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Retinol-Induced Protein Phosphorylation and Emergence of a New Protein Species in Endothelial Cells

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Retinol and vitamin C stimulate the synthesis of plasminogen activator in cultured endothelial cells (*Biochem. Biophys. Res. Commun.*, **130**, 182, 1985). During the course of investigations to clarify the mechanism, the following results were obtained. An 86 kD protein appeared in the cytosol after incubation of the cells with retinol. Simultaneously, two cytosolic proteins of 78 kD and 100 kD were phosphorylated by endogenous protein kinases. The former was not associated with cyclic adenosine monophosphate-, cyclic guanosine monophosphate- or Ca^{2+}-dependent kinases, while the latter was associated with Ca^{2+}-dependent kinase. These stimulative phenomena are discussed in relation to the synthesis of plasminogen activator induced by retinol.

Keywords——retinol; phosphorylation; protein kinase; plasminogen activator; endothelial cell

Endothelial cells make up the surface of blood vessels and synthesize plasminogen activators which catalyze the activation of plasminogen. The activated plasminogen, plasmin, dissolves fibrin, which is the final product of the blood coagulation cascade. Using cultured bovine endothelial cells, we demonstrated that retinol, vitamin A, induces not only synthesis of plasminogen activator in cells but also excretion of plasminogen activator into the medium of the cultured cells.[1] A similar stimulation of plasminogen activator production by retinol was reported with human synovial fibroblasts,[2] chick fibroblasts[3] and human chondrocytes.[4] However, the mechanism of this phenomenon is still unclear. Recently, it has been reported that retinoic acid stimulates the alkaline phosphatase activity in various mammalian cells.[5,6]

Various regulatory agents such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), Ca^{2+} and hormones are known to produce their effects through the regulation of specific protein kinases.[7,8] It was expected that the action of retinol might also be mediated through protein phosphorylation in endothelial cells. To elucidate the mechanism of the stimulation of plasminogen activator production by retinol, we studied whether specific cytosolic proteins might be phosphorylated or synthesized in association with the production of plasminogen activator of endothelial cells after retinol treatment.

Experimental

Endothelial cells from bovine carotid artery were cultured by the method described previously.[9,10] Cells which had been passaged 12—14 times (13—15 generations) were used in the present study. The cells were subcultured with the culture medium in the presence of retinol at various concentrations (0—10 μM) dissolved in ethanol (final concentration 1%). This concentration of ethanol did not affect the secretion of plasminogen activator from the cells in this study. Several days after confluency had been visually confirmed the monolayers were subjected to analyses of protein kinase activity and fibrinolytic activity. [γ-^{32}P]Adenosine triphosphate (ATP) was purchased from the Radiochemical Centre, Amersham, England. The phosphorylation reaction was conducted as described pre-

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viously.[11,12] A 20 μl aliquot of 0.5 mM [γ-^{32}P]ATP (6×10^4 cpm/nmol) was added to 60 μl of the reaction mixture containing cytosol (50 μg protein), 42 mM Tris–HCl (pH 7.5) and 8.3 mM $MgCl_2$ in the presence or absence of 5 μM cAMP, 5 μM cGMP or 2 mM ethylene glycol bis-(2-aminoethylether)-*N*,*N*-tetraacetic acid (EGTA). The reaction mixture was incubated for 5 min at 37 °C with mild shaking. At a given time, 20 μl of a solution containing 10% sodium dodecyl sulfate (SDS), 0.31 M Tris–HCl (pH 6.8), 50% glycerol, 25% 2-mercaptoethanol and 0.05% bromophenol blue was added to the mixture to stop the reaction. The sample solution was run on slab gel with 12% polyacrylamide in the presence of 0.1% SDS according to the method of Laemmli.[13] Gel was stained with Coomassie Brilliant Blue R-250. The gel was dried, placed on an X-ray film (Kodak RP X-OmatAR), and exposed for 2 d to identify phosphorylated proteins.[11,12]

The assay of fibrinolytic activity was carried out by the method described previously.[9,10,14]

Results and Discussion

To determine the effect of retinol on the protein profile and phosphorylation of cytosolic proteins in endothelial cells, the cytosol fraction prepared from the cells treated with 5 μM retinol for 120 h was incubated with [γ-^{32}P]ATP in the presence or absence of cAMP, cGMP or EGTA. The samples were subjected to SDS polyacrylamide gel electrophoretic analyses (Fig. 1). Figure 1a shows the electrophoretic patterns visualized with Coomassie Brilliant Blue of cytosolic proteins of cells treated with and without retinol. A strong and distinct band appeared at the position corresponding to a molecular weight of 86 kD after the retinol treatment (lane B), while this band was hardly visible in the cytosol of untreated cells (lane A). These results indicate that an 86 kD protein appears in the cytosol after the addition of retinol. This protein is quite different from plasminogen activators of 56 kD, 105 kD and 120 kD synthesized in endothelial cells[1] or from cellular retinol-binding protein of 14.6 kD.[15,16] Figure 1b shows a radioautogram of phosphorylated proteins in cytosol of cells treated with (lane B) and without (lane A) retinol. In the presence of retinol, a 78 kD protein was extensively phosphorylated; this was not observed in the absence of retinol. This

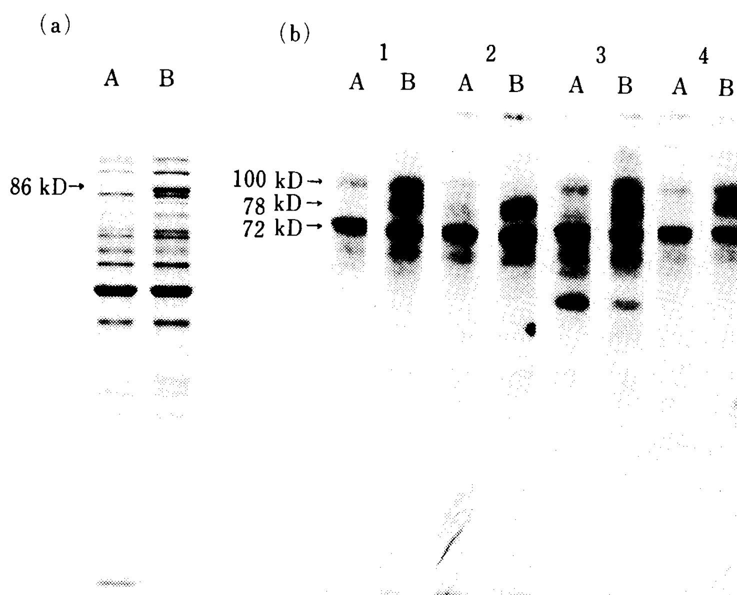

Fig. 1. Electrophoretic Profiles and Radioautogram of Cytosolic Proteins of Endothelial Cells Treated with (Lane B) or without (Lane A) Retinol

Cytosolic proteins (50 μg) were phosphorylated and analyzed by gel electrophoresis, then the gel was radioautographed.

(a) Electrophoretic profile visualized with Coomassie Brilliant Blue of cytosolic proteins. (b) Radioautogram of phosphorylated proteins in the absence (1) and in the presence of 2 mM EGTA (2), 5 μM cAMP (3) or 5 μM cGMP (4).

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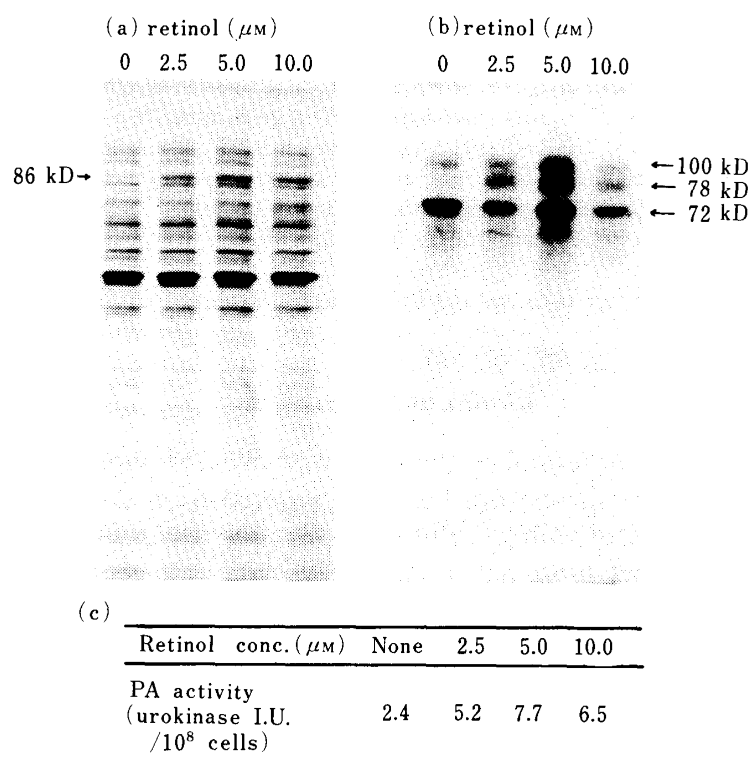

Retinol conc. (μM)	None	2.5	5.0	10.0
PA activity (urokinase I.U. / 10^8 cells)	2.4	5.2	7.7	6.5

Fig. 2. Electrophoretic Profiles (a) and Radioautogram (b) of the Cytosolic Proteins of Endothelial Cells after Treatment with Various Concentrations of Retinol (0—10 μM), together with the Activity of Plasminogen Activator (PA) Excreted into the Medium of Cultured Endothelial cells (c)

phosphorylation was not associated with cAMP-, cGMP- or Ca^{2+}-dependent kinase. A similar phosphorylation was observed for a 100 kD protein, which was phosphorylated by Ca^{2+}-dependent kinase. From these results, it can be concluded that retinol induces phosphorylation of 78 kD and 100 kD proteins in endothelial cells.

The next experiments were conducted to see whether the appearance of 86 kD protein and the phosphorylation of 78 kD and 100 kD proteins are induced by retinol in a dose-dependent manner. The concentration of retinol was varied and the amounts of cytosolic proteins formed and phosphorylated were estimated by electrophoresis and radioautography. At the same time, the enzymic activity of plasminogen activator in the medium was also determined. The plasminogen activator activity was enhanced by increasing the retinol concentration and reached the maximum level, 7.7 urokinase I.U./10^8 cells, at 5 μM retinol. With further increase of the retinol concentration above 5 μM, the activity was rather decreased (Fig. 2c). Similar trends were observed in the amounts of 86 kD protein (Fig. 2a) and of phosphorylation of 78 kD and 100 kD proteins (Fig. 2b) induced by retinol at various concentrations.

From the results obtained above, it can be said that the formation of 86 kD protein and the phosphorylation of 78 kD and 100 kD proteins may be related to the appearance of plasminogen activator.

Experiments are in progress to clarify the physiological function of 86 kD protein and to examine in more detail the causal relation between the phosphorylation of 78 kD and 100 kD proteins and the production of plasminogen activator.

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