethanol or acetone. This indicates that HA might adsorb the fat-soluble vitamins by the other mechanism in the case of the HA to protein.

3.2 Release of vitamins from HA

Figure 2 shows the ratio of the released vitamins from HA. The release ratio increased with the incubation time. The order of the release ratio was: $25(OH)D_3 > K_2 > D_3$, which was proportional to that of the adsorption amount. As stated above, $25(OH)D_3$ has more affinity to water than K_2 and D_3. $25(OH)D_3$ might be easily released from HA and dissolve in saline. The fat-soluble vitamins of K_2 and D_3 may only minimally dissolve in saline.

However, the released ratio even from the $25(OH)D_3$/HA was still low (0.21%) after 48 h. This indicates that HA has a high affinity to the vitamin D3, K2, and 25(OH)D3, consistent with the idea that these vitamins remains on HA in the living body for a long period of time.

3.3 ALP activity of MC3T3-E1 cells with HA/vitamins

Figure 3 shows the ALP activities of the MC3T3-E1 cells after 48 h incubations. $25(OH)D_3$ and HA/$25(OH)D_3$ showed higher ALP activity than any other samples. The ALP expression is associated with osteoblastic differentiation and the level of ALP activity is an indication of the

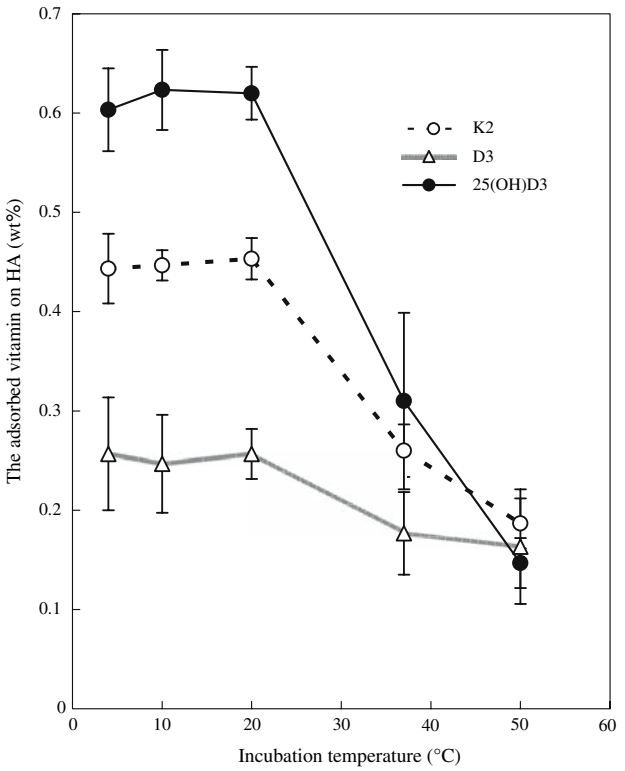

Fig. 1 The adsorption of vitamins on HA ($N = 5$)

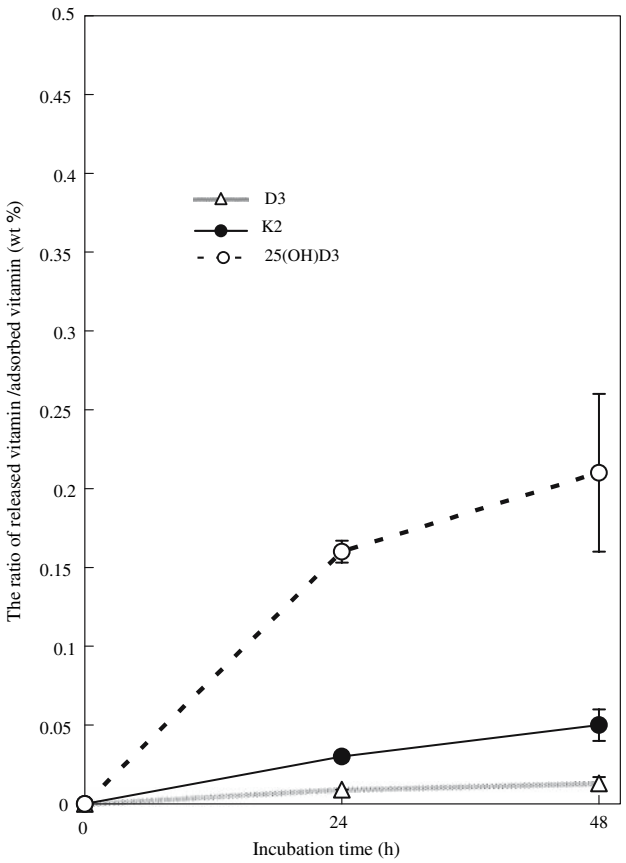

Fig. 2 The ratio of released vitamin from HA ($N = 5$)

stage of osteoblastic differentiation. HA/25(OH)D_3 has higher ALP activity than 25(OH)D_3, suggesting a synergistic effect of HA and 25(OH)D_3. HA only had higher ALP activity than the control. There have been many reports showing that MC3T3-E1 cells on HA had high ALP activity [22–24].

25(OH)D_3 has a higher ALP activity than D_3. Some studies have shown increase in the expression of ALP of osteoblastic cells in the presence of 1,25$(OH)_2D_3$ or 24,25$(OH)_2D_3$ [16, 17, 25]. Dziak et al. reported that the uptake of calcium from the medium is increased in rat bone cells in the presence of 25(OH)D_3 [26]. This might be related to the biological effect of 25(OH)D_3 to osteoblastic cells.

D_3 showed higher ALP activity than the control. Barroga et al. evaluated ALP activity of osteosarcoma cells in the presence of 10^{-8} M of D_3 [27]. In this experiment, the D_3 concentration [6.5×10^{-7} M (0.25 μg/mL)] used might be sufficient to stimulate cells. However, HA/D_3 is similar ALP activity to HA. There is no synergistic effect of HA and D_3 but further investigation is needed.

There was no significant difference between K_2 and control, which may be caused by the low amount of K_2. Yamaguchi et al. reported that various concentrations of K_2 (10^{-5}–10^{-7} M) increased ALP activity. The activity increased in the presence of more than 10^{-6} M of K_2, and not in the presence of 10^{-7} of K_2 [28]. In this experiment, the K_2 concentration used was 4.3×10^{-7} M (0.25 μg/mL).

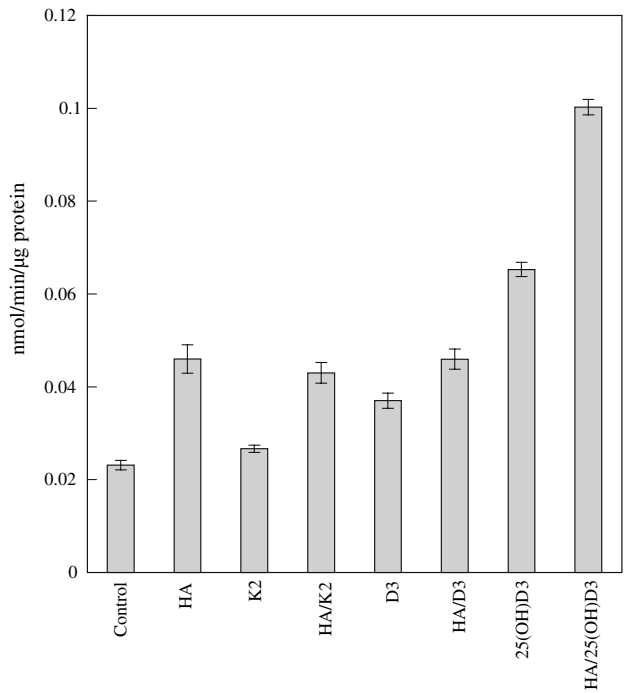

Fig. 3 The ALP activities of the MC3T3-E1 cells after 48 h culture time ($N = 3$)

4 Conclusions

The fat-soluble vitamins of D_3, 25(OH)D_3 and K_2 were adsorbed on HA powder. The adsorption amount was constant below 20 °C, and decreased with increasing the incubation temperature over 20 °C. The order of the adsorption amount was: 25(OH)D_3 > K_2 > D_3. In the release test, the ratio of all released vitamins increased with incubation time. The order of the release ratio was: 25(OH)D_3 > K_2 > D_{3e}, which was proportional to that of the adsorption amount. In cell culture, HA/25(OH)D_3 showed higher ALP activity than any other samples. However, HA/K_2 and HA/D_3 showed similar ALP activity to HA. There was no synergistic effect of HA and vitamins in HA/K_2 and HA/D_3.

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