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Increase in cell surface wheat germ agglutinin binding in a rat hepatoma cell line dRLa 74 treated with concanavalin A.

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Abstract

Wheat germ agglutinin binding to a rat hepatoma cell line dRLa 74 treated with concanavalin A was studied. It increased depending on the concanavalin A concentration in the culture medium. The cells exhibited about twofold increase in wheat germ agglutinin-binding when pretreated with 50 micrograms/ml of concanavalin A for 48 h. The wheat germ agglutinin binding sites were shown to be localized at the cell surface by lectin-histochemistry. Wheat germ agglutinin blotting of microsomal membrane proteins showed a broad wheat germ agglutinin-reactive band with an apparent molecular weight of 90 to 100 kDa. Loss of wheat germ agglutinin binding to dRLa 74 cells and the glycoprotein after neuraminidase treatment suggested that wheat germ agglutinin reacted with cell surface sialyl residues of dRLa 74 cells. The induced change was reversible. Increased wheat germ agglutinin binding returned to the pretreatment level when the concanavalin A-treated cells were subcultured in the absence of concanavalin A. These observations suggest that environmental factors interacting with tumor cell surface sugar moieties may induce reversible epigenetic changes on cell surface carbohydrate structures.

KEYWORDS: lectin, glycoprotein, hepatoma, cell line, rat

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Eukaryotic cells carry complex carbohydrates on their cell surfaces in the form of glycoproteins, glycolipids, and polysaccharides. Although not definitely proved, they have been supposed to be involved in cell-cell and cell-substratum interactions (1, 2). They play a key role in social behavior of normal cells, their differentiation and development (3, 4). Abnormal behavior of malignantly transformed cells may be related to the cancer-associated changes of cell surface carbohydrate structures (5, 6), especially negatively charged sialic acid (7-10).

Lectins have been used to characterize the changes in cell surface complex carbohydrates associated with malignant transformation of cells (11, 12). They have been also used to select tumor cell subclones resistant to the cytotoxic levels of specific lectins *i.e.*, mutants lacking the

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List of abbreviations used in this paper: Con A, Concanavalin A; DBA, Dolichos biflorus agglutinin; FITC: Fluorescein isothiocyanate; GlcNAc, N-acetylglucosamine; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; kDa, Kilodaltons; MEM, Modified Eagle's medium; NeuNAc: N-acetylneuraminic acid; PAGE, Polyacrylamide gel electrophoresis; PBS, Phosphate buffered saline; PNA, Peanut agglutinin; RCA 1, Ricinus communis agglutinin 1; SBA, Soybean agglutinin; SDS, Sodium dodecyl sulfate; UEA, Ulex europaeus agglutinin; WGA, Wheat germ agglutinin.

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lectin reactivity (13, 14). Cell surface carbo-hydrate phenotype of these variants is stable and generally believed to be associated with genetic changes.

The change of tumor cell surface complex carbohydrates, however, may not necessarily require genetic changes. Cell surfaces may be modulated epigenetically (15). Cell surface carbohydrate phenotype of tumor cells has been shown to be dependent on the sites where they grow (8, 9). Interaction between tumor cells and soluble or immobilized ligands specific for sugar chains such as animal lectins and antibodies may transiently modulate tumor cell surface structures for successful survival, invasion, and metastasis of tumor cells. In the present study, we examined whether the lectin treatment of a rat hepatoma cell line dRLa 74 induced the epigenetic change in the cell surface sialylation. Con A was used as a modulator and WGA binding was used to examine the change in cellular sialylation. Pretreatment with Con A induced increased WGA binding, *i.e.*, cell surface sialylation. The change was reversible. Both genetic and epigenetic changes in cell surface complex carbohydrates must be important to determine cellular behavior of tumor cells.

Materials and Methods

Cells and Con A treatment. A rat hepatoma cell line dRLa 74 was established from 4-dimethylaminoazobenzene-induced Donryu rat hepatoma at the Department of Pathology, Cancer Institute, Okayama University Medical School by Dr. J Sato (16). It has been continuously cultured in MEM containing 10 % fetal calf serum.

dRLa 74 cells were plated at 10^5 cells/ml/well in a NUNCLON 24-well multiwell-dish (Nunc, Denmark) and cultured for 24 h at 37 °C. They were treated with Con A (Vector Lab., Inc., Burlingame, CA) at final concentrations of 0 to 50 μg/ml and further cultured to be confluent for 48 h. When indicated, the Con A-treated cells were subcultured in the presence or absence of Con A to examine the reversibility of the change induced by the first lectin treatment.

Radio-labelled WGA binding to Con A-treated dRLa 74 cells. Nonfixed dRLa 74 cells attached to the plastic plate were used in the binding assay, since vigorous pronase treatment to make dRLa 74 cells single cell suspension might artificially modify the WGA binding sites.

We washed the cells two times with 50 mM Tris-HCl buffer, pH 7.4 containing 50 mM HEPES, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 2 mM EDTA, 5 mM KCl, 55 mM NaCl, and 0.5 % bovine serum albumin (Buffer L) and added 0.5 ml of Buffer L containing 33.8 ng of ^{125}I-labelled WGA to them. After incubation for one hour at room temperature, cells were washed two times with the same buffer and three times with PBS, and solubilized with 2 % SDS in water. Radioactivity and protein concentration were determined on the aliquots. Results were expressed as the bound WGA per mg protein.

Inhibition of WGA binding with ovalbumin. dRLa 74 cells were cultured in the absence or presence of 50 μg/ml of Con A. Ovalbumin (Sigma Chem. Co., St. Louis, MO) at a final concentration of 2 mg/ml was included in the assay mixture.

Effect of posttreatment of Con A-pretreated cells with methyl-α-D-glucopyranoside on their WGA binding. Fetal calf serum glycoproteins reactive with both Con A and WGA may affect the results if Con A attached to the cell surfaces binds such glycoproteins, which in turn bind WGA. To exclude this possibility, the bound Con A was eluted before the assay. Con A-treated cells were washed with MEM, incubated in 25 mM methyl-α-D-glucopyranoside (Sigma Chem. Co.) in the same medium at room temperature for one hour, and subjected to the binding assay.

SDS-PAGE and WGA blotting. Microsome fraction of dRLa 74 cells was prepared as previously described (17). SDS-PAGE in the presence of mercaptoethanol was performed according to the procedure of Laemli (18) in a 7.5 % separating gel. Proteins separated were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad Lab., Richmond, CA) according to the procedure of Towbin *et al.* (19). The membrane was washed with PBS, blocked with 5 % bovine serum albumin in PBS, washed again with PBS, and incubated with ^{125}I-labelled WGA (3 x 10^5 cpm/ml) in PBS containing 2 % polyvinylpyrolidone K-30 (Nakarai Chemicals, Ltd., Tokyo) at room temperature for 6 h. After thoroughly washing with the same buffer, the membrane was subjected to autoradiography with the use of a Du Pont CRONEX Lightening Plus screen and

Fuji-RX x-ray film (Fuji Photo Film Co., Ltd., Tokyo).

Lectin histochemical procedure. dRLa 74 cells were cultured in the presence or absence of 50 μg/ml of Con A on a cover glass as described. After washing with PBS, they were reacted with 50 μg/ml of FITC-WGA (Vector Lab. Inc.) at 4 °C for 60 min. They were then mounted in PBS: glycerin (9: 1) and observed using a fluorescein microscope (Model BH-RFL, Olympus, Tokyo). The fixed cells were digested with neuraminidase at room temperature overnight and immunostained as previously described (20).

Other procedures. WGA (Vector Lab., Inc.) was radioiodinated in a chloramine T method as described previously (21). Carrier free Na^{125} I was obtained from Radiochemical Center (Amersham, Bucks, UK). Protein concentration was determined by a Lowry method (22) using bovine serum albumin as standard. Data were expressed as means ± SD, and the differences between groups were analyzed with the unpaired *t*-test.

Results

Increased WGA binding to dRLa 74 cells after the treatment with Con A. dRLa 74 cells were reactive with various lectins including Con A, WGA, RCA 1, DBA, PNA, and UEA (data not shown). Con A and WGA showed relatively higher binding activities. To see the change in cell surface sialylation induced by the binding of sugar specific molecules to cell surfaces, effects of Con A-pretreatment on the WGA binding of dRLa 74 cells were examined in the following studies. Cells cultured in the presence of Con A did not differ significantly in microscopic appearance and cell size from those grown in the absence of Con A. Inhibition of cell growth by Con A was less than 10 % in concentrations used in the present study.

Cells grown in the presence of 20 μg/ml or more of Con A showed the significant increase in WGA binding activity compared with the nontreated cells (Fig. 1). At lower concentrations, *i. e.*, less than 10 μg/ml, no apparent increase in the binding was observed. Maximum binding was attained at 40 μg/ml of Con A. It was about twice of that of the untreated cells.

The binding at 50 μg/ml of Con A was not significantly different from that at 40 μg/ml of Con A. More than 50 μg/ml of Con A was cytotoxic to dRLa 74 cells.

Reversibility of Con A-effect on WGA binding. The increase in WGA binding induced with Con A pretreatment was reversible (Table 1). Cells cultured in the presence of 25 μg/ml of Con A exhibited 45 % increase in WGA binding (p < 0.01). When these cells were subcultured in the absence of Con A, WGA binding returned to the level observed in the nontreated dRLa 74 cells, *i. e.*, 145 ± 8 % to 104 ± 15 %. However, when the treated cells were further subcultured in the presence of Con A, the binding increased further to 226 % (p < 0.01). Percent increase in the second Con A-treatment was 56 % and comparable with that in the first treatment, *i. e.*, 45 %. When dRLa 74 cells not treated with Con A in the first culture were subcultured in the presence of Con A, the 36 % increase was observed (p < 0.05). The cells subcultured in the absence of Con A did not show any significant change in
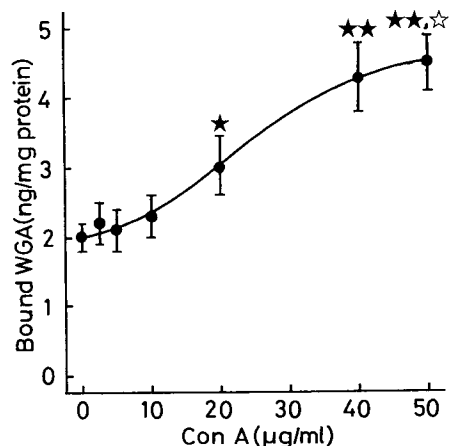

Fig. 1   Effect of Con A treatment on WGA binding to dRLa 74 cells. dRLa 74 cells were cultured to be confluent in the presence of 0 to 50 μg/ml of Con A and assayed for their WGA binding as described in "Materials and Methods". "Mean ± SD" of three determinations are presented. ★: p < 0.05, ★★: p < 0.01 compared with the value in the absence of Con A. ☆: not significant compared with the value at 40 μg/ml of Con A.

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WGA binding.

Effect of ovalbumin on WGA binding to dRLa 74 cells. The WGA binding to the Con A-treated and -nontreated cells was effectively inhibited with ovalbumin (Table 2). When 2 mg/ ml of ovalbumin were included in the assay mixture, the WGA binding of the Con A-treated and -nontreated cells were inhibited 79 % and 87 %, respectively.

Effect of pretreatment of Con A-treated cells with methyl-α-D-glucopyranoside on WGA binding. Treatment of the Con A-pretreated cells with 25 mM methyl-α-D-glucopyranoside, which

was previously shown to inhibit Con A binding to human erythrocytes completely (21), did not affect the results significantly (data not shown).

WGA staining of SDS-PAGE separated membrane proteins. There was no appreciable difference between the SDS-PAGE separated protein staining patterns of the microsome fractions from dRLa 74 cells nontreated and treated with Con A (Fig. 2, A). WGA staining of the nontreated cells revealed a major WGA reactive broad bands with an apparent molecular weight of 90 to 100 kDa, *i.e.*, 90–100 kDaGP (Fig. 2, B, lane 1). Con A treatment of the cells resulted in
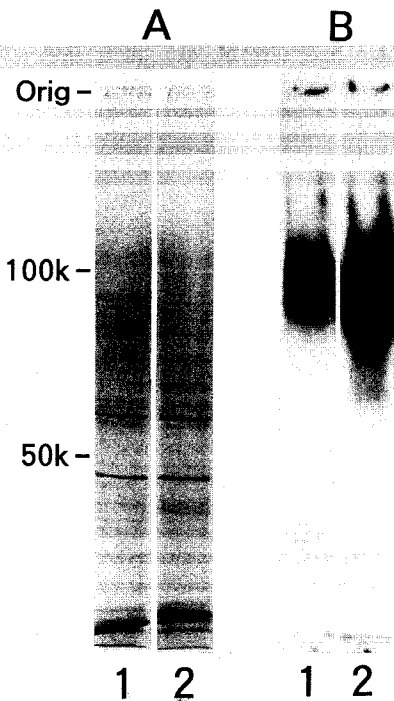
Fig. 2 WGA staining of SDS-PAGE-fractionated microsomal fractions. B: Microsomal fractions from dRLa 74 cells cultured in the absence (lane 1) and presence (lane 2) of 50 μg/ml of Con A were fractionated by SDS-PAGE, transferred to a nitrocellulose sheet, reacted with ^{125}I-labelled WGA, and subjected to autoradiography.
A: Protein staining with coomassie brilliant blue. 100 μg of membrane protein per lane were applied. Positions for the separating gel origin and the molecular weights of 100 and 50 kDa are shown.

Table 1 Reversibility of the increased WGA binding induced by Con A treatment[a].

Con A in the 1st culture	WGA binding after the 1st culture	Con A in the 2nd culture	WGA binding after the 2nd culture
—	100 ± 9 %	—	100 ± 14 %
		+	136 ± 10 %[c]
+	145 ± 8 %[b]	—	104 ± 15 %[d]
		+	226 ± 23 %[b,d]

[a]dRLa 74 cells were cultured in the absence or presence of 25μg/ ml of Con A for 24 h and determined for their WGA binding (WGA binding after 1st culture). The cells were subcultured after the first culture in the absence or presence of Con A and assayed for the WGA binding (WGA binding after 2nd culture). WGA binding of the cells after the first culture without Con A was taken 100 %. Results are expressed as "mean ± SD" of three determinations.
[b]p < 0.01 and [c]p < 0.05 compared with the WGA binding after the first culture in the absence of Con A.
[d]p < 0.01 compared with the WGA binding after the first culture in the presence of Con A.

Table 2 Inhibitory effect of ovalbumin on WGA binding to the Con A-treated dRLa 74 cells[a]

Ovalbumin (2 mg/ml)	Bound WGA (ng/mg protein)	
	Con A treatment	
	(−)	(+)
(−)	2.2 ± 0.2	4.2 ± 0.5
(+)	0.3 ± 0.1 (87 %)[b]	0.8 ± 0.3(81 %)[b]

[a]WGA binding was determined in the absence or presence of 2 mg/ml of ovalbumin as described in "Materials and Methods". Results are presented as "mean ± SD" of three determinations.
[b]Values in the parenthesis are percent inhibition by ovalbumin.
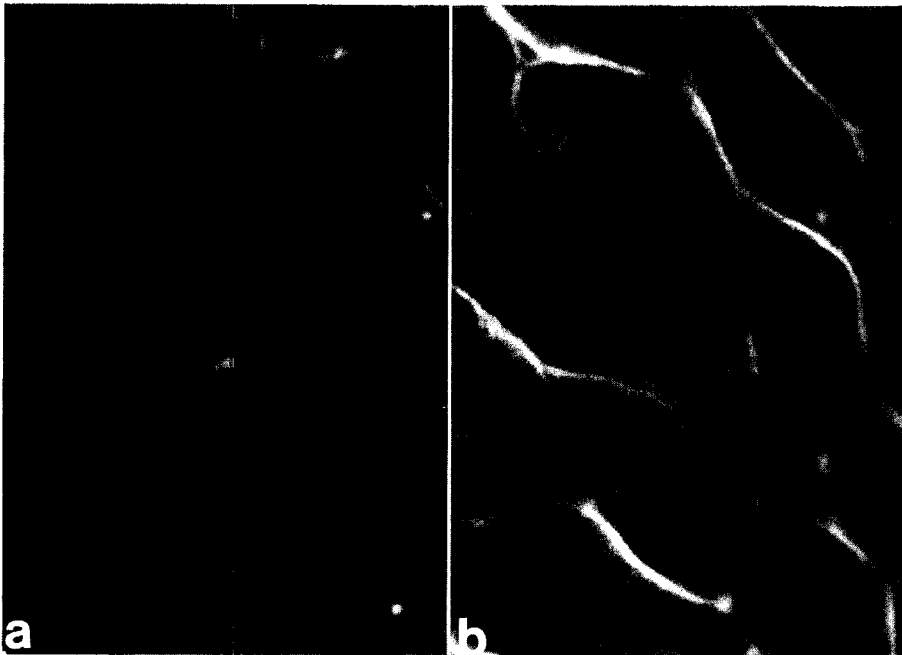
Fig. 3 Lectin histochemistry of dRLa 74 cells. dRLa 74 cells cultured in the absence (a) or presence (b) of 50 μg/ml of Con A were stained with FITC-labelled WGA as described in "Materials and Methods".

the enhanced WGA binding to this band (Fig. 2, B, lane 2). There was no WGA reactive glycoprotein specific for the Con A-treated cells. WGA binding was completely abolished when the nitrocellulose membrane was treated with 0.5 units/ml of vibrio cholerae neuraminidase (Test-neuraminidase, Behringwerke AG, Marburg) in 0.1 M sodium acetate buffer, pH 4.5 containing 0.1 % $CaCl_2$ at room temperature for 16 h (data not shown). WGA did not react with Con A on a nitrocellulose membrane.

Cellular localization of WGA binding sites by lectin histochemistry. When dRLa 74 cells treated with Con A were stained with FITC-labelled WGA, surface membranes were positively stained (Fig. 3, b). The number of positively stained cells was much greater and the intensity of membrane fluorescence was stronger in the Con A treated cells compared with the untreated cells (Fig. 3 a, *vs.* b). Although the untreated cells were only faintly stained generally, those cells piling up

after becoming confluent were stained rather strongly. Neuraminidase treatment of the fixed cells reduced the WGA stainability (data not shown).

Discussion

In the present study, we showed a lectin induced change in cell surface carbohydrate structures of a rat hepatoma cell line dRLa 74. WGA binding activity of this cell line increased in the presence of Con A. The observed change was not due to the selection of Con A resistant mutants, since Con A at the concentrations used in the present study was not cytotoxic to dRLa 74 cells. Moreover, the WGA binding returned to the level of the nontreated cells promptly on the removal of Con A from the culture medium. These observations suggest that the interaction of molecules with cell surface carbohydrate antigens

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may epigenetically modulate the latter structures. This type of cell surface modulation may make tumor cells escape from the selection pressure of their environment and increase their tissue adaptability *in vivo.*

Metastatic site specific differences in cell surface sialylation (8) and WGA reactivity (9) of tumor cells have been reported. Although these *in vivo* studies suggested the epigenetic modulation of cellular complex carbohydrates, mechanisms governing these inductions has not been elucidated. We demonstrated that a ligand reactive with cell surface glycoconjugates, *i. e.,* Con A, induced the change in WGA reactivity. And it was related with cell surface sialylation. It should be noted that two above reports also indicated the possible epigenetic changes in cell surface sialylation.

WGA binds specifically to N-acetyl-D-glucosamine and its β 1→4 oligomers (23), and N-acetylneuraminic acid and neuramin 2→3 lactose (24). It was suggested previously that in the interaction between the cell surface and WGA, non reducing terminal NeuNAc residues of glycocojugates play a more important role than the usually internal GlcNAc residues (24). Interaction of WGA with dRLa 74 cells was sugar specific, since it was inhibited by ovalbumin which has a high affinity for WGA. We showed furthermore that neuraminidase treatment of SDS-PAGE-fractionated glycoproteins completely abolished the WGA reactivity of 90–100 kDaGP from both the untreated and Con A-treated dRLa 74 cells. And desialylation of the fixed cells profoundly reduced the WGA reactivity. These results suggested that NeuNAc was responsible for WGA binding to dRLa 74 cells. Mechanisms for the increased WGA binding by Con A-pretretment may be i) enhanced synthesis of 90–100 kDaGP, ii) hypersialylation of the glycoprotein, or iii) increased affinity of WGA binding sites due to the reorganization of the cell surface glycoproteins. The last possibility seems unlikely as the WGA binding to the glycoproteins separated by SDS-PAGE after the

disorganization of membrane glycoproteins with the detergent was also higher in the Con A-treated cells than the nontreated cells. Further study is needed to elucidate whether the observed change is associated with the enhanced de novo synthesis of the peptide portion of 90–100 kDaGP or the change in the glycosylation.

Cell surface carbohydrate structures have been implicated in cell-cell and cell-substratum interactions (1, 3). Their expression must be strictly regulated in embryogenesis and normal development (4). Aberration of their expression is often observed in malignant transformation of cells (5, 6). Difficulty in ordinary cell-cell interaction due to the uncontrolled expression of cell surface carbohydrate antigens may be responsible for abnormal tumor cell growth and metastasis. Changes in cell surface sialylation have been especially correlated with metastasis (7, 10, 13, 25 –27). On the other hand, proteins specifically reactive with carbohydrate antigens such as animal lectins and antibodies play a complementary role in carbohydrate mediated recognition system (28). Interaction of the carbohydrate antigens and the animal lectins must be one of the important factors governing social behavior of cells (28). Present results suggested that cell surface carbohydrates of malignantly transformed cells can change epigenetically. When tumor cells invade or metastasize, they may change their cell surface carbohydrate antigens for adaptation. Similarly, antibodies reacting with cell surface carbohydrate antigens may also induce the phenotypic change of the cell surface. Cell surface carbohydrate phenotype, in normal and pathological conditions, must be dually regulated, genetically and epigenetically.

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