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Effects of Vitamin D_3 and Related Compounds on Angiotensin Converting Enzyme Activity of Endothelial Cells and on Release of Plasminogen Activator from Them

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Subculture of bovine endothelial cells with 33 μM vitamin D_3 or 25-(OH)-D_3 for 48 h caused marked morphological change. Angiotensin converting enzyme (ACE) activity of the morphologically changed cells was decreased to approximately one-third and plasminogen activator (PA) activity released from them increased approximately 12-fold as compared to the control cells. Fibrin autography revealed both urokinase-type (53 kDa) and tissue-type (80 kDa) PAs in the culture medium of the cells treated with vitamin D_3. Neither 1,25-$(OH)_2$-D_3 nor 24,25-$(OH)_2$-D_3 had any effect on PA activity released from the cells, while ACE activity of the cells was decreased by the treatment with these dihydroxylated vitamins. Provitamin D_3 or cholesterol had no effect on PA or ACE activities of endothelial cells.

Keywords——vitamin D_3; 25-hydroxyvitamin D_3; angiotensin converting enzyme; plasminogen activator; endothelial cell

Endothelial cells constitute the luminal surface of blood vessels and play an important role in fibrinolysis by secretion of plasminogen activator (PA) and in the regulation of blood pressure through the catalytic action of angiotensin converting enzyme (ACE). PA catalyzes the conversion of plasminogen to plasmin, which dissolves fibrin clots formed in blood vessels. ACE exists on the surface of endothelial cells and converts angiotension I to angiotensin II, which is a potent vasopressor. ACE also releases dipeptides from bradykinin, thereby inactivating this vasodepressing molecule. Our purpose is to find a reagent capable of decreasing ACE activity of endothelial cells or increasing the amount of PA secreted from them.

In a previous paper, we reported that vitamins A and C,[1,2] and phytosterols such as sitosterol[3] and fucosterol[4] enhance PA production of bovine endothelial cells. However, fucosterol is able to decrease ACE activity of the endothelial cells,[5] while sitosterol cannot. It is not known whether vitamins A and C affect ACE activity of the endothelial cells.

In the present study, we found that endothelial cells cultured in the presence of 33 μM vitamin D_3 or 25-hydroxyvitamin D_3 (25-OH-D_3) underwent marked morphological change. ACE activity of the morphologically changed cells was significantly decreased and PA activity released from them was markedly increased. The effects of provitamin D_3 and derivatives of vitamin D_3 on ACE and PA activities of endothelial cells were also examined.

Materials and Methods

Materials——Vitamin D_3 was purchased from Nakarai Chemicals Ltd. (Tokyo, Japan). 25-OH-D_3, 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$-D_3), and 24,25-dihydroxyvitamin D_3 (24,25-$(OH)_2$-D_3) were kindly supplied by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Provitamin D_3 was obtained from Sigma Chemical Co. (St. Louis,

Mo.). Benzoyl-Gly–His–Leu and benzyloxycarbonyl-Gly–Gly–Arg–7-amino-4-methylcoumarin (Z–Gly–Gly–Arg–7AMC) were products of Peptide Institute, Inc. (Osaka, Japan) and Cambridge Research Biochemicals Ltd. (Harston, England), respectively. Eagle's minimum essential medium (MEM) was from GIBCO Laboratories (Grand Island, N. Y.).

Cell Culture——Endothelial cells obtained from bovine carotid artery were cultured by the method described previously.[2,3] Cells which had been passaged 10—18 times were used in the present study.

Assay of ACE Activity——ACE activity was measured by the fluorimetric method using benzoyl-Gly–His–Leu as a substrate as described previously.[1] The cells were subcultured in Petri dishes (21 cm^2) in the presence of vitamin D_3 or its derivatives at various concentrations dissolved in ethanol (final concentration 1%). This concentration of ethanol did not affect the viability of endothelial cells, ACE activity or PA activity. After 48 h of subculture, the cells were washed with MEM without calf serum. The washed cells were incubated with 1.5 ml of reaction mixture containing benzoyl-Gly–His–Leu, 150 mM NaCl and 10 mM Tris–HCl (pH 7.9) at 37 °C. From the resulting medium, 100 μl of the sample solution was periodically withdrawn and added to 1.4 ml of 0.5 N NaOH to stop the reaction. To this solution was added 100 μl of *o*-phthalaldehyde (10 mg/ml) dissolved in ethanol, and the mixture was incubated at 25 °C for 5 min. After addition of 250 μl of 6 N HCl to this solution, fluorescence was measured with a Hitachi F-4000 spectrofluorimeter (excitation at 365 nm and emission at 495 nm). The standard curve was obtained using known concentrations of His–Leu. ACE activity was expressed as nanomoles of substrate hydrolyzed per hour per 10^6 cells.

Assay of PA——PA activity was measured fluorimetrically according to the method of Zimmerman, *et al.*[6] The endothelial cells subcultured in the presence of vitamin D_3 or its derivatives were washed with MEM without calf serum and incubated in this medium for 8 h. To 500 μl of this conditioned medium was added 500 μl of Tris–HCl containing 0.5 mM Z–Gly–Gly–Arg–7AMC. After incubation for 1 h at 25 °C, the fluorescence was measured (excitation at 388 nm and emission at 455 nm). Cell extracts were prepared by extraction of endothelial cells with 0.5% Triton X-100.[3] PA activity was expressed as nanomoles of substrate hydrolyzed per hour per 10^6 cells. The standard curve was obtained using known concentrations of urokinase and the activity was expressed as urokinase international units.

Fibrin Autography——Fibrin autography was conducted by the method of Granelli-Piperno and Reich.[7] The sample solution containing PA secreted from endothelial cells was run on slab gels of 10% polyacrylamide in the presence of 0.1% sodium dodecyl sulfate (SDS) according to the method of Laemmli.[8] After electrophoresis, the SDS gel was soaked in 2.5% Triton X-100 for 1.5 h to remove the SDS and applied to the surface of a fibrin-agar indicator gel. Fibrin-agar indicator gel was prepared by mixing 5 ml of a 2% solution of agarose with 4.9 ml of phosphate-buffered saline (PBS) containing fibrinogen (8.2 μg/ml), plasminogen (1 U/ml), and thrombin (0.4 U/ml). The indicator gel was allowed to incubate at 37 °C in a moist chamber, and then photographed. The dark bands of the indicator gel correspond to the lytic zones that resulted from the interaction of PA in the SDS gel and plasminogen in the indicator gel.

Results

Effect on ACE Activity

When endothelial cells were subcultured for 48 h in culture medium containing 33 μM vitamin D_3, they underwent dramatic morphological change, as shown in Fig. 1. Addition of 25-OH-D_3 instead of vitamin D_3 to the culture medium of endothelial cells also caused the

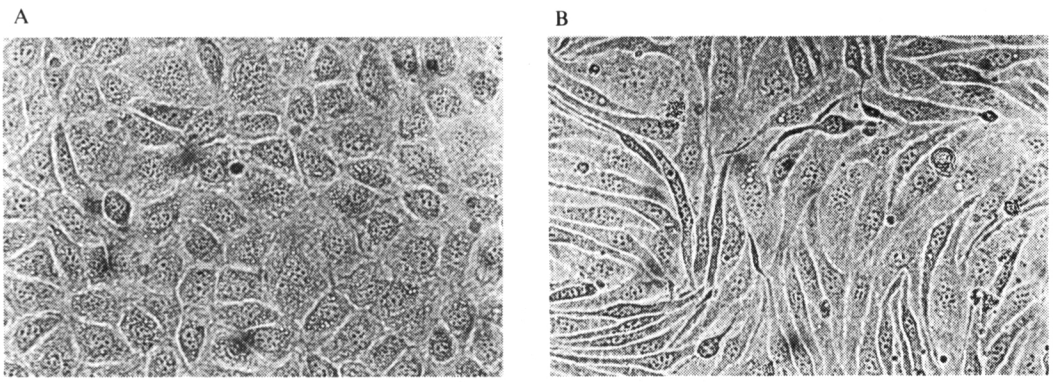

Fig. 1. Morphological Change of Endothelial Cells Induced by Vitamin D_3

Endothelial cells were subcultured in the presence of 33 μM vitamin D_3 for 48 h. ×200.

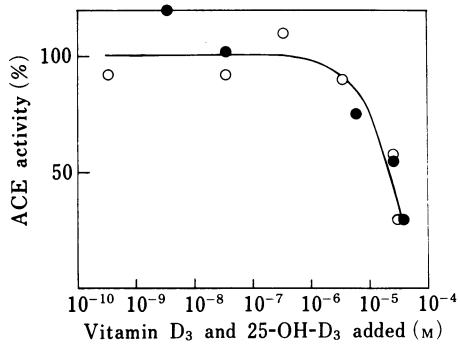

Fig. 2. ACE Activity of Endothelial Cells Treated with Vitamin D_3 or 25-OH-D_3

Endothelial cells were subcultured in culture medium containing various concentrations of vitamin D_3 (—○—) or 25-OH-D_3 (—●—) for 48 hr. ACE activity was measured using benzoyl-Gly-His-Leu as a substrate. ACE activity of endothelial cells without vitamin D_3 treatment was 44.9 ± 13.3 nmol/h/10^6 cells (means ± S.E. for 3 determinations).

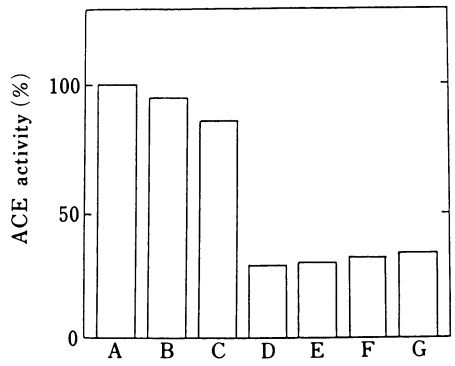

Fig. 3. ACE Activity of Endothelial Cells Treated with 33 μM Vitamin D_3 or Its Derivatives

ACE activity was measured as in Fig. 2.
A, control; B, cholesterol; C, provitamin D_3; D, vitamin D_3; E, 25-OH-D_3; F, 24,25-$(OH)_2$-D_3; G, 1,25-$(OH)_2$-D_3.

same morphological change as vitamin D_3. In contrast, no morphological change was induced by treatment with 33 μM cholesterol, provitamin D_3, 1,25-$(OH)_2$-D_3, or 24,25-$(OH)_2$-D_3. The morphological change of endothelial cells caused by vitamin D_3 or 25-OH-D_3 was reversible. Removal of vitamin D_3 or 25-OH-D_3 from the morphologically changed endothelial cells caused them to revert gradually to their regular form.

ACE activities of endothelial cells cultured in the presence of various concentrations of vitamin D_3 or 25-OH-D_3 for 48 h are shown in Fig. 2. With increasing concentrations of vitamin D_3 or 25-OH-D_3 above 10^{-6} M, the ACE activity of endothelial cells was gradually decreased. The ACE activity of endothelial cells treated with 33 μM vitamin D_3 or 25-OH-D_3 for 48 h was decreased to approximately 30% of that of the control cells (without treatment). 1,25-$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 had almost the same effect as vitamin D_3 on ACE activity of endothelial cells (results not shown). Figure 3 summarizes the effects of cholesterol, provitamin D_3, and its derivatives on ACE activity of endothelial cells when the cells were cultured in the presence of these compounds at 33 μM for 48 h. 24,25-$(OH)_2$-D_3 also caused the decrease of ACE activity of endothelial cells, while cholesterol or provitamin D_3 had no significant effect.

In order to examine whether the decrease of ACE activity of endothelial cells treated with vitamin D_3 or its derivatives is due to a decrease in the total amount of ACE in the cells or due to a decrease in the specific activity of the enzyme, the cells were lysed with 0.5% Triton X-100 and the total ACE activity of the cells was measured. As shown in Fig. 4, the total ACE activity of the cells decreased gradually with increasing concentration of vitamin D_3 or 25-OH-D_3, suggesting that the total amount of ACE in the cells is decreased by the treatment with these vitamins.

Effect on PA Activity

Next, we examined whether PA activity of the endothelial cells treated with vitamin D_3 and 25-OH-D_3 changed or not. Curve A in Fig. 5 and Fig. 6 show PA activities released from endothelial cells treated with various concentrations of vitamin D_3 or its derivatives. With increasing concentrations of either vitamin D_3 or 25-OH-D_3 above 2×10^{-5} M, PA activity released from the cells markedly increased. As described above, ACE activity decreased

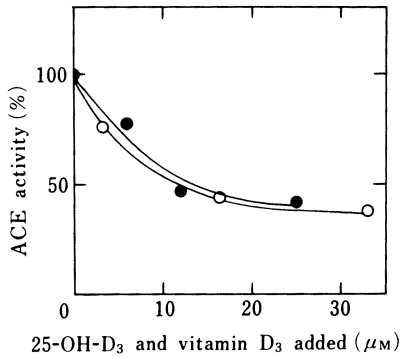

Fig. 4. ACE Activity in the Cell Extract of Endothelial Cells Treated with Vitamin D_3 or 25-OH-D_3

Endothelial cells were subcultured in culture medium containing various concentrations of either vitamin D_3 (—○—) or 25-OH-D_3 (—●—) for 48 h. The cell extract was prepared by solubilization of the cells with 0.5% Triton X-100 and ACE activity of the cell extract was measured as in Fig. 2. Total ACE activity of endothelial cells without vitamin D_3 treatment was 27.0 ± 17.0 nmol/h/10^6 cells (mean ± S.E. for 3 determinations).

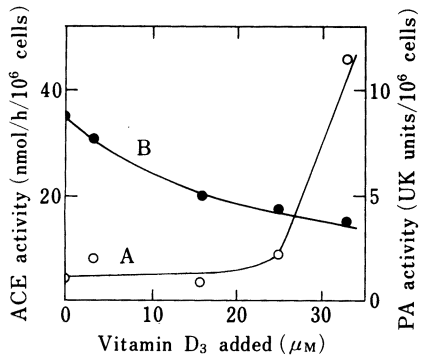

Fig. 5. Effects of Vitamin D_3 on PA Activity Released from Endothelial Cells and on Their ACE Activity

Endothelial cells were subcultured in culture medium containing various concentrations of vitamin D_3 for 48 hr. PA activity released from the cells (curve A) and their ACE activity (curve B) was measured as described in Materials and Methods.

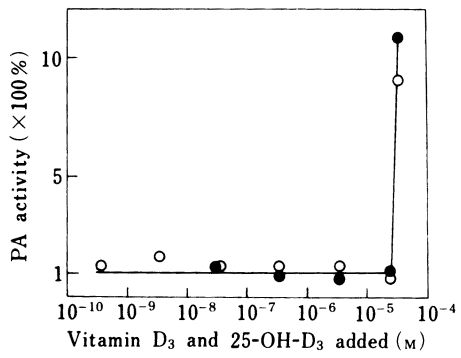

Fig. 6. PA Activity Released from Endothelial Cells Treated with Vitamin D_3 or 25-OH-D_3

Endothelial cells were subcultured in culture medium containing various concentrations of either vitamin D_3 (—○—) or 25-OH-D_3 (—●—) for 48 hr. PA activities were measured as described in Materials and Methods. PA activity released from endothelial cells without vitamin D_3 treatment was 0.70 ± 0.34 nmol/h/10^6 cells (mean ± S.E. for 4 determinations).

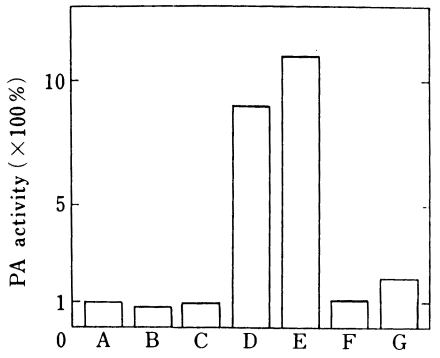

Fig. 7. Effect of Vitamin D_3 and Its Derivatives on PA Activity Released from Endothelial Cells

Endothelial cells were subcultured in the presence of 33 μM vitamin D_3 and its derivatives for 48 hr. PA activities were measured as described in Materials and Methods.

A, control; B, cholesterol; C, provitamin D_3; D, vitamin D_3; E, 25-(OH)-D_3; F, 24,25-$(OH)_2$-D_3; G, 1,25-$(OH)_2$-D_3.

gradually with increasing concentration of vitamin D_3 (curve B in Fig. 5). Note that the values plotted in Fig. 5 correspond to a small but expanded section of Fig. 2. Treatment of endothelial cells with 33 μM vitamin D_3 or 25-OH-D_3 increased the PA activity released to approximately 12 times that from untreated cells. The viability of endothelial cells treated with more than 33 μM vitamin D_3 decreased abruptly as determined by trypan blue staining. In contrast to vitamin D_3 and 25-OH-D_3, treatment with 1,25-$(OH)_2$-D_3 or 24,25-$(OH)_2$-D_3 from 2×10^{-10} to 3.3×10^{-5} M caused no change in PA activity released from the cells (results not shown). As shown in Fig. 7, neither cholesterol nor provitamin D_3 had any effect on PA

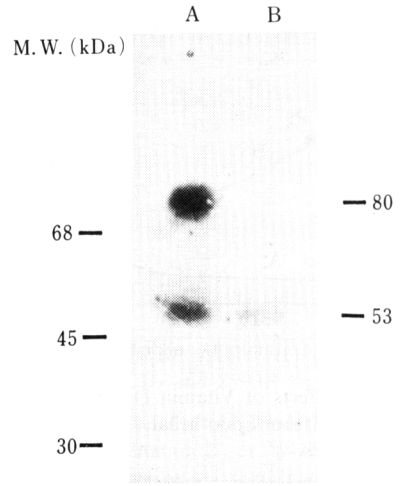

Fig. 8. Fibrin Autogram of PA Released from Endothelial Cells

Endothelial cells were treated with vitamin D_3 for 48 hr and the conditioned medium was prepared as described in Materials and Methods. The conditioned medium was fractionated by SDS polyacrylamide gel electrophoresis and PA activity was localized on fibrin-agar gels. The dark bands correspond to fibrin lysis zones. Lane A, treated with vitamin D_3; lane B, without treatment. M.W. = molecular weight.

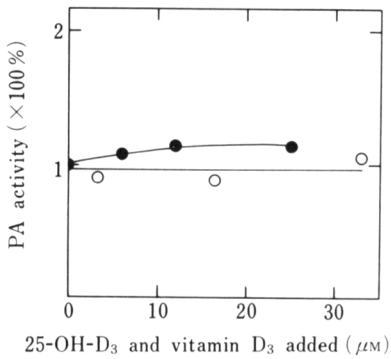

Fig. 9. PA Activity in the Cell Extract of Endothelial Cells Treated with Vitamin D_3 or 25-OH-D_3

Endothelial cells were subcultured in culture medium containing various concentrations of either vitamin D_3 (—○—) or 25-OH-D_3 (—●—) for 48 h. Then, cell extract was prepared by solubilization of the cells with 0.5% Triton X-100 and PA activity of the cell extract was measured as in Materials and Methods. Total PA activity of endothelial cells without vitamin D_3 was 76.6 ± 24.9 nmol/h $\times 10^6$ cells (mean ± S.E. for 3 determinations).

activity released from endothelial cells.

Figure 8 shows the result of fibrin autography of PAs released from endothelial cells. Levin and Loskutoff[9] reported that two types of PAs, a urokinase-type of 53 kDa and a tissue-type of 81 kDa, were released in the conditioned medium of bovine aortic endothelial cells. In accordance with their report, endothelial cells without vitamin D_3 treatment secreted two types of PAs, one of which was a urokinase-type of 53 kDa (lane A in Fig. 8); the tissue-type of 81 kDa was undetectable on the photograph under these conditions but became visible upon longer incubation of the fibrin plate. We have previously shown that the activities of urokinase-type PA (53 kDa) and tissue-type PA (81 kDa) were neutralized by anti-urokinase and anti-tissue PA antibodies, respectively.[4] As is evident from lane A in Fig. 8, both types of PA's with molecular weight of 53 and 81 kDa were increased markedly in the conditioned medium of endothelial cells treated with vitamin D_3.

In order to examine whether the increase in PA activity released from endothelial cells by treatment with vitamin D_3 or 25-OH-D_3 is due to an increase in the total amount of PA in the cells or to an increase in the release of PA from the cells, the cells were solubilized with Triton X-100 and the total PA activity in the cells was measured. As shown in Fig. 9, the total activity of PA in endothelial cells was not significantly changed by treatment of the cells with vitamin D_3 or 25-OH-D_3.

Discussion

Vitamin D_3 and its derivatives affected endothelial cell morphology, ACE activity, and release of PA. Morphological change of the cells was induced by treatment with 33 μM vitamin D_3, or 25-OH-D_3 but not with the same concentration of provitamin D_3, 1,25-$(OH)_2$-D_3, or 24,25-$(OH)_2$-D_3. The physiological concentrations of vitamin D_3, 25-OH-D_3, and 1,25-

$(OH)_2$-D_3 were reported to be 2×10^{-8} to 5×10^{-8} M,[10] 3×10^{-8} to 1×10^{-7} M, [11-13] and 7×10^{-11} M,[14] respectively. The concentration of vitamin D_3 and 25-OH-D_3 used in the present study for causing morphological change of endothelial cells was 3×10^{-5} M. This is 660 to 1700 and 11 to 330 times the respective concentrations of vitamin D_3 and 25-OH-D_3 *in vivo*. However, the concentrations of vitamin D_3 and 25-OH-D_3 specified in the present paper were the amounts added to the culture medium, so actual end concentrations of these vitamins in the solutions were much smaller by an unknown factor. Since these are both oil-soluble substances, much of the vitamin D_3 and 25-OH-D_3 added to the culture medium was not dissolved and part may have bound to the surface of the culture dish.

The treatment of endothelial cells with vitamin D_3, 25-OH-D_3, 1,25-$(OH)_2$-D_3, or 24,25-$(OH)_2$-D_3 at concentrations higher than 10^{-6} M inhibited ACE activity of the endothelial cells. In contrast, provitamin D_3 did not affect ACE activity. Since the total ACE activity of the endothelial cells treated with vitamin D_3 or 25-OH-D_3 was decreased by an amount similar to the decrease of ACE activity in the cell membranes, these vitamins may affect ACE synthesis in the cells. Hagiwara *et al.*[3] demonstrated that fucosterol also lowers the level of ACE of endothelial cells by affecting the biosynthesis of the enzyme.

Vitamin D_3 and 25-OH-D_3 also stimulated PA activity released in the culture medium of endothelial cells when the cells were treated with these vitamins at 33 μM, while provitamin D_3, 1,25-$(OH)_2$-D_3, or 24,25-$(OH)_2$-D_3 at the same concentration had no significant effect. Sitosterol, fucosterol, and vitamins A and C are known to stimulate PA activity release from endothelial cells by stimulating PA synthesis in the cells.[4] On the other hand, hydrocortisone inhibits PA activity secreted in the culture medium of endothelial cells by stimulating the synthesis of PA inhibitors or by stabilizing the plasma membrane of the cells, which in turn affects the release of PA.[15] In human breast cancer cells (MCT-7), estradiol increased the level of PA in the culture medium by stimulating the production of PA.[16] In the present experiment treatment of endothelial cells with vitamin D_3 or 25-OH-D_3 did not affect the total PA activity of the cells but increased PA activity release from the cells. This result suggests that plasma membrane permeability to PA, but not its synthesis in the cells, is changed by vitamin D_3 or 25-OH-D_3, and this is what leads to increased release of PA from the cells. Further studies are needed to clarify in more detail the mechanisms of the change in ACE and PA activities of endothelial cells caused by vitamin D_3 and its derivatives as well as by steroids.

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