Concanavalin A-Induced Decrease in Cell Surface Charge and its Modification by Sulfhydryl Blocking Agents

Kiyohide Kojima, Chikako Sato*, Kimiko Nishizawa* and Satoru Shimizu**

*Department of Oncological Pathology, Nara Medical University, Kashihara 634, and Laboratories of *Experimental Radiology and **Ultrastructure Research, Aichi Cancer Center Research Institute, Nagoya 464, Japan*

ABSTRACT. The electric surface charge of mouse melanoma B16-C2W cells treated with various concentrations of Con A was determined by cell electrophoresis. At Con A concentrations higher than 5 $\mu\text{g/ml}$, the cellular electrophoretic mobility decreased significantly at 10 minutes incubation, but this effect was reversed by α -methyl-D-mannoside. At these higher Con A concentrations, more than 30% of Con A receptors were bound with lecitin. Pretreatment of cells with sulfhydryl blocking agents, 10^{-5} M p-chloromercuribenzoic acid or 10^{-5} M N-ethylmaleimide, completely prevented the subsequent Con A-induced mobility reduction without changing the amount of Con A-binding to the cell surface. Similar effects were found with pretreatment with protein linkage agents, such as 2% glutaraldehyde and 10^{-6} M fluorescein mercuric acetate. The removal of chondroitin sulfate with the specific enzyme resulted in a 30% decrease in electrophoretic mobility of intact cells but produced no change in Con A-treated cells. These results suggest that Con A treatment may induce an alteration in chondroitin sulfate distributions at the cell surface and that sulfhydryl residues of membrane proteins may play an important role in this surface alteration.

Many cancer cells are aggregated by concanavalin A (Con A) (8) or wheat germ agglutinin (1), while their ancestral normal cells do not show this effect. This reactivity difference to lectins between neoplastic and normal cells is probably related to the rearrangement of lectin receptors on the cell surface (9, 11-13, 20). Electron microscopic studies have demonstrated in most neoplastic cells that Con A receptors form cluster easily in bindings with Con A, resulting in cell aggregation (11, 12). Other cell surface alterations induced by Con A, however, are not yet fully elucidated.

To clarify details on cell surface changes induced by Con A, we studied alterations of electric surface charge in mouse melanoma cells after treatment with Con A by cell electrophoresis coupled with chemical modifications of the cell surface.

MATERIALS AND METHODS

The mouse melanoma cell line B16-C2W (2) was used. The cells were maintained *in vitro* in Ham's F12 medium supplemented by 10% fetal calf serum as described previously (15).

Crystallized Con A in saturated sodium chloride (carbohydrate free) was obtained from Miles Yeda, Illinois. The lectin had no any proteolytic enzyme or glycosidase activity in our

screening tests. Con A was labeled with Na^{125}I (carrier-free) by slight modification (17) of the Sonoda method (21). The newly prepared iodinated Con A was immediately used. Following treatment with Con A at different concentrations, the cells were washed twice with Hank's balanced salt solution, and then mechanically stripped off from the culture dish with a rubber policeman.

Cell electrophoresis was performed in 66 mM phosphate-buffered saline supplemented with 0.25 M sorbitol (pH, 7.3; ionic strength, 0.167), as previously reported (10). Measurements of cellular electrophoretic mobility were made at $25 \pm 0.5^\circ\text{C}$ with a cytopherometer (Zeiss).

For chemical modifications of the cell surface, sulfhydryl-blocking agents, such as 10^{-5} M p-chloromercuribenzoic acid (PCMB) and 10^{-5} M N-ethylmaleimide (NEM), and protein linkage agents, such as 2% glutaraldehyde and 10^{-6} M fluorescein mercuric acetate (FMA) in phosphate buffered saline, were used.

For enzymatic modification, neuraminidase from *Vibrio cholerae* (General Biochemicals, Chagrin Falls, Ohio), chondroitinase-ABC from *Proteus vulgaris* (Seikagaku Kogyo, Tokyo), and hyaluronidase from *Streptomyces* (Seikagaku Kogyo, Tokyo) were used at a concentration of 20 units/ml in Hank's solution at pH 7.0, 1 unit/ml in Veronal-buffered saline supplemented with 0.1% egg albumin at pH 8.0 and 10 turbidity-reducing units/ml in Veronal-buffered saline with 0.1% egg albumin at pH 6.0, respectively. Neuraminidase showed no chondroitinase or hyaluronidase activities, and the chondroitinase and hyaluronidase showed no neuraminidase activity. *Streptomyces* hyaluronidase (14) digested only hyaluronic acid, and the chondroitinase-ABC (23) was specific for the respective chondroitin sulfates.

RESULTS

Con A-induced alteration in the electric surface charge of cells. Fig. 1 shows changes in electrophoretic mobility of melanoma cells treated with Con A at different concentrations. Cell mobility was significantly decreased at concentrations higher than $5\text{ }\mu\text{g/ml}$, while no significant change in mobility was observed at concentrations

Fig. 1. Changes in electrophoretic mobility of C2W cells after treatment with Con A at different concentrations. Cell electrophoretic mobility was measured after treatment with Con A at 37°C for 30 min. Each point indicates the mean mobility value of 40 cells. The vertical lines represent standard deviations.

lower than $1 \mu\text{g/ml}$. The decrease in negative surface charge by the lectin was induced within 10 min of incubation and reverted by addition of α -methyl-D-mannoside (Fig. 2).

Fig. 2. Time course changes in electrophoretic mobility of C2W cells after Con A treatment and their reversibility after treatment with α -methyl-D-mannoside. The cells were treated with Con A at $20 \mu\text{g/ml}$ in phosphate-buffered saline at 37°C and the electrophoretic mobility of more than 40 cells was measured at the indicated time intervals. After Con A treatment for 30 min, the cells were washed, and the reversibility of mobility was observed by addition of 0.1 N α -methyl-D-mannoside. The vertical lines represent standard deviations. Dotted line indicates cell mobility changes by combined treatment with Con A and α -methyl-D-mannoside. αMM , α -methyl-D-mannoside.

Fig. 3. Correlation between amount of Con A bound to cell surface and concentration of Con A used. The cells were treated with ^{125}I -Con A at 37°C for 30 min, and the radioactivity bound on the cell surface was counted in a liquid scintillation counter.

The amount of Con A bound on the cell surface increased in direct proportion with the increase in Con A concentration in the media to 20 $\mu\text{g/ml}$ (Fig. 3). This shows that Con A binding sites were saturated at concentrations higher than 20 $\mu\text{g/ml}$. The results suggest that cell surface charge decreased when more than 30% of Con A receptors were bound with lectin (Figs. 1 and 3).

TABLE 1. EFFECTS OF PROTEIN LINKAGE AND SULFHYDRYL BLOCKING AGENTS ON CON A INDUCED DECREASE OF ELECTROPHORETIC MOBILITY IN B16C2W CELLS

Treatment	Electrophoretic mobility ($\mu\text{m}\cdot\text{sec}^{-1}\cdot\text{v}^{-1}\cdot\text{cm}$)	^{125}I -Con A binding (cpm/ 10^5 cells)
No treatment	-1.061 ± 0.060	
Con A only	-0.720 ± 0.071	2296 ± 197
Glutaraldehyde, 3°C 10 min	-1.438 ± 0.122	
Glut→Con A	-1.420 ± 0.103	2346 ± 341
Con A→Glut	-1.149 ± 0.104	
FMA 10^{-6}M , 10 min	-1.057 ± 0.070	
FMA 10^{-6}M , 10 min→Con A	-1.060 ± 0.074	2173 ± 320
PCMB 10^{-5}M , 10 min	-1.049 ± 0.069	
PCMB→Con A	-1.051 ± 0.054	2270 ± 274
NEM 10^{-5}M , 10 min	-1.047 ± 0.049	
NEM→Con A	-1.054 ± 0.070	2073 ± 296

All values are shown as $\text{M} \pm \text{SD}$. Concanavalin A (Con A) was used at a concentration of 20 $\mu\text{g/ml}$. The cells were incubated at 37°C for 30 min. Glut, glutaraldehyde (2% solution); FMA, fluorescein mercuric acetate; PCMB, p-chloromercuribenzoic acid; NEM, N-ethylmaleimide.

Effects of cell surface modification on Con A alteration. Table 1 shows the effects of various chemical modifications on the Con A-induced mobility reduction of the cell surface. When cells were treated with 10^{-5} M PCMB or NEM, sulfhydryl blocking agents, for 10 min at 37°C, the Con A-induced mobility reduction was completely prevented without changing the number of Con A-binding sites at the cell surface. Similar results were obtained by pretreatment of cells with protein linkage agents, such as glutaraldehyde, 2% solution at 3°C, or FMA, 10^{-6} M at 37°C, for 10 min. However, when cells were pretreated with Con A at 20 $\mu\text{g/ml}$ at 37°C and subsequently treated with glutaraldehyde, a decrease in cellular mobility was observed

TABLE 2. EFFECT OF ENZYMIC TREATMENT ON ELECTROPHORETIC MOBILITY OF B16C2W CELLS ($\text{M} \pm \text{SD}$)

Treatment		Intact cells			Con A-treated cells		
		Electrophoretic mobility ($\mu\text{m}\cdot\text{sec}^{-1}\cdot\text{v}^{-1}\cdot\text{cm}$)	Difference %	P	Electrophoretic mobility ($\mu\text{m}\cdot\text{sec}^{-1}\cdot\text{v}^{-1}\cdot\text{cm}$)	Difference %	P
Neuraminidase	Control	-1.073 ± 0.094					
	Experiment	-0.802 ± 0.106	25.3	<0.01			
Chondroitinase-ABC	Control	-1.046 ± 0.107			-0.896 ± 0.058		
	Experiment	-0.711 ± 0.059	32.0	<0.01	-0.941 ± 0.038	5.0	>0.05
Hyaluronidase	Control	-1.087 ± 0.097					
	Experiment	-1.088 ± 0.086	0.1	>0.05			

Concentrations were: neuraminidase, 20 units/ml of Hank's salt solution at pH 7.0; chondroitinase-ABC, 1 unit/ml of 0.05 M acetate-Veronal-buffered saline at pH 8.0; and hyaluronidase, 10 turbidity-reducing units/ml of 0.02 M acetate-Veronal-buffered saline at pH 6.0. The cells were incubated in each enzymic solution at 37°C for 30 min.

as compared with glutaraldehyde treatment alone.

Ionogenic groups of the cell surface. Changes in cell electrophoretic mobility were estimated after treatment with specific enzymes for sialic acid and acid mucopolysaccharides to examine the distribution of ionogenic groups at the cell surface. The results are shown in Table 2. When intact cells were treated with neuraminidase and chondroitinase-ABC, the electrophoretic mobility was significantly reduced about 25% and 30% in neuraminidase and chondroitinase, respectively. On the contrary, when the cells were pretreated with Con A at 20 $\mu\text{g/ml}$ at 37°C for 30 min, no further reductions in cell mobility were found by subsequent treatment with chondroitinase-ABC.

DISCUSSION

Reported Con A effects on cell surface include cell agglutination (1, 8, 13), clustering of agglutinin-binding sites (9, 11–13), changes in electrophoresis (22), and resistance to trypsinization (18). The present experiment has also demonstrated that cell electrophoretic mobility is significantly reduced by Con A treatment at concentrations of more than 5 $\mu\text{g/ml}$. The reduction in electrophoretic mobility is a specific surface alteration for Con A binding on the cell surface since the Con A-induced alteration was reverted by addition of α -methyl-D-mannoside which removed more than 60% of Con A bound from the C2W cell surface (17). Cell agglutination and resistance to trypsinization were found in C2W cells at these concentrations of Con A (18). Furthermore, the result from the binding experiment of labeled Con A indicated that more than 30% of Con A receptors were bound with lectin at these higher Con A concentrations.

The surface modification with protein linkage agents inhibited the Con A-induced mobility reduction, although cells treated with glutaraldehyde had a higher mobility value than intact cells, suggesting the presence of potential cationogenic groups at the cell surface (7, 10). Since cross-linkage of proteins inhibits their lateral movement on the cell surface (19), the Con A-induced reduction in the surface charge seems to be a phenomenon associated with the rearrangement of Con A receptor proteins at the cell surface. The present study has also demonstrated that pretreatment of cells with sulfhydryl-blocking agents inhibits the Con A-induced mobility reduction without changing the number of Con A-binding sites on the cell surface. The detailed mechanism of its inhibitory effect on mobility reduction is unknown from the present results. We previously reported a reduction in cell electrophoretic mobility after X-irradiation and its protection by pretreatment of cells with sulfhydryl blocking agents (15) or small amounts of Con A (17). This seems to imply a configurational change in receptor proteins by binding of Con A and the participation of sulfhydryl residues of membrane components in the configurational change. Thus, the present results may suggest that sulfhydryl residues of membrane components play an important role in the rearrangement of receptor proteins by binding of Con A.

The study of negatively charged sugars on the cell surface has demonstrated the lack of negative charge due to chondroitin sulfates in Con A-treated cells, since chondroitinase-ABC treatment was not effective in reducing the electrophoretic mobility of Con A-pretreated cells while effective in intact cells. The Con A used had no proteolytic enzyme and glycosidase activities by our tests. Furthermore, it is unlikely

that the enzyme was not accessible to the cell surface for cell aggregation produced by Con A treatment, because Con A and the subsequent enzyme treatment were performed without stripping off the cells from the culture dish. We have observed in other cultured cells that Con A treatment of cells resulted in the removal of a larger amount of chondroitin sulfates from the cell surface by enzyme digestion without changing the electrophoretic mobility, as compared with intact cells (Kojima and Sato, in preparation). Under the measuring conditions used, the cell electrophoretic method detects charged groups only within 10 Å of the periphery of the cell surface (6). The present results, therefore, suggest the dislocation of chondroitin sulfate from the peripheral zone to the deeper zones of the cell surface by Con A treatment. Similar alterations in cell surface distributions of acid mucopolysaccharides were also reported in lymphoblastic cells treated with phytohemagglutinin (16).

The results thus indicated that Con A treatment of cells produced not only clustering of the lectin-binding sites but also architectural alterations of other membrane components, such as acid mucopolysaccharides on the cell surface, and suggested the possible involvement of sulfhydryl residues in the architectural membrane alteration. Chondroitin sulfate-protein complex is different from the Con A receptor proteins. The mechanism of information transmission from one receptor protein to another protein is quite obscure. The possible involvement of contractile proteins of the membrane was suggested in the mitogenic effects of Con A (3, 5, 24). It was also shown that phosphorylation of membrane proteins plays an important role in the rearrangement of membrane molecules (4). Further study is required to elucidate the characteristics of the conformational change in glycoproteins and the information transmission mechanism induced by binding of Con A.

Acknowledgment. This work was partially supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. BURGER, M.M. and A.R. GOLDBERG. Identification of a tumor-specific determinant on neoplastic cell surfaces. *Proc. Natl. Acad. Sci. USA* **57**, 359—366, 1967
2. CLAUNCH, C., A. OIKAWA, T.T. TCHEN and F. HU. Advances in biology of skin. Vol. 8, *The Pigmentary System*. ed. MONTAGNA W. and F. HU, Pergamon press, Oxford, pp. 479—488, 1967
3. EDELMAN, G.M., I. YAHARA and J.L. WANG. Receptor mobility and receptor-cytoplasmic interactions in lymphocytes. *Proc. Natl. Acad. Sci. USA* **70**, 1442—1446, 1973
4. GAZITT, Y., I. OHAD and A. LOYTER. Phosphorylation and dephosphorylation of membrane proteins as a possible mechanism for structural rearrangement of membrane components. *Biochim. Biophys. Acta* **436**, 1—14, 1976
5. GUNTHER, G., J.L. WANG, I. YAHARA, B. CUNNINGHAM and G.M. EDELMAN. Concanavalin A derivatives with altered biological activities. *Proc. Natl. Acad. Sci. USA* **70**, 1012—1016, 1973
6. HAYDON, D.A. The surface charge of cells and some other small particles as indicated by electrophoresis. I. The zeta potential-surface charge relationships. *Biochim. Biophys. Acta* **50**, 450—457, 1961
7. HEARD, D.H. and G.V.F. SEAMAN. The action of lower aldehydes on the human erythrocytes. *Biochim. Biophys. Acta* **53**, 336—374, 1961
8. INBAR, M. and L. SACHS. Interaction of the carbohydrate-binding protein concanavalin A with normal and transformed cells. *Proc. Natl. Acad. Sci. USA* **63**, 1418—1425, 1969
9. INBAR, M. and L. SACHS. Mobility of carbohydrate containing sites on the surface membrane in relation to the control of cell growth. *FEBS Lett.* **32**, 124—128, 1973
10. KOJIMA, K. and A. MAEKAWA. A difference in the architecture of surface membrane between

- two cell types of rat ascites hepatomas. *Cancer Res.* **32**, 847—852, 1972
11. MARTINEZ-PALOMO, A., R. WICKER and W. BERNHARD. Ultra-structural detection of concanavalin surface receptors in normal and in polyoma-transformed cells. *Int. J. Cancer* **9**, 676—684, 1972
 12. NICOLSON, G.L. Difference in topology of normal and tumour cell membranes shown by different surface distributions of ferritin-conjugated concanavalin A. *Nature New Biol.* **233**, 244—246, 1971
 13. NICOLSON, G.L. Temperature-dependent mobility of concanavalin A sites on tumour cell surfaces. *Nature New Biol.* **243**, 218—220, 1973
 14. OHYA, T. and T. KANEKO. Novel hyaluronidase from *Streptomyces*. *Biochim. Biophys. Acta* **198**, 607—609, 1970
 15. SATO, C. and K. KOJIMA. Changes in electrophoretic mobility of cultured cells after X-irradiation and their modification by SH-blocking agents and hemagglutinin. *Radiat. Res.* **60**, 506—515, 1974
 16. SATO, C. and K. KOJIMA. Phytohemagglutinin-induced change in the distribution of acidic sugars in surface membrane of lymphoid cells and blocking of the radiation effect. *Exp. Cell Res.* **98**, 90—94, 1976
 17. SATO, C., K. KOJIMA, K. NISHIZAWA, S. SHIMIZU and M. INOUE. Small amount of concanavalin A modifies radiation-induced alteration in cell-surface depending on its binding condition. *Biochim. Biophys. Acta* **448**, 379—387, 1976
 18. SATO, C. and K. TAKAZAWA-NISHIZAWA. Changes in cell-to-dish contact of cultured mammalian cells induced by concanavalin A. *Exp. Cell Res.* **89**, 121—126, 1974
 19. SILVA, P.P. Translational mobility of the membrane intercalated particles of human erythrocyte ghosts. *J. Cell Biol.* **53**, 777—787, 1972
 20. SINGER, S.J. and G.L. NICOLSON. The fluid mosaic model of the structure of cell membranes. *Science* **175**, 720—731, 1972
 21. SONODA, S. and M. SCHLAMOWITZ. Studies of ¹²⁵I trace labeling of immunoglobulin G by chloramine-T. *Immunochemistry* **7**, 885—898, 1970
 22. YAMADA, T. and M. YAMADA. Changes in distribution of electric charges in the surface membrane of rat ascites hepatocarcinoma cells induced by Con A. *Nature* **244**, 297—299, 1973
 23. YAMAGATA, T., H. SAITO, O. HABUCHI and S. SUZUKI. Purification and properties of bacterial chondroitinases and chondrosulfatases. *J. Biol. Chem.* **243**, 1523—1535, 1968
 24. WANG, J.L., G.R. PIRAS and G.M. EDELMAN. Inhibition by colchicine of the the mitogenic stimulation of lymphocytes prior to the S phase. *J. Cell Biol.* **66**, 128—144, 1975

(Received for publication, February 15, 1977)