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SYNERGISM OF VITAMINS A AND C ON FIBRINOLYSIS

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SUMMARY: A hitherto unknown synergism exerted by retinol(vitamin A) and L-ascorbic acid(vitamin C) was discovered using endothelial cells. Retinol stimulated the extracellular and intracellular activities of plasminogen activator up to approximately 8- and 4-fold from the control values, respectively. L-Ascorbic acid enhanced the extracellular and intracellular activities up to approximately 1.5-fold. Above all it was demonstrated that their effects on extracellular activity were synergistic; simultaneous administration of these two vitamins enhanced the extracellular activity up to a 20- to 50-fold. Synthesis of plasminogen activator induced with vitamins A and C was inhibited by a protein synthesis inhibitor, cycloheximide.

Every vitamin is vital as the name implies; there are many different diseases associated with deficiency of different vitamins. The biochemical functions of some of these, however, are not necessarily understood in detail, although many act as specific cofactors in various enzymic reactions. Vitamin C(L-ascorbic acid) is involved in the hydroxylation of certain prolyl and lysyl residues in a structural protein, collagen(1,2). This may not be the sole physiological function of this vitamin, and recently different roles have been implicated to it(3). It is well documented that vitamin A(retinol) participates in the visual cycle. Lately, other effects of this vitamin such as cell differentiation have been investigated(4), and its chemotherapeutic effect on certain kinds of cancer has been discussed(5).

The present study deals with a synergism on the enhancement of plasminogen activator production in endothelial cells with vitamins A and C.

MATERIALS AND METHODS

Materials

Eagle's minimum essential medium and antibiotics(penicillin-streptomycin solution) were purchased from GIBCO, N.Y., and fetal bovine serum was pur-

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chased from Flow Laboratories Inc., Va. Fibrinogen(93% clottable), thrombin, urokinase and plasminogen, all obtained from human, were supplied by Green Cross Co., Osaka, Japan. L-Ascorbic acid and retinol were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan and Sigma Chemical Co., St. Louis, Mo., respectively.

Culture of Endothelial Cells

Endothelial cells were obtained from bovine carotid artery and were cultured by the method of Hagiwara et al.(6) previously described. The cells which had been passaged 13-16 times(14-17 generations) were used in the present study.

Preparation of Conditioned Media and Cellular Extracts

Preparation of conditioned media and cellular extracts were carried out by the method described previously(6,7). The cells which were harvested from one petri dish(55 cm^2) were seeded in twelve petri dishes(9 cm^2). The cells were subcultured with the culture medium in the presence and the absence of retinol dissolved in 1% ethanol(final concentration), L-ascorbic acid, or both combined. This concentration of ethanol did not affect the synthesis of plasminogen activator by the cells in this study. Several days after visual confluency was reached, the monolayers were washed with cold minimum essential medium. The washed monolayers were incubated with 2 ml of the serum-free medium for 8 hr, and conditioned media and cellular extracts(0.5% Triton X-100 treatment) were subjected to analyses. The conditioned media and cellular extracts were stored at -20°C until assayed.

Assay of Fibrinolytic Activity

Fibrinolytic activity was measured by the method which was explored by Saito et al.(8). This method is based on the nephelometric measurement of turbidity decrease of fibrin suspension, which is a natural substrate for plasmin formed from plasminogen by plasminogen activator. To 700 µl of fibrin suspension(35 µg) were added 100 µl of plasminogen solution(7.0 I.U./ml) and 200 µl of conditioned media with plasminogen activator activity. Turbidity decrease caused by fibrinolysis was recorded at 37°C with time with a Kyoto Daiichi Kagaku nephelometer Model DZ-2111. A standard curve was obtained for the rate of the turbidity decrease against urokinase concentrations, so that plasminogen activator activity was expressed as urokinase international units(6,7).

Fibrin Autography

Fibrin autography was carried out by the method described previously(7). Samples containing plasminogen activators were subjected to electrophoretic analyses by the method of Laemmli(9) and the sample gels prepared were applied to the surface of fibrin-agar indicator gels according to Granelli-Piperno et al.(10). The plasminogen activators were identified by observing the lysis zones on the indicator gels and their approximate molecular weights were estimated from the position of lysis zones on the gels.

RESULTS AND DISCUSSION

Uninterrupted blood flow is ensured by endothelial cells which make up the surface of blood vessels. The failure will result in cerebrovascular and heart diseases. Endothelial cells excrete prostacyclin PGI_2 to inhibit platelet aggregation(11,12). They also provide blood flow with a protease, plasminogen activator, which activates a zymogen form of a protease, plasminogen, normally present in blood(13). The activated plasminogen, plasmin,

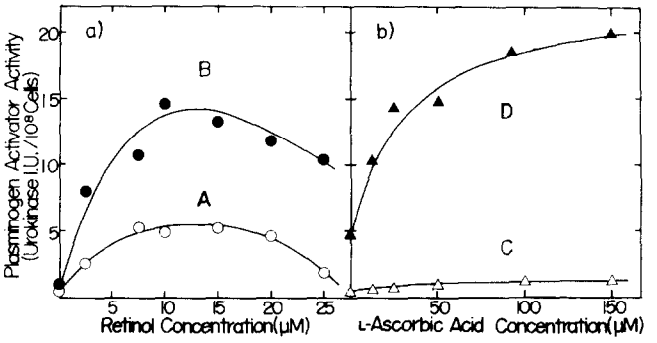

Figure 1. Synergistic effect of retinol and L-ascorbic acid on the activity of plasminogen activator produced by endothelial cells.
a) Curve A, varied amounts of retinol alone were added; Curve B, varied amounts of retinol were added with a fixed amount of L-ascorbic acid(50 μM).
b) Curve C, varied amounts of L-ascorbic acid alone were added; Curve D, varied amounts of L-ascorbic acid were added with a fixed amount of retinol(10 μM).

readily dissolves fibrin, which is the final product of blood coagulation cascade. Using cultured bovine carotid endothelial cells, we found that two phytosterols, sitosterol and fucosterol, enhanced the production of plasminogen activator(6,7). We also reported about the details of the enhancement and the characteristics of the induced plasminogen activators(7). We have continued to search for chemicals to enhance the production of plasminogen activator. Over 60 different compounds tested including various vitamins, hormones, neurotransmitters and reducing agents, only retinol and L-ascorbic acid were effective. The stimulative effects of retinol and L-ascorbic acid were not observed with cancer cells tested: AH-66; rat hepatoma cell, L 929; mouse fibroblast cancer cell, NS-1; mouse myeloma cell and HL-60; human myelogenous leukemia. All the experiments shown below were conducted with endothelial cells. Cells were cultured with and without retinol and L-ascorbic acid for several days in serum-containing culture medium. They were washed and incubated for 8 hr in the minimum essential medium without these vitamins and serum. The activity of plasminogen activator excreted to the medium during this 8 hr-incubation period was determined by a nephelometric method using fibrin suspension as the substrate(8). As is depicted in Fig.1 by Curves A and C, the activity was enhanced quite well with retinol, and rather moderately with L-ascorbic acid, respectively. The effect of retinol

peaked at about 10 to 15 μM, which was comparable to that obtained with fucosterol(7), when the ratios of elevated levels to those of the control were compared. Beyond 15 μM the effect of retinol was steadily decreased. We do not have any good explanation for this at the present. Although the morphology was altered from the typical cobble-stone to spindle shape, the cell density after grown with 25 μM of retinol for a week was about the same as that grown without it. We have further set up experiments to see the synergism between retinol and L-ascorbic acid. To our very surprise, simultaneous additions caused dramatic increment in plasminogen activator activity. When varied amounts of retinol with a fixed amount of L-ascorbic acid(50 μM) were added, the activities obtained throughout the concentration range tested were always higher than the mere addition of the effects obtained with retinol or L-ascorbic acid alone. This was shown by the shift of Curve A to B. Even more dramatic effect was shown by Curve D, when varied amounts of L-ascorbic acid were added with a fixed amount of retinol(10 μM). Very obvious synergism between these two vitamins persisted throughout the concentration range examined. This may suggest that the mechanisms through which these vitamins enhance the activity of plasminogen activator should be different each other. The effectiveness of the synergism varied from experiment to experiment, probably due to the change of physiological condition of cells. The maximum activity obtained by the synergism, however, is always 20- to 50-fold higher than the control value. These vitamins themselves neither had fibrinolytic activity nor directly activated plasminogen activator.

In the next set of experiments, we determined the activity of plasminogen activator, intracellular as well as extracellular. Endothelial cells were cultured for a week in medium containing serum, retinol and/or L-ascorbic acid, and the cells were washed and incubated for 8 hr in the medium free from serum and these vitamins. The results are shown in Table 1. As is shown in column B, the intracellular activity was already elevated by the culture for a week with retinol and/or L-ascorbic acid. After incubation these cells in

Table 1
Stimulative Production of Extra- and Intra-cellular Activities of Plasminogen Activator by Retinol and L-Ascorbic Acid-Treated Endothelial Cells

Treatment	Plasminogen Activator Activity (Urokinase I.U./10^8 Cells)			
	Extracellular after Incubation for 8 hr (A)	Intracellular after Incubation for 0 hr (B)	8 hr (C)	8 hr +cycloheximide (D)
Control	1.7	1.1	1.4	0.0
Retinol(10 μM)	6.3	4.0	5.2	0.0
L-Ascorbic acid(50 μM)	2.4	1.6	1.7	0.0
Retinol + L-ascorbic acid	35.3	5.1	7.1	0.0

Endothelial cell monolayers obtained in the medium with and without 50 μM L-ascorbic acid, 10 μM retinol and both combined were washed and were incubated for 8 hr with serum- and these vitamins-free medium in the presence or absence of 10 μg/dish cycloheximide. After incubation, conditioned media and cellular extracts were prepared and assayed by the method described in MATERIALS AND METHODS.

medium free from serum and these vitamins for 8 hr, the intracellular activity was slightly increased, as is shown in column C. The extracellular activity was, however, greatly enhanced, as shown in column A as well as Fig. 1. These data suggest that the synthesis of plasminogen activator in endothelial cells is stimulated by retinol, L-ascorbic acid and both combined. In order to ascertain this point, the effect of one of protein synthesis inhibitors, cycloheximide, was examined. Cycloheximide was added to the culture medium free from serum and these vitamins, and the activity of plasminogen activator in cellular extracts were measured after 8 hr-incubation. As shown in column D, the activity in cells was completely disappeared by the treatment with cycloheximide. The enhancement of the activity can also be explained by the suppression of inhibitor, if there is any. The enhanced synthesis of plasminogen activator was, however, further supported by fibrin autography, in which the activity was determined after plasminogen activators were separated from other proteins with SDS polyacrylamide gel electrophoresis. These vitamins uniformly enhanced the plasminogen activators with molecular weights of 56,000, 105,000 and 120,000, with the one with the smallest molecular weight being the most predominant. These indicate that these vitamins stimulate the protein synthesis of plasminogen activator in endothelial cells.

It was reported that vitamin C increased the fibrinolytic activity in man when it was administered in a large quantity(14). It was also shown that vitamin A induced the differentiation of certain cancer cells and made them produce plasminogen activator(5). Nevertheless, it has never been shown before that these vitamins enhanced the synthesis of plasminogen activator by cultured endothelial cells, which are the physiological producer of plasminogen activator in blood vessels. The synergism reported in this article has of course never been observed with any kind of cells, and may reinforce the importance of taking balanced amounts of multiple kinds of vitamins. We believe that combination of vitamins A and C should be effective in preventing thrombosis. The detailed mechanisms for the enhancement and the synergism remain to be elucidated.

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