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Griffonia simplicifolia Agglutinin-2-binding Glycoprotein as a Novel Carbohydrate Antigen of Human Colonic Carcinoma

Jun Nakayama,[1,4] Akira Okano,[2] Hiroshi Maeda,[1] Mami Miyachi,[1] Hiroyoshi Ota,[1] Tsutomu Katsuyama[3] and Masamitsu Kanai[3]

[1]*Central Clinical Laboratories, Shinshu University Hospital, Asahi 3-1-1, Matsumoto 390,* [2]*Department of Lipid Biochemistry, Institute of Cardiovascular Disease, Shinshu University School of Medicine and* [3]*Department of Laboratory Medicine, Shinshu University School of Medicine*

***Griffonia simplicifolia* agglutinin-2-binding glycoprotein (GBG) in human colonic carcinoma was examined immunochemically and histochemically. GBG was extracted from colonic carcinoma as a serum-type glycoprotein of 160 kilodaltons. GBG was not identical with carcinoembryonic antigen (CEA), since its molecular weight and localization in tissue sections were different from those of CEA. The non-reducing terminals of GBG probably carry N-acetylglucosamine, but not blood group determinants. Furthermore, GBG was released by phosphatidylinositol-specific phospholipase C from cell membrane. GBG was suggested to be anchored to the membrane via linkage to a glycosyl-phosphatidylinositol molecule. Among colonic carcinoma-associated antigens, serum-type glycoproteins having N-acetylglucosamine at non-reducing terminals have not previously been reported. GBG is a novel carbohydrate antigen of human colonic carcinoma.**

Key words: Colonic carcinoma — Lectin — Carcinoma-associated antigen

Sugar moieties of glycoprotein or glycolipid associated with mammalian cell membrane exhibit various alterations accompanying neoplastic transformation. Loss of antigenicity due to incomplete synthesis with or without precursor accumulation, enhanced- or neo-synthesis of sugar chains, and the appearance of abnormal antigens due to incompatible synthesis are well-known events occurring in neoplastic cells.[1-4] For clarification of the metabolism of complex carbohydrates in neoplastic cells, analysis of the sugar moieties of the cell surface membrane is essential. To detect these changes more precisely, lectins and, more recently, monoclonal antibodies raised against various sugar chains are powerful tools for immunochemical[5-7] as well as histochemical analysis.[8,9]

We have studied the mucosubstances produced in normal and neoplastic conditions of the human colon and elucidated their histochemical properties using various lectins and anti-blood group substance antibodies.[10-14] Among the many probes which showed affinities for colonic carcinoma, a lectin, GSA-2,[5] bound specifically to the apical surface of the carcinoma cells.[11] This study was undertaken to elucidate the biochemical properties of a carcinoma-specific glycoconjugate reactive with GSA-2, that is, GSA-2-binding glycoprotein (GBG) by immunochemical and histochemical techniques. Analyzing GBG is expected to help in developing a novel tumor marker for serum diagnosis.

In this paper, we describe the extraction of GBG and its structural characterization, comparing it with various carcinoma-associated antigens, such as carcinoma-specific UEA-1-binding glycoprotein,[5] blood group substances and CEA. Enzymatic digestions of GBG were also performed. The results indicated that GBG is a serum-type glycoprotein of 160 kDa. The non-reducing terminal of GBG might carry GlcNAc, but not blood group determinants.

MATERIALS AND METHODS

Cases Specimens from fifteen cases of invasive colonic carcinoma operated on at Shinshu University Hospital, Matsumoto, and Maruko Central General Hospital, Nagano prefecture, were used in this study. Patients' clinicopathological profiles are listed in Table I.

Immunochemical study Immunochemical procedures were as follows.

1. Tissue preparation: Freshly removed carcinoma tissues (about 500 mg of wet tissue) were taken from the luminal edge of medullary portions, avoiding possible contamination of collagenous tissues. The normal mucosae (about 500 mg of wet tissue) were stripped from the muscularis propria at the cut end. The samples

[4] To whom correspondence should be addressed.

[5] The abbreviations used are: CEA, carcinoembryonic antigen; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; GSA-2, *Griffonia simplicifolia* agglutinin-2; HRP, horseradish peroxidase; kDa, kilodaltons; 2-ME, 2-mercaptoethanol; NeuAc, N-acetylneuraminic acid; PAGE, polyacrylamide gel electrophoresis; PI-PLC, phosphatidylinositol-specific phospholipase C; PNGase F, peptide: N-glycosidase F; SDS, sodium dodecyl sulfate; UEA-1, *Ulex europaeus* agglutinin-1.

Table I. Clinicopathological Status of the Cases Examined

Case No.	Sex	Age	Blood group	Location of carcinoma	Histological type[a)]	Stage[b)]
1	M	67	AB	cecum	muci	B_2
2[c)]	F	74	A	ascending	por	C_2
3[c)]	F	40	O	ascending	mod	C_2
4	M	57	O	transverse	mod	B_2
5	F	72	O	transverse	mod	B_2
6	M	56	B	descending	mod	C_2
7	F	73	A	sigmoid	well	B_2
8	M	66	A	sigmoid	mod	B_2
9[c)]	F	80	A	sigmoid	well	B_2
10[c)]	F	75	B	sigmoid	well	C_2
11	F	63	O	sigmoid	muci	C_2
12	M	67	A	rectum	mod	C_2
13	F	52	O	rectum	well	B_2
14[c)]	F	73	O	rectum	well	B_2
15	M	79	A	rectum	well	B_1

a) Abbreviations: well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma; muci, mucinous adenocarcinoma.
b) Astler-Coller classification: B_1, penetration of muscularis mucosae without complete penetration of the muscularis propria; B_2, complete penetration of muscularis propria; C_2, B_2+ nodal involvement.
c) Cases examined immunochemically as well as histochemically.

were stored at −80°C till the extraction of glycoproteins.
2. Extraction: Five hundred mg of tissue was homogenized with saline and centrifuged. Two percent SDS containing 10% 2-ME in 125 m*M* Tris-HCl buffer, pH 6.8, was added to the pellets, and they were heated in boiling water for 3 min.
3. SDS-PAGE: Following the method of Laemmli,[15] an aliquot of each sample, which contained about 10 μg as protein, was applied to 7 or 8% polyacrylamide gels. Molecular weight protein standards were purchased from Bio-Rad Laboratories, Richmond, CA.
4. Western blotting analysis: Following the method of Towbin *et al.*,[16] proteins on a gel were transferred to nitrocellulose membrane. The membrane was then incubated with lectins or antibodies. The lectins used in this study were HRP-labeled GSA-2 and UEA-1 (E-Y Laboratories, San Mateo, CA), and their sugar specificities were α/β-GlcNAc and α-Fuc, respectively. The antibodies employed here were anti-CEA polyclonal antibody (MILAB, Malmö, Sweden) and anti-CEA monoclonal antibody (Takara Shuzo, Kyoto). Neither reacted with nonspecific cross-reacting antigen. Anti-A, B and H (type 2 chain) antibodies were purchased from Dako, Santa Barbara, CA, and the anti-Le^a and Le^b antibodies were obtained from Chembiomed, Edmonton, AB, Canada. Immunostaining was carried out by the avidin-biotin-peroxidase complex method.[17] Biotinylated anti-rabbit IgG antibody (for anti-CEA polyclonal antibody), biotinylated anti-mouse IgG antibody (for the other antibodies) (Medical Biological Laboratories, Nagoya) and streptoavidin-peroxidase complex (Dako) were used. The peroxidase reactivity was visualized by using diaminobenzidine-H_2O_2 as the substrate.
5. Effect of PNGase F treatment: To determine the type of carbohydrate chains in glycoprotein, digestion of extracted carcinoma tissues with PNGase F was carried out.[18] Twenty μg of homogenized sample in 0.5% SDS containing 0.1 *M* 2-ME was incubated overnight with (or without as a control) 10 U/ml of PNGase F from *Flavobacterium meningosepticum* (Genzyme, Boston, MA) in 200 m*M* sodium phosphate buffer, pH 8.6, containing 10 m*M* 1,10-phenanthroline, 0.17% SDS and 1.25% Nonidet P-40 at 37°C. The samples were analyzed by SDS-PAGE-Western blotting-GSA-2 stain.
6. Effect of PI-PLC treatment: To determine whether GBG was attached to the cell membrane via a glycosylphosphatidylinositol linkage, digestion with PI-PLC from *Bacillus thuringiensis* (Funakoshi, Tokyo) was performed.[19, 20] Fresh samples from the carcinoma tissues were homogenized with 10 m*M* Tris-HCl buffer, pH 7.6, and centrifuged at 15,000 rpm for 15 min. The residue was digested with 3 U/ml of PI-PLC in the same buffer for 6 h at 37°C. As a control, the residue was incubated in the same buffer without PI-PLC. After the digestion, the sample was centrifuged at 15,000 rpm for 1 h at 0°C. The supernatant and the pellet were analyzed by SDS-PAGE-Western blotting-GSA-2 stain.

Histochemical study Histochemical procedures were as follows.
1. Tissue preparation: Histochemical evaluation was performed for 5 cases randomly selected from the fifteen cases examined (Table I). As soon as the fresh materials were resected for immunochemical studies, the remaining tissues were fixed for 24 h in a cold 4% paraformaldehyde solution buffered with 0.1 *M* phosphate buffer, pH 7.4. Tissue blocks of carcinoma and of normal mucosa at the cut end were prepared. Serial or mirror paraffin sections of 3 μm thickness were stained by the following sequences including hematoxylin and eosin.
2. Staining: Lectin stains with HRP-labeled GSA-2 or UEA-1 were carried out following the procedure described previously.[11, 12, 14] Immunostaining with the anti-CEA antibodies (polyclonal and monoclonal) and the anti-blood group H, Le^a, Le^b antibodies was also performed by employing the avidin-biotin-peroxidase complex method.[17] To facilitate comparison of the distributions of two antigens, one of the mirror sections was

stained for GSA-2 and the other for antigens other than GSA-2.[13]

3. Controls: For the lectin stains, control sections were incubated in lectin solutions containing 0.1 *M* GlcNAc or GalNAc for GSA-2 and 0.1 *M* Fuc for UEA-1. Monosaccharides used here were all obtained from E-Y Laboratories. For the immunostains, control sections were processed without incubating in primary antisera.

RESULTS

Detection of GBG in tissue extracts With SDS-PAGE-Western blotting and lectin staining with GSA-2, a major band (GBG) could be detected only in the carcinoma tissues, but not in the normal mucosae in all cases examined, regardless of the locations of the carcinoma, the clinicopathological stages and the patients' blood group phenotypes. The reactive band approximately corresponded to a molecular weight of 160 kDa (Fig. 1).

Immunochemical comparison of GBG with carcinoma-specific UEA-1-binding glycoprotein, blood group substances and CEA Since there were no immunochemical differences of GBG depending on the clinicopathological

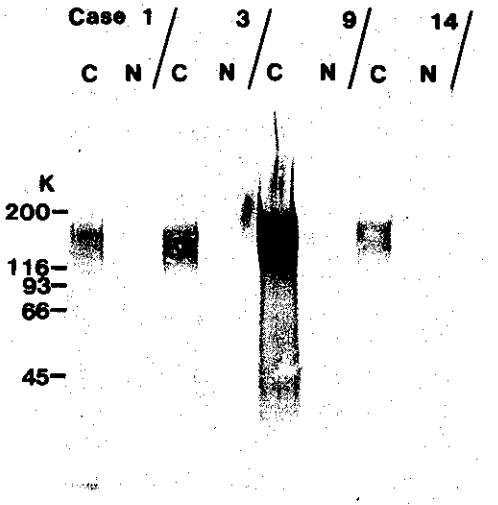

Fig. 1. Western blot (SDS-PAGE, 8% gel) analysis of the extracts from the normal colonic mucosae (lane N) and from the colonic carcinoma tissues (lane C), with GSA-2-HRP staining. GSA-2 identified one major moiety of 160 kDa only in the extracts from the carcinoma tissues.

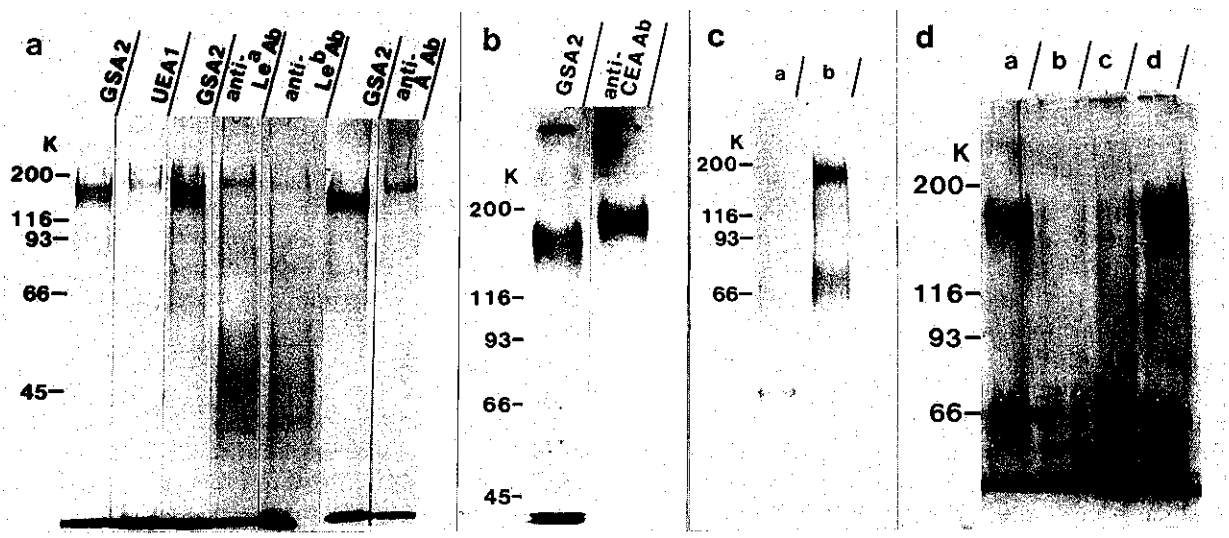

Fig. 2. Western blot (SDS-PAGE, 8% gel in a and c, and 7% gel in b and d) analysis of the extracts from the colonic carcinoma tissues. a: Immunostains with various lectins and anti-blood group substance antibodies. The migration point of GBG was apparently different from those of the other lectin- or monoclonal antibody-defined moieties. b: Immunostains with GSA-2 and anti-CEA antibody. GBG ran faster than CEA, and their molecular weights were 160 kDa and 180 kDa, respectively. c: Immunostain with GSA-2 before (lane b) and after (lane a) the digestion with PNGase F. GSA-2 reactivity was completely eliminated after the enzymatic digestion. d: Immunostain with GSA-2 before (lanes c and d) and after (lanes a and b) the digestion with PI-PLC. Before the digestion, GBG was detected in the residue (lane d), but not in the supernatant (lane c). After the digestion, GBG was demonstrated in the supernatant (lane a), but not in the residue (lane b).

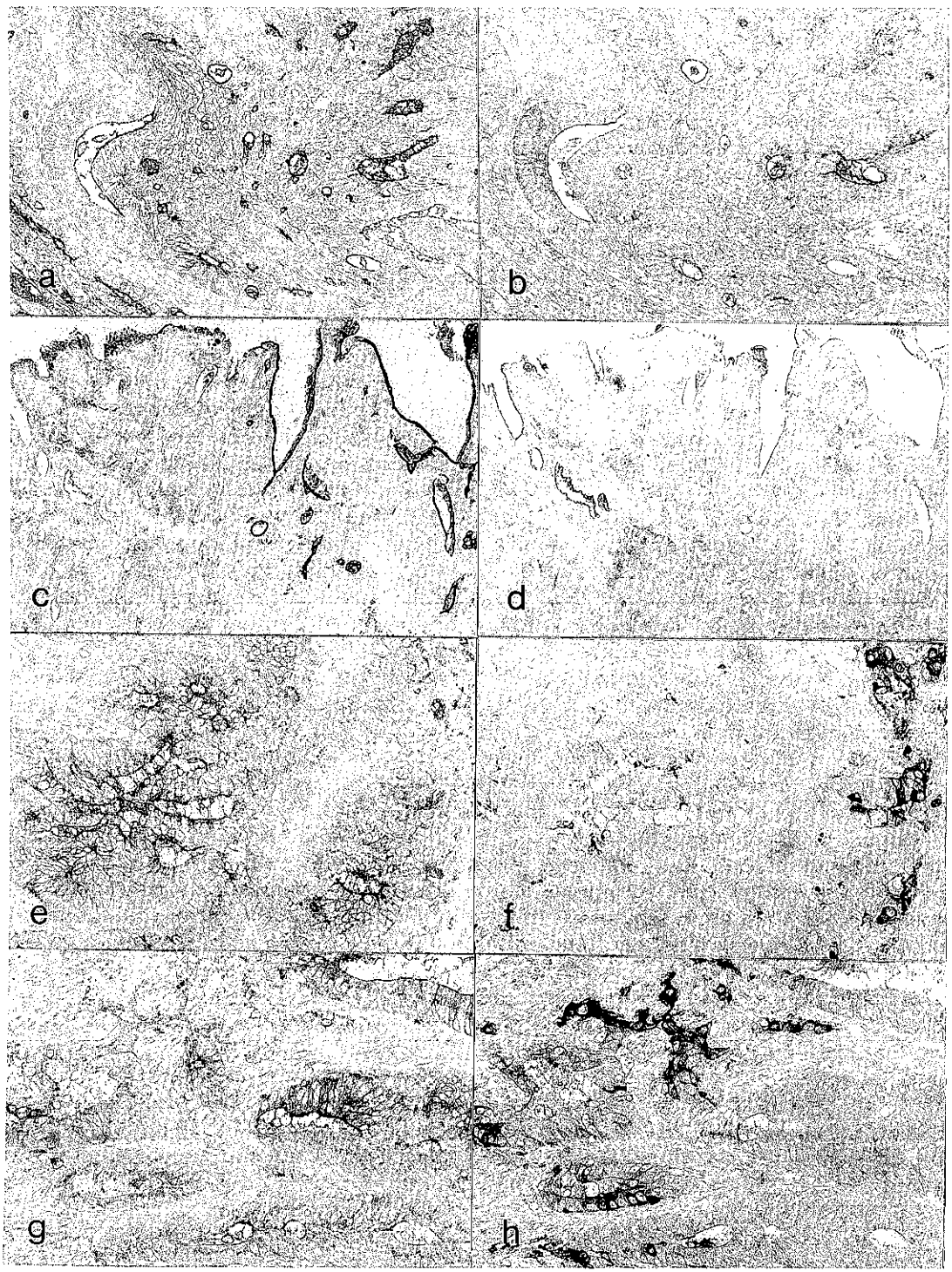

Fig. 3. Comparative histochemistry of the colonic carcinoma tissues with GSA-2-HRP stain and other stains. Four sets of figures (a and b, c and d, e and f, and g and h) were prepared from mirror sections; b, d, f and h are printed inversely to facilitate comparison. a, c, e and g: GSA-2-HRP stain. b: UEA-1-HRP stain. d: Immunostain with anti-CEA polyclonal antibody. f: Immunostain with anti-Le^a antibody. h: Immunostain with anti-Le^b antibody. (×288). Note that the distribution of the carcinoma cells that reacted with GSA-2 was distinct from that of the carcinoma cells that reacted with the other probes.

status including the patients' blood group phenotypes, the samples from the carcinoma tissues were mixed together for the following immunochemical analysis.

Carcinoma-specific UEA-1-binding glycoprotein[5] was detected, in the carcinoma tissues by SDS-PAGE-Western blotting with UEA-1 stain. Its molecular weight (180 kDa), however, was larger than that of GBG, and there was no overlap between them (Fig. 2a).

Immunostains with the anti-Le^a, anti-Le^b and anti-A antibodies gave reactive bands at the same migration point as the UEA-1-reactive band. The anti-B and anti-H antibodies, however, showed no reaction with the extracted glycoprotein (Fig. 2a).

Immunostaining with the anti-CEA antibodies revealed a single broad band, and there were no differences between the reactivities of the polyclonal and the monoclonal antibodies. CEA ran more slowly than GBG, so the molecular weight of CEA (180 kDa) is greater than that of GBG (Fig. 2b).

Effect of PNGase F digestion After digestion with PNGase F, the major GSA-2-reactive band of 160 kDa was completely eliminated on SDS-PAGE-Western blotting with GSA-2 stain (Fig. 2c).

Effect of PI-PLC digestion Before the digestion, GBG could be extracted as a glycoprotein of 160 kDa from the residue of the colonic carcinoma tissues, but not from the supernatant. After the digestion with PI-PLC, GBG could conversely be extracted from the supernatant, but not from the residue, which indicated that GBG bound to the cell membrane in the residue was released into the supernatant by PI-PLC. Both bands were, furthermore, detected at the same migration point (Fig. 2d).

Histochemical comparison of GSA-2-HRP stain with UEA-1-HRP stain and immunostains with various antibodies against CEA and blood group substances In the normal mucosae, GSA-2-reactive sites were confined to the Golgi regions of the epithelial cells; the apical surface of the columnar cells and mucin in the goblet cells never showed GSA-2 reactivity, as reported previously.[11] The probes other than GSA-2 were not carcinoma-specific, because the apical surface of the columnar cells or the mucin in the goblet cells in the normal mucosae were stained to some extent with UEA-1, anti-CEA polyclonal, anti-CEA monoclonal, anti-H, anti-Le^a and anti-Le^b antibody in five, five, one, two, five, and five of 5 cases examined, respectively.

In the carcinoma tissues, on the other hand, GSA-2 reactivities to the carcinoma tissues agreed well with those described previously.[11] Namely, GSA-2-reactive sites were found along the apical surface of columnar-type carcinoma cells in all cases. The ratio of GSA-2-reactive cells to total carcinoma cells varied considerably from case to case, but the average was approximately 65%.

With UEA-1-HRP stain, most of the carcinoma cells were stained. Positive reactions on the cell surface and/or the cytoplasm of the columnar-type carcinoma cells were observed in all cases. About 80% of the carcinoma cells reacted with this lectin. As compared with the GSA-2-HRP stain with mirror sections, the carcinoma cells stained by UEA-1 usually predominated over those stained by GSA-2. In one case, however, GSA-2-reactive carcinoma cells were more abundant than UEA-1-reactive ones (Fig. 3a, b).

Most of the carcinoma cells in all cases were stained with the polyclonal and the monoclonal antibodies to CEA. The staining patterns obtained with the two antibodies were roughly similar, but carcinoma cells reactive for the former antibody prevailed over those for the latter. The cell surface of columnar-type carcinoma cells showed the most intense reaction. As the carcinoma cells invaded, cytoplasmic staining was noted. The mirror section study for the comparison of the distributions of GBG and CEA, revealed that carcinoma cells positive for CEA were usually observed more frequently than GSA-2-reactive carcinoma cells. But some carcinoma cells were stained only for GSA-2 (Fig. 3c, d).

The apical surface of the carcinoma cells was stained with the anti-H antibody in two of 5 cases, and the distribution of the carcinoma cells positive for H antigen did not correlate with that of the carcinoma cells positive for GSA-2 in the mirror section study.

With immunostain for Le^a antigen or for Le^b antigen, the cell surface and/or the cytoplasm of the carcinoma cells showed reactivities in all cases examined. The ratio of the positive cells to total carcinoma cells varied considerably from case to case and gland to gland, but the average was approximately 85% for Le^a antigen or 70% for Le^b antigen. The mirror section study disclosed that there were no correlations between carcinoma cells reactive for GSA-2 and those for anti-Le^a or anti-Le^b antibody (Fig. 3e, f, g, h).

Control sections for the immunostains showed no specific staining.

Effect of adding a monosaccharide to lectin solution The GSA-2 binding to the carcinoma cells was completely eliminated by incubation with 0.1 *M* GlcNAc added to the GSA-2 solution. No inhibition of GSA-2 binding by incubation with 0.1 *M* GalNAc added to the GSA-2 solution was seen.

The UEA-1 binding to the carcinoma cells was completely abolished by adding 0.1 *M* Fuc to the UEA-1 solution.

DISCUSSION

In the previous papers, we described the selective binding of GSA-2 to the apical surface of colonic carci-

noma cells, but not to the epithelial cells lining either normal mucosae or adenomas except for faint reactivity in the Golgi regions of the epithelial cells.[11, 14] This study with histochemical techniques confirmed that all probes other than GSA-2 reacted with not only the carcinoma cells, but also to some extent with the apical surface of the epithelial cells in the normal mucosa.

GBG, which was located in the apical surface of the carcinoma cells, was extracted as a glycoprotein of 160 kDa from the colonic carcinoma tissues. Its occurrence was not related to the patients' blood group phenotypes, the locations of the carcinomas or the clinicopathological stages. GBG was never extracted from normal colonic mucosae. It thus appears to have great potential as a new colonic carcinoma-associated antigen.

Many monoclonal antibodies raised against colonic carcinoma-associated antigens have been reported, and most of them recognized a variety of glycoconjugates including blood group antigens and various epitopes of CEA.[21] Distinct differences of the migration points in the gel between GBG and the other antigens including blood group antigens, carcinoma-specific UEA-1-binding glycoprotein and CEA were supported by the histochemical analysis using mirror sections. The obvious differences in distribution between GBG and the other antigens indicated that GBG was distinct from the other antigens. Furthermore, the presence of carcinoma cells positive for GSA-2 and negative for the anti-CEA antibodies suggested that GBG did not share a common protein with CEA, since the anti-CEA antibodies used here mainly recognize the protein parts rather than sugar moieties.

The highly selective affinity of GSA-2 toward GlcNAc[22] and the complete inhibition of GSA-2 binding to the carcinoma cells by prior addition of GlcNAc, but not by the addition of GalNAc, suggested that the carbohydrate chains of GBG had GlcNAc at the non-reducing terminals. Previously, we envisaged that GBG might have GlcNAc in a penultimate or inner position of the carbohydrate chains at the non-reducing terminal, since GSA-2 binding to the carcinoma cells was not eliminated by prior 90-min oxidation with 1% periodate.[11] The 90-min oxidation procedure is known to cleave most of the ordinary vicinal glycols.[23] Later, we confirmed that GSA-2 binding to lactotriaosylceramide (GlcNAcβ(1-3)-Galβ(1-4)Glcβ(1-1)ceramide) developed on a thin layer plate was not affected by the 90-min oxidation with 1% periodate (unpublished data). Our previous histochemical data with periodate oxidation, therefore, should not necessarily imply that GlcNAc was located elsewhere than at the non-reducing terminal.

PNGase F is an enzyme which cleaves N-glycosidically linked carbohydrate chains from the amide nitrogen of asparagine residues.[18] By the digestion of GBG with PNGase F, GSA-2-reactive sugar residues were completely released from the protein parts of GBG, indicating that GBG belonged to the category of serum-type glycoproteins. Colonic carcinoma-associated antigens having GlcNAc at non-reducing terminals have not been reported so far, except for an oligosaccharide of GlcNAcβ(1-3)(NeuAcα(2-6))GalNAc,[24] which had, however, a mucin-type glycoprotein linkage.

In the biosynthesis of serum-type glycoproteins, glycoproteins which have GlcNAc at non-reducing terminals appeared as intermediates in the conversion of the high-mannose structures to the complex structures of the glycoproteins ("processing").[25] GBG might correspond to such intermediates, and its localization in the cell coat of the carcinoma cells suggested that GBG was immediately transferred from the Golgi apparatus to the cell membrane as the intermediate that it was, due to the incomplete synthesis of the glycoprotein. In normal cells, the processing is known to occur in the Golgi apparatus. To confirm this, further study of the subcellular localization of GBG would be needed using electron microscopy with lectin staining.

Recently, another membrane attachment form, that is glycosyl-phosphatidylinositol-anchoring linkage, was proposed.[19] Here, the C-terminal of the protein was bound to a series of ethanolamine-glycan-intramembranous phosphatidylinositol. PI-PLC specifically digested phosphatidylinositol to diacylglycerol and myo-inositol-cyclic 1,2-phosphate. Some of the cell surface proteins were anchored to cell membranes via glycosyl-phosphatidylinositol.[19, 26] Alkaline phosphatase, acetylcholine esterase, and CEA are examples of the glycosyl-phosphatidylinositol-anchoring protein. In this study, our data suggested that GBG is another example of a glycosyl-phosphatidylinositol-anchoring protein. Presumably, GBG was released from diacylglycerol integrated in the cell membrane. The release associated with no alteration of the molecular weight of GBG supported this possibility. The physiological roles of glycosyl-phosphatidylinositol anchoring are not well known yet. Certain kinds of cellular metabolism and release of the protein from the membrane might be controlled by the endogenous activity of phospholipase. GBG was expected to be released into patients' sera by endogenous phospholipase activity, as has been suggested in the case of CEA.[26]

The identification of GBG originally started from the histochemical observation of the highly selective reactivity of GSA-2 to colonic carcinoma cells,[11] and the biochemical characterization of GBG was performed by applying immunochemical methods. This might be a useful approach to studying carbohydrate antigens.

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