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AN α-GALACTOSYL RESIDUE IN THE LARGE CARBOHYDRATES OF TERATOCARCINOMA CELLS: THE ANTIGENIC DETERMINANT RECOGNIZED BY SERA FROM PATIENTS WITH OVARIAN GERM CELL TUMORS

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SUMMARY—— Certain patients with ovarian germ cell tumors develop a specific antibody reacting with glycoprotein-bound large carbohydrates of murine teratocarcinoma cells. The antigenic determinant was found to involve an α-galactosyl residue, since α-galactosidase from coffee bean, but not other glycosidases abolished the antigenic activity of the large glycan isolated from F9 and OTT6050 cells. Several evidences excluded the possibility that the antigen is blood group B or P_1 antigen. These results indicate tumor-associated expression of an unusual α-galactosyl residue in human ovarian germ cell tumors.

Patients with malignant ovarian tumors of germ cell origin, such as yolk sac tumor and teratocarcinoma, frequently developed an antibody (1) reacting with the glycoprotein-bound large carbohydrates isolated from murine teratocarcinoma cells (2). The large carbohydrates termed "embryoglycan" have complex core structures composed of galactose and N-acetylglucosamine (3). In no case, did sera from normal human subjects or patients with ovarian benign cystic teratoma or ovarian adenocarcinoma react with the glycan (1). The result suggests that the human malignant tumors express a unique carbohydrate sequence which is shared by the glycan from murine teratocarcinomas. Because of the strong antigenicity of the sequence, and its apparently specific expression in tumors of germ cell origin, the biochemical nature of the determinant is of significant interest from the view point of tumor-associated

Abbreviations ; DBA, Dolichos biflorus agglutinin ; PBS, Dulbecco's phosphate buffered saline.

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alteration of cell surface. In order to identify the non-reducing sugar involved in the antigenicity, we attempted to inactivate the antigen by a specific exoglycosidase which acts on the non-reducing terminus of glycans.

Materials and Methods

Preparation of Radioactively-labeled Embryoglycan Embryoglycan labeled with 6-[^{3}H]-galactose (4.0 Ci/mmol, New England Nuclear) was prepared as described previously (4) from teratocarcinoma stem cell F9 (5) or teratocarcinoma OTT6050 (6) grown in vitro (7). Embryoglycan was also prepared from receptors for Dolichos biflorus agglutinin (DBA) isolated from teratocarcinoma OTT6050 (8) : the receptors were either metabolically labeled with 6-[^{3}H]-fucose (15 Ci/mmol, New England Nuclear) or externally labeled by the galactose oxidase-NaB[^{3}H]$_4$ method (9). In the latter case, 0.2 mg of the receptors prepared as described previously (7) was reacted with 5 units of galactose oxidase (Worthington) at 37°C for 30 min. in 0.4 ml of Dulbecco's phosphate buffered saline (10) containing 0.1 % Triton X-100, reduced with 400 μCi of NaB[^{3}H]$_4$ (100 mCi/mmol, New England Nuclear) in 40 μl dimethylsulfoxide for 1 h at room temperature, and was purified again by affinity chromatography on DBA-agarose (8). Acid hydrolysis (2 N HCl, 100°C, 4 h) and paper chromatography in butanol-pyridine-H_2O (6:4:3) revealed that 76 % of the radioactivity in the galactose oxidase-NaB[^{3}H]$_4$ labeled glycan was in galactose and the rest in N-acetylgalactosamine.
Glycosidase Digestion Embryoglycan (60,000 cpm) was incubated with glycosidases in 0.06 ml of reaction mixture for 24 h at 37°C with a small amount of toluene. The source of the glycosidase, its amount in the reaction mixture, and the composition of the reaction medium were as follows : α-galactosidase [coffee bean, Boehringer Mannheim, 0.48 U, 0.15 M citrate-phosphate buffer, pH 5.4 containing 0.03 M NaCl] ; β-galactosidase [jack bean, Seikagaku Fine Chemicals, heated at 60°C for 30 min. before use to inactivate contaminating α-galactosidase, 0.18 U, 0.15 M citrate-phosphate buffer, pH 4.0 containing 0.03 M NaCl] ; β-N-acetylhexosaminidase [jack bean, Seikagaku Fine Chemicals, 0.28 U, 0.15 M citrate-phosphate buffer, pH 5.0] ; α-L-fucosidase [C. lampas, Seikagaku Fine Chemicals, 24 mU, 0.15 M citrate-phosphate buffer, pH 4.0 containing 0.3 M NaCl] ; α-N-acetylgalactosaminidase [C. perfringens, Bethesda Research Lab., 60 mU, 0.1 M potasium phosphate buffer, pH 6.0, containing 1 mM $CaCl_2$] ; neuraminidase [C. perfringens, Worthington Biochemical Co., 18 mU, 0.1 M sodium acetate buffer, pH 5.0].
Immunoprecipitation The antigenic activity of embryoglycan was determined by immunoprecipitation [modified Farr's assay (11)] as described previously (1). The sera used were from a teratocarcinoma patient (H.N., 26 yeard old Japanese), and a patient with a yolk sac tumor (M.K., 31 years old Japanese) (1).

Results and Discussion

Sera from two patients, one with yolk sac tumor and the other with teratocarcinoma, were used to detect the unique antigen in embryoglycan. About 1/4 of the total galactose-labeled glycan from F9 cells (teratocarcinoma stem cells) and OTT6050 teratocarcinoma cells were precipitated by the two sera in a modified Farr assay (Table I).

Digestion with β-galactosidase, β-N-acetylhexosaminidase, α-N-acetylgalactosaminidase or with α-L-fucosidase did not significantly alter the

Table I Effect of glycosidase digestion on the antigenicity of embryoglycan detectable by sera from the patients.

Sources of the glycan and glycosidases used	Per cent of the labeled embryoglycan precipitable by the sera from a patient with	
	teratocarcinoma	yolk sac tumor
A. Galactose-labeled glycan from F9 cells		
No glycosidase	25.0	21.8
α-Galactosidase	2.0	2.0
α-Galactosidase, in the presence of galactonolactone (10 mg/ml)	2.4	2.3
β-Galactosidase	23.5	23.7
α-N-Acetylgalactosaminidase	24.1	21.9
β-N-Acetylhexosaminidase	25.2	20.7
α-L-Fucosidase	19.2	16.7
Neuraminidase	27.9	22.9
B. Galactose-labeled glycan from OTT6050 cells		
No glycosidase	24.7	27.4
α-Galactosidase	3.4	4.6
β-Galactosidase	20.8	25.5
α-N-Acetylgalactosaminidase	19.6	26.6
β-N-Acetylhexosaminidase	21.9	29.7
α-L-Fucosidase	18.8	21.3
C. Galactose oxidase-NaB[^{3}H]$_4$ labeled glycan from DBA receptors of OTT6050 cells		
No glycosidase	19.5	22.9
α-Galactosidase*	2.7	3.4
D. Fucose-labeled glycan from DBA receptors of OTT6050 cells		
No glycosidase	16.3	21.2
α-Galactosidase**	1.9	3.8

* As shown in Fig. 1 B, 58 % of the label was released as free galactose, but the rest still attached to the glycan.

** No release of fucose was confirmed by Sephadex G-25 column chromatography performed as described in Fig. 1 B.

antigenic activity. However, α-galactosidase from coffee bean was found to abolish the antigenic activity detectable by the both sera (Table I). α-Galactosidase released 10 % of galactose-label from the glycan of both F9 and OTT6050 cells as revealed by paper chromatography of the reaction products in butanol-pyridine-H_2O (6:4:3). Thus, these results apparently indicated that significant amounts of α-galactosyl termini were present in embryoglycan and that some of them contained immunodeterminant group detectable by the patients' sera.

The α-galactosidase preparation from coffee bean is a highly purified one. The level of contaminating glycosidases measured by using p-nitrophenyl glycosides as substrates were less than 0.1 % of the principal activity. However, it was necessary to exclude the possibility that a contaminating glycosidase which does not act on p-nitrophenyl glycosides but acts on natural substrates is responsible for the inactivation. α-L-Fucosyl, β-galactosyl and α-N-acetylgalactosaminyl residues have been identified as non-reducing sugars in embryoglycan (4, 8). Galactonolactone, a specific inhibitor of β-galactosidase, showed no effect on the inactivation (Table I, A). Thus, possible contamination with β-galactosidase is not responsible for the inactivation. The following experiments were performed to examine the possibility of contamination with α-N-acetylgalactosaminidase. Receptors for DBA were prepared from teratocarcinoma OTT6050. Since DBA is a lectin specific for N-acetylgalactosamine terminus, isolation of the receptors is helpful in concentration of glycoproteins with the terminus. Embryoglycan from the receptors was also reactive to the patients' sera, and the reactivity was abolished by α-galactosidase digestion (Table I, C, D). After the α-galactosidase digestion, no N-acetylgalactosamine was released (Fig. 1 A), while around 75 % of the galactose terminus labeled by the galactose oxidase -$NaB[^3H]_4$ method was released (Fig. 1 A, B). As above, embryoglycan was inactivated without any detectable release of N-acetylgalactosamine. Since no fucose-label was released from the glycan upon α-galactosidase treatment (Table I, D), possible contamination with α-L-fucosidase was also considered unlikely.

From all these results, the immunodominant group of the antigen was concluded to involve an α-galactosyl reisue. As α-galactosidase did not inactivate TC antigen, the previously described antigen on embryoglycan (12), the present antigen is different from TC antigen (data not shown). Although blood group B antigen is determined by an α-galactosyl residue, no correlation existed between anti-B activity measured by hemagglutination and the reactivity to embryoglycan (Table II). In order to further comfirm the

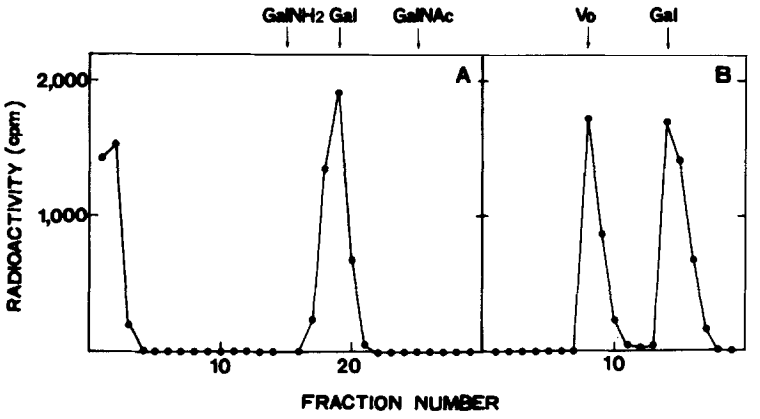

Fig. 1. α-Galactosidase digestion of galactose oxidase-NaB[^{3}H]$_4$ labeled embryoglycan isolated from DBA receptors of teratocarcinoma OTT6050. The glycan was digested with α-galactosidase as described in Materials and Methods.

(A) Analysis of the products by descending paper chromatography on Whatman 3 MM paper in butanol/pyridine/water (6:4:3) for 24 h. The chromatogram was cut into 1 cm strips and counted. Fraction 1 represents -1 to 0 cm from the origin. The elution position of standard monosaccharides is shown at the top of the figure. $GalNH_2$, D-galactosamine ; Gal, D -galactose ; GalNAc, N-acetyl-D-galactosamine.

(B) Analysis of the products on a column of Sephadex G-25 (superfine, 1 x 23 cm). The column was equilibrated and eluted with 0.05 M ammonium acetate buffer, pH 6.0. Fractions, 1 ml, were collected and counted. The elution position of standard substances is shown at the top of the figure. Vo, blue dextran ; Gal, galactose.

Degree of hydrolysis was more accurately determined in (B), since [^{3}H] -labeled embryoglycan was more efficiently counted in (B). From the experiment described in B, we could calculate that 58 % of the total label was released. Since 76 % of the label in the glycan was in the form of galactose, we concluded that about 3/4 of the labeled galactose termini was released by the enzyme.

difference from blood group B antigen, the patients' sera were absorbed by type B erythrocytes. Indeed, a portion of the antibody activity survived the massive absorption, after which no reactivity to type B erythrocytes was observed. Although the absorption removed the majority of the activity from the serum of a teratocarcinoma patient, only a slight decrease of the activity occurred in the serum of a patient with a yolk sac tumor. These results were interpretated as follows. The antigenic structure is somewhat similar to blood group B determinant, although is distinct from it. The antibodies in the teratocarcinoma patient have rather loose specificity, and thus a majority of them was removed by cross reaction to B antigen upon the absorption. The antibodies in the other patient have strict specificity, and thus

Table II Examination of the possible relationship between the patients' antibodies and the antibody against blood group B.

Sera from patients or normal volunteers	Per cent of the [^{3}H]-galactose-label precipitated from F9 cells	Per cent of the [^{3}H]-galactose-label precipitated from OTT6050 cells	Hemagglutination titer against blood group B erythrocytes**
A patient with teratocarcinoma			
No treatment	24.7	24.3	60
Absorption on type O erythrocytes*	20.5	16.4	60
Absorption on type B erythrocytes*	8.2	7.5	0#, §
A patient with yolk sac tumor			
No treatment	22.1	27.1	8
Absorption on type O erythrocytes*	18.2	21.0	4#
Absorption on type B erythrocytes*	15.4	16.6	0#, §
Normal volunteers			
Blood group O	3.3	4.9	40
Blood group A	2.1	3.1	40
Blood group B	1.5	2.8	0
Blood group AB	1.4	2.9	0

* Human sera were diluted 10-fold with PBS and mixed with the same volume of packed human erythrocytes of indicated blood group types. After 1 h on ice, the mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was used for immunoprecipitation.

** Hemagglutination titer was determined in a microtiter V-shaped multitest plates. Twenty μl of serially diluted human sera was added to a well and then 20 μl of human erythrocytes of blood group type B (1 % v/v) in PBS was added. After skaking the dish, the erythrocytes were allowed to settle for 1 h at room temperature. The result was expressed by the highest dilution of the serum still capable of hemagglutination.

\# Hemagglutination titer of these samples was determined after the concentration of the samples to the original volume.

§ Microscopic observation also revealed no hemagglutination.

were not absorbed significantly more as compared to absorption using type O erythrocytes (Table II).

The present antigen is also different from P_1 antigen, which is another α-galactosyl antigen (13), since absorption with P_1 positive erythrocytes (type O) did not remove the antibody activities (data not shown). Furthermore, the antisera did not agglutinate the P_1 positive erythrocytes.

These results indicate that an unusual α-galactosyl residue is expressed on human germ cell tumors and murine teratocarcinomas. This knowledge might

be helpful in considering diagnosis and immunotherapy of the malignant tumor. Furthermore, the same determinant can be present in early embryos of the human. The antibodies in the patients' sera may be helpful to follow the fate and to clarify biological meaning of the unusual carbohydrate linkage during early stages of embryogenesis.

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