Dimeric Le^a (Le^a-on-Le^a) status of ß-haptoglobin in sera of colon cancer, chronic inflammatory disease and normal subjects

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Abstract. The glycosyl epitope dimeric Le^a (Le^a-on-Le^a), defined by mouse monoclonal antibody NCC-ST-421, was identified previously as tumor-associated antigen, expressed highly in various human cancer tissues and cell lines derived therefrom, but with minimal expression in various normal tissues. In the present study, we observed clearly higher expression of this epitope, defined by ST421, in ß-haptoglobin (ß-Hap) from sera of patients with colorectal cancer, compared to normal, healthy subjects or patients with chronic inflammatory processes (Crohn's disease, ulcerative colitis). We focused, therefore, on biochemical characterization of glycosyl epitope status expressed in ß-Hap. We concluded that the dimeric Le^a epitope is carried by O-linked but not by N-linked structure, based on the following observations: i) Treatment of ß-Hap with α-L-fucosidase reduced its reactivity with ST421, but did not affect its reactivity with anti-Hap antibody. In contrast, treatment of purified ß-Hap with PNGase F, which releases N-linked glycans, had no effect on reactivity with ST421, but changed molecular mass from 40 kDa to 30 kDa. ii) Strong reactivity of Colo205 supernatant with ST421 was reduced clearly by pre-incubation of cells with benzyl-α-GalNAc.

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Abbreviations: BSA, bovine serum albumin; C/M, chloroform/methanol; C/M/W, chloroform/methanol/water; GSL, glycosphingolipid; Hap, haptoglobin; HRP, horseradish peroxidase; mAb, monoclonal antibody; MWCO, molecular weight cutoff; PBS, phosphate-buffered saline (10 mM phosphate buffer/2.7 mM potassium chloride/137 mM sodium chloride, pH 7.4); PNGase F, Peptide: N-glycosidase F (mixture of endo-N-acetylglucosaminidase F and peptide-N4-(N-acetylglucosaminyl)asparagine amidase F); PVDF, polyvinylidene difluoride; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TLC, thin-layer chromatography; T-TBS [TBS (140 mM NaCl, 10 mM Tris-HCl, pH 8.0)/0.05% Tween-20]

Key words: dimeric Le^a, haptoglobin, colon cancer, lectin blotting, O-linked glycan

Introduction

Glycosylation is one of the well-known post-translational modifications. Altered glycosylations in glycolipids and glycoproteins, defined by specific lectin or monoclonal antibodies (mAbs), have been considered as useful markers indicating tumor development and its possible inhibition (1). Tumor-associated carbohydrate antigens have been utilized for diagnosis of tumor development, or prediction of recurrence (2-5). These antigens have also been found to be useful targets for immunotherapy in various pre-clinical studies (6-10) (reviewed in refs. 11,12). One type of glycosyl epitope expressed highly in human cancers, but much less in normal human cells and tissues, is repeated lacto-series type 1 chain. The epitopes are dimeric Le^a (Le^a-on-Le^a), defined by mAb ST421 (13,14), and Le^b-on-Le^a, defined by mAb IMH2 (15,16).

Haptoglobin (Hap) is one of the acute-phase serum components. Its elevated level, with altered glycosylation status, is closely associated with cancer, inflammation and other diseases (reviewed in ref. 17). While altered glycosylation status of ß-Hap has been studied in detail (18-20), no studies have addressed possible association of dimeric Le^a or Le^b-on-Le^a, expressed in serum ß-Hap, with development of colorectal cancer.

We report here a higher level of dimeric Le^a expression in ß-Hap in sera of patients with colorectal cancer, than that of patients with chronic inflammatory processes, or normal subjects, providing a basis for diagnostic application for colorectal cancer.

Materials and methods

Cell lines and cell culture. Colo205 cells (21), originally purchased from ATCC, were cultured and maintained in RPMI-1640 medium with 10% fetal calf serum at 37˚C in 5% CO_2/95% air.

Materials. Rabbit anti-human Hap antibody was purchased from Dako (Carpinteria, CA). Goat anti-mouse IgG-HRP was from Southern Biotech (Birmingham, AL). Mouse IgG3 mAb ST-4-39 (or NCC-ST-421), showing high reactivity with various human cancers, but very low reactivity with various normal tissues (22), was kindly donated by Setsuo Hirohashi and Masahiko Watanabe (National Cancer Center Research Institutue, Tokyo, Japan). Another mouse IgG3 mAb, IMH2, was established at the Biomembrane Institute after immunization of Balb/c mice with purified Le^b-on-Le^a glycosphingolipid (GSL) antigen adsorbed on *Salmonella minnesotae* (15). The immunization method was the same as often used previously by us (23). Benzyl 2-acetamido-2-deoxy-α-D-galacto-pyranoside, bovine kidney α-L-fucosidase, which preferentially hydrolyzes Fucα1-2,3,4GlcNAc rather than Fucα1-6GlcNAc, commercial human Hap, and Extr Avidin®-peroxidase were from Sigma (St. Louis, MO). Peptide: N-glycosidase F (PNGase F) was from QA Bio (Palm Desert, CA).

Glycosphingolipid extraction, thin-layer chromatography (TLC) and immunostaining. For glycosphingolipid (GSL) extraction, 5x10^6 cells were cultured in 150-mm round culture plate until confluent. After harvested by rubber policeman, cell pellet in glass tube was dissolved in 1 ml chloroform/methanol (C/M, v/v) 2:1 in glass tube, sonicated, centrifuged, and transfer the supernatant to a new tube. The cell pellet was mixed with 1 ml isopropanol/hexane/water (55:25:20, v/v/v), sonicated and centrifuged (24). The supernatant was combined with C/M 2:1 extract, evaporated under N_2 stream and subjected to alkaline hydrolysis of phospholipids for isolation of GSL as described previously (25). Briefly, the dried extract was dissolved in 0.1 M NaOH/methanol, incubated for 2 min at 40˚C, neutralized with 0.2 ml 1 N HCl and allowed to stand to separate the upper layer from the lower layer following adding 2 ml of hexane. The lower layer was separated, dried under N_2 stream, dissolved in 1 ml distilled water and subjected to SepPak C_{18} cartridge (Varian, Palo Alto, CA). After rinsing with 10 ml distilled water, C_{18} cartridge-bound fraction was eluted twice by 1 ml C/M 2:1 and evaporated under N_2 stream, dissolved in chloroform/methanol/water (C/M/W) 30:60:8, and applied onto DEAE-Sephadex A25 column chromatography. Neutral fraction, mono-, di-, tri- and multi-sialoganglioside were eluted by C/M/W 30:60:8, 0.03 M ammonium acetate in C/M/W 30:60:8, 0.13 M ammonium acetate in C/M/W 30:60:8, 0.45 M ammonium acetate in C/M/W 30:60:8, and 0.8 M ammonium acetate in C/M/W 30:60:8, respectively. Each fraction was concentrated under vacuum rotary evaporator, dialyzed in dialysis membrane (MWCO 3,500; Spectrum Labs, Rancho Dominguez, CA) against distilled water, lyophilized, dissolved in C/M 2:1 and subjected to TLC developed in a solvent system of C/M/0.5% aqueous $CaCl_2$ 5:4:1. After air drying, TLC plate was stained by orcinol/H_2SO_4 for detection of glycosyl residue (26).

For immunostaining using IMH2 and ST421 antibodies, TLC plates were soaked in 0.5% plastique in hexane/chloroform (9:1 by vol.) for 90 sec, air dried and blocked with 1% BSA/T-TBS [TBS (140 mM NaCl, 10 mM Tris-HCl, pH 8.0)/0.05% Tween-20] for 2 h at RT. After rinsed with T-TBS, plates were incubated with 1:1,000 diluted IMH2 or ST421 antibodies supplemented 1% goat serum for 2 h at RT, rinsed with T-TBS, incubated with 1:3,000 diluted goat anti-mouse IgG-HRP for 1 h at RT, rinsed with T-TBS and developed by Supersignal West Pico chemiluminescence substrate kit (Pierce, Rockford, IL).

Western blotting using IMH2 and ST421 antibodies. To explore whether Le^b-on-Le^a, or dimeric Le^a (Le^a-on-Le^a) oligosaccharide is present in Hap, we performed Western blotting using IMH2 directed to Le^b-on-Le^a and ST421 antibody directed to dimeric Le^a, in concentration-dependent manner. Briefly, commercial Hap was subjected to electrophoresis on 12.5% acrylamide gel and transferred onto PVDF membrane (Millipore). After blocking with 5% skim milk in T-TBS for 2 h at RT, the membrane was rinsed with T-TBS, incubated with 1:1,000 diluted IMH2 antibody supplemented 1% goat serum for 2 h at RT, rinsed 5 times with T-TBS, incubated with 1:3,000 diluted goat anti-mouse IgG-HRP for 1 h at RT, rinsed with T-TBS and developed by Supersignal West Pico Chemiluminescence substrate kit.

The same blot after Western blot using IMH2 antibody was subjected to reblotting with ST421 antibody (27). Briefly, membrane was stripped by stripping buffer (2% SDS, 62.6 mM Tris-HCl, pH 6.7, 0.78% 2-mercaptoethanol) by incubation for 20 min at 60˚C, rinsed and blocked with 5% skim milk in T-TBS for 2 h at RT. Membrane was incubated with 1:1,000 diluted ST421 antibody supplemented 1% goat serum for 2 h at RT, incubated with 1:3,000 diluted goat anti-mouse IgG-HRP for 1 h at RT and developed as described above.

After reblotting with ST421 antibody, the same blot was subjected to another reblotting using 1:50,000 diluted rabbit anti-human Hap antibody followed by 1:3,000 diluted goat anti-rabbit IgG-HRP as described above.

Treatment with fucosidase and PNGase F. Hap was subjected to defucosylation and de-N-glycosylation by treatment of bovine kidney α-L-fucosidase and PNGase F, respectively. For defucosylation, 5 μg of Hap was incubated with/without 1 mU bovine kidney α-L-fucosidase in 50 mM sodium acetate buffer (pH 5.5) for 24 h at 37˚C as described previously (28). The reaction of de-N-glycosylation was performed by incubation of 5 μg Hap with/without 15 mU PNGase F according to the manufacturer's instructions. After incubation of de-N-fucosylation and de-N-glycosylation, the reaction

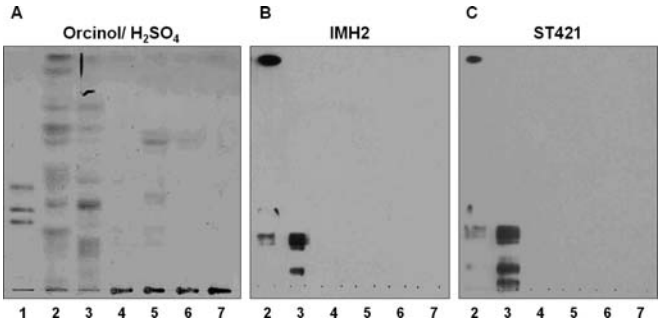

Figure 1. TLC pattern of glycolipids extracted from Colo205 cells. Glycolipids extracted from Colo205 cells were subjected to TLC and stained by orcinol/H_2SO_4 spraying or blotted by immunostaining using IMH2 and ST421 antibody as described in Materials and methods. (A) Orcinol/H_2SO_4 spraying; (B) immunostaining using IMH2 antibody; (C) immunostaining using ST421 antibody. Lane 1, mixture of GM1, GM2 and GM3; lane 2, total GSL; lane 3, neutral fraction from total GSL; lane 4, monosialogangliodise from total GSL; lane 5, disialogangliodise from total GSL; lane 6, trisialogangliodise from total GSL; lane 7, multi-sialogangliodise from total GSL.

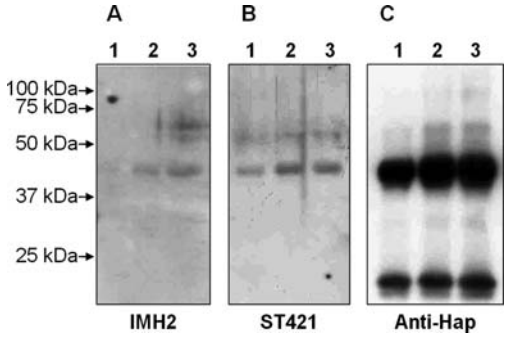

Figure 2. Western blotting of haptoglobin. Various concentrations of commercial Hap were subjected to Western blotting using IMH2, followed by reblotting with ST421, and anti-Hap antibodies as described in Materials and methods. (A) Blotting using IMH2 antibody; (B) reblotting using ST421 antibody; (C) reblotting using anti-Hap antibody. Lane 1, 1 μg Hap; lane 2, 2 μg Hap; lane 3, 5 μg Hap.

mixture was subjected to Western blotting using ST421 antibody followed by reblotting with anti-Hap antibody as described above.

Inhibition of synthesis of O-linked glycosylation by treatment with benzyl-α-GalNAc. To inhibit the synthesis of O-linked glycosylation, Colo205 cells were grown in culture medium supplemented with 2 mM benzyl-α-GalNAc (BzαGalNAc) (29). After incubation for 72 h, the same amount of secretary protein in culture medium quantified by protein assay was subjected to Western blotting using ST421 antibody as described above.

Sera from colon cancer patients, chronic inflammatory disease and healthy subjects. We used 37 cases sera for this study, of which 14 cases were obtained from colon cancer patients, 5 cases of Crohn's disease (mean age 34 years), and 4 cases of ulcerative colitis (mean age 49 years) which were classified by a pathologist based on biopsy results. The 14 cases of colon cancer were subclassified by ascending colon cancer (n=5, mean age 75 years), sigmoid colon cancer (n=3, mean age 71 years), rectal cancer (n=3, mean age 66 years), descending colon cancer (n=2, mean age 78 years) and cecal cancer (n=1, age 63 years). Note that the histologic types of all colorectal cancers were adenocarcinoma. Serum collection and colon biopsy for 14 cases of colon cancer, one case of Crohn's disease, and 2 cases of ulcerative colitis were performed in Chungnam National University College of Medicine, Daejeon, Republic of Korea. The sera collection and colon biopsy for 4 cases of Crohn's disease and 2 cases of ulcerative colitis were carried out in Mount Sinai School of Medicine, New York. Fourteen sera from healthy volunteer subjects (mean age 28 years) were obtained in our laboratory. This study was approved by the Ethics Committees of the participating hospitals, Korea Advanced Institute of Science and Technology (KAIST), and Pacific Northwest Research Institute (PNRI).

Purification of Hap from sera using anti-Hap-affinity chromatography. Purification of Hap was performed as described previously (28). Briefly, each serum was diluted 4 times with PBS, subjected into rabbit anti-Hap antibody-conjugated sepharose 4B equilibrated with PBS, and incubated for 2 h at room temperature by shaking. After eliminating unbound components by PBS, column-bound Hap was eluted by elution buffer (0.1 M glycine/0.5 M NaCl, pH 2.8). Eluted solution was neutralized by neutralization buffer (1.0 M Tris-Cl, pH 9.0), concentrated using a concentrifugal filter (MWCO 10,000, Amicon Ultra, Millipore), quantified the Hap by Quant-iT™ Assay Kit (Invitrogen, Carlsbad, CA), lyophilized and kept at -20˚C until analysis.

Western blot of Hap purified from sera of normal, chronic inflammatory disease and colon cancer using ST421 antibody. To determine dimeric Le^a oligosaccharide structure in ß-Hap purified from sera of healthy subjects, chronic inflammatory disease patients and colon cancer patients, we performed Western blotting using ST421 antibody. Briefly, 5 μg purified Hap was subjected to SDS-PAGE (12.5% acrylamide gel) and transferred onto PVDF membrane. PVDF membrane was subjected to blocking with 5% skim milk for 2 h, rinsed with T-TBS, blotted with 1:1,000 diluted ST421 for 2 h at RT, rinsed with T-TBS and incubated with 1:3,000 diluted goat anti-mouse IgG-HRP for 1 h at RT. After rinsed with T-TBS, the membrane was developed by Supersignal West Pico chemiluminescence substrate kit. For quantity control of Hap, the same blot was subjected to reblotting with anti-Hap antibody as described above.

Results

Reactivity of mAbs ST421 and IMH2. a) Reactivity with glycosphingolipids and Hap. On TLC immunostaining, both ST421 and IMH2 displayed clear reactivity with neutral GSLs, but not with mono-, di-, tri-, or multi-sialogangliosides extracted from Colo205 cells (Fig. 1). Le^b-on-Le^a and dimeric Le^a oligosaccharide was observed over 2 μg of Hap on Western blot using IMH2 and ST421, respectively (Fig. 2). These results show that dimeric Le^a on ß-Hap was present and able to be detected by Western blot using IMH2, and ST421 antibodies.

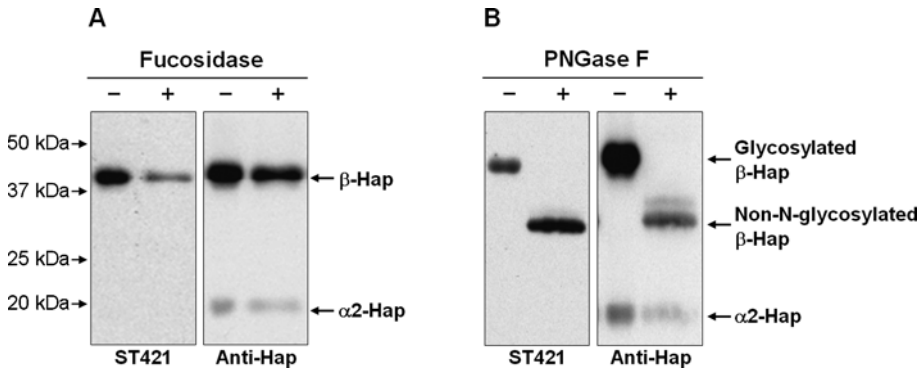

Figure 3. Effect of treatment of fucosidase and PNGase F on ST421 antibody reactivity to ß-Hap. Hap (5 μg) was incubated with or without 1 mU bovine kidney α-L-fucosidase for 24 h at 37˚C (A) or 15 mU PNGase F (B) *in vitro*, and subjected to Western blotting using ST421 as described in Materials and methods. The same blots were reblotted with anti-Hap antibody.

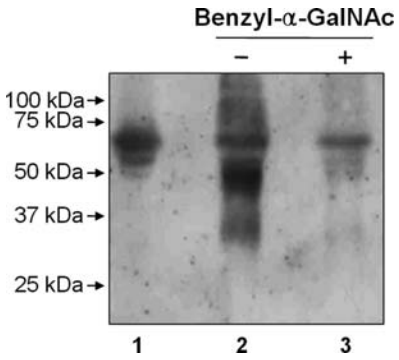

Figure 4. Effect of O-linked glycosylation inhibition on expression of glycoprotein with dimeric Le[a] (Le[a]-on-Le[a]) structure in Colo205 cells. Colo205 cells were grown with/without the inhibitor of O-linked glycosylation, 2 mM benzyl-α-GalNAc, for 72 h, and the cell culture medium was subjected to Western blotting using ST421 antibody as described in Materials and methods. Lane 1, 1/10 diluted fetal calf serum (negative control); lane 2, culture medium without treatment of benzyl-α-GalNAc; lane 3, cultured medium with treatment of benzyl-α-GalNAc.

b) Effect of treatment of ß-Hap with α-L-fucosidase, and PNGase F, on expression of ST421 epitope. In order to confirm the presence of ST421 epitope, ß-Hap was treated with α-L-fucosidase as described in Materials and methods. ST421 reactivity of ß-Hap was greatly decreased, as expected (Fig. 3A, left panel), whereas binding to anti-Hap was unchanged (Fig. 3A, right panel). In contrast, treatment with PNGase F, releasing all N-linked glycans, did not change reactivity of ß-Hap with ST421, but molecular mass of the band was greatly reduced to ~30 kDa (Fig. 3B, left panel), and this band also bound to anti-Hap (Fig. 3B, right panel). These results suggest that ST421 epitope could be present as O-linked glycan, since it is not present in PNGase F-sensitive glycan.

c) Effect of treatment of Colo205 cells with benzyl-α-GalNAc on expression of ST421 epitope. Based on common knowledge that cellular O-linked glycans are metabolically inhibited when cells are cultured in the presence of benzyl-α-GalNAc (29), ST421 reactivity in culture supernatant of Colo205 cells was determined by culturing cells in the presence vs. absence of benzyl-α-GalNAc. The strong ST421 reactivity detected in culture supernatant was greatly reduced in the presence of benzyl-α-GalNAc (Fig. 4).

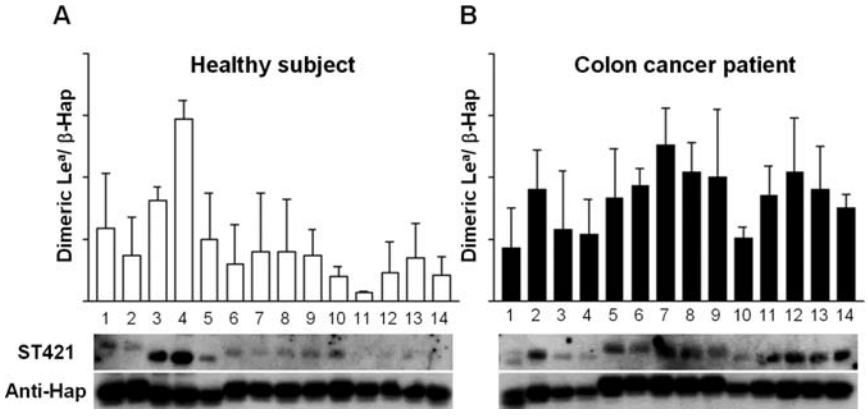

Figure 5. Expression of dimeric Le[a] (Le[a]-on-Le[a]) structure on purified ß-Hap from sera of healthy subjects and colon cancer patients. The defined quantity (5 μg) of purified Hap from 14 healthy subjects (A) and 14 colon cancer patients (B) was subjected to Western blotting using ST421 antibody and then reblotted with anti-Hap antibody as described in Materials and methods. Densitometric comparison of each band corresponding to ß-Hap was performed by Scion image program. The ratio of density by ST421 to density by anti-Hap antibody was expressed as 'dimeric Le[a]/ß-Hap' (ordinate). Mean ± SD is presented in each panel. (A) Dimeric Le[a] reactivity of ß-Hap from healthy subjects; (B) dimeric Le[a] reactivity of ß-Hap from colon cancer patients.

Table I. Information on sera from normal subjects, chronic inflammatory diseases and colorectal cancers.

Status	No. of sera collected	Diagnosis based on biopsy	Age (years)	Sex	AJCC	Case No.
Normal	14 (total)					
	10		30±10	M		
	4		26±4	F		
Chronic inflammatory diseases	9 (total)					
	2	Ulcerative colitis	35±17	M		
	2	Ulcerative colitis	63±6	F		
	5	Crohn's disease	33±6	M		
Colorectal cancers	14 (total)					
		Ascending colon cancer	79	M	II	2
		Ascending colon cancer	80	F	II	4
		Ascending colon cancer	74	M	II	7
		Ascending colon cancer	69	F	III	8
		Ascending colon cancer	72	M	II	13
		Cecal cancer	67	M	III	12
		Descending colon cancer	82	F	III	5
		Descending colon cancer	74	F	III	6
		Rectal cancer	50	M	II	1
		Rectal cancer	85	F	III	3
		Rectal cancer	63	F	I	14
		Sigmoid colon cancer	82	F	I	9
		Sigmoid colon cancer	66	F	I	10
		Sigmoid colon cancer	65	F	III	11

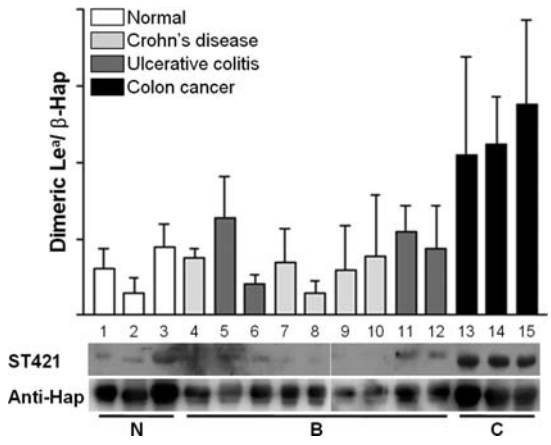

Figure 6. Expression of dimeric Le^a (Le^a-on-Le^a) on purified ß-Hap from sera of healthy subjects, and patients from chronic inflammatory disease, and from colon cancer. Hap from 3 cases of normal, 5 cases of Crohn's disease, 4 cases of ulcerative colitis and 3 cases of colon cancer was subjected to Western blot using ST421 antibody. The ratio of density by ST421 to density by anti-Hap antibody was expressed as 'dimeric Le^a/ß-Hap' (ordinate). Mean ± SD is presented in each panel. Lanes 1-3, normal subjects (N); lanes 4-12, chronic inflammatory disease (B); lanes 13-15, colon cancer subjects (C). White panel, normal; light gray panel, Crohn's disease; dark gray panel, ulcerative colitis; black panel, colon cancer.

Reactivity of ST421 in serum ß-Hap from patients with colorectal cancer, with chronic inflammatory disease and from healthy subjects. Sera of patients with colorectal cancer, with chronic inflammatory diseases (either ulcerative colitis or Crohn's disease) and normal subjects are listed in Table I. ST421-binding activities based on equal 5 μg purified Hap from sera of patients with colorectal cancer, with chronic inflammatory diseases (either ulcerative colitis or Crohn's disease) and normal subjects were analyzed, as described in Materials and methods. Expression of dimeric Le^a structure blotted with ST421 antibody was significantly higher in ß-Hap from colon cancer patients (Fig. 5B) than in that from healthy subjects (Fig. 5A).

To study whether the expression of dimeric Le^a on ß-Hap has cancer specificity, we carried out Western blot using normal, chronic inflammatory disease, and colon cancer subjects (Fig. 6). Analysis was made together for 3 normal subjects (N; lanes 1-3), 5 cases of Crohn's disease (B; lanes 4, 7-10), 4 cases of ulcerative colitis (B; lanes 5, 6, 11, 12), and 3 cases of colorectal cancer (C; cases 13-15). Interestingly, ST421 binding for Crohn's disease and ulcerative colitis was much lower than for colorectal cancer.

Discussion

Lacto-series structures are abundantly expressed in GSLs, and in O-linked or N-linked glycans in many types of glycoproteins. These lacto-series structures are based on the backbone Galß1-4GlcNAcß1-3Galß1-4GlcNAcß1-3Gal-R as core, and occasionally repeated, with branching (30), and termed 'lacto-series type 2 chain'. These structures, highly expressed in embryonal carcinoma or other rapidly-growing

cells, were termed 'embryoglycan' or 'lactosaminoglycan' (31). While these groups of structures are functionally important in defining cell adhesion, motility and signal transduction (32,33), type 2 chain *per se* may not be strongly immunogenic, particularly as tumor-associated antigen.

In contrast, there are a much smaller number of lacto-series structures termed 'lacto-series type 1 chain', having the basic structure Galß1-3GlcNAcß1-3Galß1-R. The chain is not repeated and not extended in normal cells. This structure is carried by GSL, or by peripheral structure of O-linked or N-linked glycan. This type 1 chain with fucosylation is well-known to be blood group Lewis A or B antigen (Le^a or Le^b). Phenotype Le^{a+b-} vs. Le^{a-b+} is ~20 vs. 75% respectively in Caucasian human population (34). Phenotypic expression of Le^a, Le^b is not correlated with cancer. However, repeated (or extended) type 1 chain occurs in human cancer, forming novel human cancer antigen. This was originally found through establishment of mAb ST-4-39, raised by immunization of Balb/c mice through xenograft of human gastric cancer cell ST4. When xenografted human tumor was grown in mice, mouse splenocytes were transfused, causing inhibition of tumor growth. Then splenocytes were fused with SP1 to clone the hybridoma, which causes strong inhibition of original ST4 growth (22). Structure of the antigen was established later, by extensive studies, as dimeric Le^a (Le^a-on-Le^a), and the antibody, termed ST421, was found to cross-react with Le^a-on-Le^x (13,14), but did not cross-react at all with Le^x nor Le^x-on-Le^x. Similary, mAb (IMH2), raised by immunization of Balb/c mice with Colo205 cells was found to be clearly directed to Le^b-on-Le^a, cross-reactive with Le^b-on-Le^x, but not with Le^y or Le^y-on-Le^x (15,16).

While these mAbs are not specifically reactive with extended type 1 chain, but also react with extended hybrid type 1 chain, they display high reactivity with various cancer tissues (gastrointestinal, colorectal, lung, pancreas, breast, kidney) and cell lines derived therefrom. Their reactivity with various normal human tissues is highly restricted. Therefore, both ST421 and IMH2 could be useful in immunotherapy in the future.

Genetic differences of Hap, based on not only α1, α2 and ß chain, but also multiple combinations of S-S linked, four N-linked and one O-linked glycans, provide the basis to distinguish individual 'genetic fingerprint' (35). Recently, increased levels of Hap, with different glycosylation types, were found to be associated with various human diseases, particularly cancer. Studies have been focused on different types of glycosylation, particularly in N-linked glycans of ß-Hap, associated with specific types of cancer (17-20,36).

The present study indicates that reactivity with mAb ST421, defining Le^a-on-Le^a, is clearly higher for serum Hap from patients with colorectal cancer, compared to patients with chronic colonic diseases, and to normal subjects. This result is similar to that of our previous study on blotting of Hap with *Aleuria aurantica* lectin (AAL), which also shows higher reactivity with colorectal cancer (28). Both Le^a-on-Le^a structure, and the unknown structure defined by AAL, contain α-L-fucosyl residue. It is therefore reasonable that both reagents react with ß-Hap from sera of cancer patients. Importantly, however, Le^a-on-Le^a epitope is linked to Hap through O-linked GalNAc, but not through N-linked GlcNAc to Asn, as clearly indicated by treatment with PNGase F, and by the inhibitory effect of benzyl-α-GalNAc. The procedure used in the present study is multi-step and complex. A simplified procedure will be required for possible application in everyday diagnosis. Further extensive studies are obviously necessary.

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References

1. Hakomori S: Glycosylation defining cancer malignancy: New wine in an old bottle. Proc Natl Acad Sci USA 99: 10231-10233, 2002.
2. Seelentag WKF, Li W-P, Schmitz S-FH, Metzger U, Aeberhard P, Heitz PU and Roth J: Prognostic value of b1,6-branched oligosaccharides in human colorectal carcinoma. Cancer Res 58: 5559-5564, 1998.
3. Dennis JW and Laferté S: Oncodevelopmental expression of -GlcNAcb1-6Mana1-6Manb1-6 branching of Asn-linked-oligosaccharides in human breast carcinomas. Cancer Res 49: 945-950, 1989.
4. Turner GA: N-glycosylation of serum proteins in disease and its investigation using lectins. Clin Chim Acta 208: 149-171, 1992.
5. Turner GA, Skillen AW, Buamah P, Guthrie D, Welsh J, Harrison J and Kowalski A: Relation between raised concentrations of fucose, sialic acid, and acute phase proteins in serum from patients with cancer: choosing suitable serum glycoprotein markers. J Clin Pathol 38: 588-592, 1985.
6. Slovin SF, Ragupathi G, Adluri S, Ungers G, Terry K, Kim S, Spassova M, Bornmann WG, Fazzari M, Dantis L, Olkiewicz K, Lloyd KO, Livingston PO, Danishefsky SJ and Scher HI: Carbohydrate vaccines in cancer: Immunogenicity of a fully synthetic globo H hexasaccharide conjugate in man. Proc Natl Acad Sci USA 96: 5710-5715, 1999.
7. Holmberg LA, Oparin DV, Gooley T, Lilleby K, Bensinger W, Reddish MA, MacLean GD, Longenecker BM and Sandmaier BM: Clinical outcome of breast and ovarian cancer patients treated with high-dose chemotherapy, autologous stem cell rescue and THERATOPE STn-KLH cancer vaccine. Bone Marrow Transplant 25: 1233-1241, 2000.
8. Livingston PO: Carbohydrate vaccines that induce antibodies against cancer. 1. Rationale. Cancer Immunol Immunother 45: 1-9, 1997.
9. Ragupathi G, Livingston PO, Hood C, Gathuru J, Krown SE, Chapman PB, Wolchok JD, Williams LJ, Oldfield RC and Hwu WJ: Consistent antibody response against ganglioside GD2 induced in patients with melanoma by a GD2 lactone-keyhole limpet hemocyanin conjugate vaccine plus immunological adjuvant QS-21. Clin Cancer Res 9: 5214-5220, 2003.
10. Gilewski TA, Ragupathi G, Dickler M, Powell S, Bhuta S, Panageas K, Koganty RR, Chin-Eng J, Hudis C, Norton L, Houghton AN and Livingston PO: Immunization of high-risk breast cancer patients with clustered sTn-KLH conjugate plus the immunologic adjuvant QS-21. Clin Cancer Res 13: 2977-2985, 2007.
11. Hakomori S: Tumor-associated carbohydrate antigens defining tumor malignancy: Basis for development of anti-cancer vaccines. Adv Exp Med Biol 491: 369-402, 2001.
12. Galonic DP and Gin DY: Chemical glycosylation in the synthesis of glycoconjugate antitumour vaccines. Nature 446: 1000-1007, 2007.
13. Watanabe M, Ohishi T, Kuzuoka M, Nudelman ED, Stroud MR, Kubota T, Kodaira S, Abe O, Hirohashi S, Shimosato Y and Hakomori S: *In vitro* and *in vivo* antitumor effects of murine monoclonal antibody NCC-ST-421 reacting with dimeric Le^a (Le^a/Le^a) epitope. Cancer Res 51: 2199-2204, 1991.

14. Stroud MR, Levery SB, Nudelman ED, Salyan MEK, Towell JA, Roberts CE, Watanabe M and Hakomori S: Extended type 1 chain glycosphingolipids: Dimeric Le^a ($III^4V^4Fuc_2Lc_6$) as human tumor-associated antigen. J Biol Chem 266: 8439-8446, 1991.
15. Ito H, Tashiro K, Stroud MR, Ørntoft TF, Meldgaard P, Singhal AK and Hakomori S: Specificity and immunobiological properties of monoclonal antibody IMH2, established after immunization with Le^b/Le^a glycosphingolipid, a novel extended type 1 chain antigen. Cancer Res 52: 3739-3745, 1992.
16. Stroud MR, Levery SB, Salyan MEK, Roberts CE and Hakomori S: Extended type-1 chain glycosphingolipid antigens: Isolation and characterization of trifucosyl-Le^b antigen ($III^4V^4VI^2Fuc_3Lc_6$). Eur J Biochem 203: 577-586, 1992.
17. Turner GA: Haptoglobin. A potential reporter molecule for glycosylation changes in disease. Adv Exp Med Biol 376: 231-238, 1995.
18. Fujimura T, Shinohara Y, Tissot B, Pang PC, Kurogochi M, Saito S, Arai Y, Sadilek M, Murayama K, Dell A, Nishimura S and Hakomori SI: Glycosylation status of haptoglobin in sera of patients with prostate cancer vs. benign prostate disease or normal subjects. Int J Cancer 122: 39-49, 2008.
19. Okuyama N, Ide Y, Nakano M, Nakagawa T, Yamanaka K, Moriwaki K, Murata K, Ohigashi H, Yokoyama S, Eguchi H, Ishikawa O, Ito T, Kato M, Kasahara A, Kawano S, Gu J, Taniguchi N and Miyoshi E: Fucosylated haptoglobin is a novel marker for pancreatic cancer: a detailed analysis of the oligosaccharide structure and a possible mechanism for fucosylation. Int J Cancer 118: 2803-2808, 2006.
20. Nakano M, Nakagawa T, Ito T, Kitada T, Hijioka T, Kasahara A, Tajiri M, Wada Y, Taniguchi N and Miyoshi E: Site-specific analysis of N-glycans on haptoglobin in sera of patients with pancreatic cancer: A novel approach for the development of tumor markers. Int J Cancer 122: 2301-2309, 2008.
21. Semple TU, Quinn LA, Woods LK and Moore GE: Tumor and lymphoid cell lines from a patient with carcinoma of the colon for a cytotoxicity model. Cancer Res 38: 1345-1355, 1978.
22. Watanabe M, Hirohashi S, Shimosato Y, Ino Y, Yamada T, Teshima S, Sekine T and Abe O: Carbohydrate antigen defined by a monoclonal antibody raised against a gastric cancer xenograft. Jpn J Cancer Res 76: 43-52, 1985.
23. Young WW, MacDonald EMS, Nowinski RC and Hakomori S: Production of monoclonal antibodies specific for distinct portions of the glycolipid asialo GM_2 (gangliotriosylceramide). J Exp Med 150: 1008-1019, 1979.
24. Kannagi R, Nudelman ED, Levery SB and Hakomori S: A series of human erythrocyte glycosphingolipids reacting to the monoclonal antibody directed to a developmentally regulated antigen, SSEA-1. J Biol Chem 257: 14865-14874, 1982.
25. Nakamura K, Suzuki M, Taya C, Inagaki F, Yamakawa T and Suzuki A: A sialidase-susceptible ganglioside, IV^3 a(NeuGca2-8NeuGc)-Gg4Cer, is a major disialoganglioside in WHT/Ht mouse thymoma and thymocytes. J Biochem 110: 832-841, 1991.
26. Skipski VP: Thin layer chromatography of neutral glycosphingolipids. Methods Enzymol 35: 396-425, 1975.
27. Yoon S, Nakayama K, Hikita T, Handa K and Hakomori S: Epidermal growth factor receptor tyrosine kinase is modulated by GM3 interaction with N-linked GlcNAc termini of the receptor. Proc Natl Acad Sci USA 103: 18987-18991, 2006.
28. Park S-Y, Yoon S-J, Jeong Y-T, Kim J-M, Kim J-Y, Bernert B, Ullman T, Itzkowitz SH, Kim J-H and Hakomori S: N-glycosylation status of ß-haptoglobin in sera of patients with colon cancer, colon inflammatory diseases and normal subjects. Int J Cancer 126: 142-155, 2010.
29. Kuan S-F, Byrd JC, Basbaum C and Kim YS: Inhibition of mucin glycosylation by aryl-N-acetyl-alpha-galactosaminides in human colon cancer cells. J Biol Chem 264: 19271-19277, 1989.
30. Hakomori S: Traveling for the glycosphingolipid path. Glycoconj J 17: 627-647, 2000.
31. Muramatsu T, Muramatsu H, Gachelin G and Jacob F. High molecular weight carbohydrates on embryonal carcinoma cells. In: Teratocarcinoma and Embryonic Cell Interactions. T. Muramatsu, G. Gachelin, A.A. Moscona and Y. Ikawa (eds). Japan Scientific Societies Press, Tokyo, pp143-156, 1982.
32. Kojima N, Fenderson BA, Stroud MR, Goldberg RI, Habermann R, Toyokuni T and Hakomori S: Further studies on cell adhesion based on Le^x-Le^x interaction, with new approaches: embryoglycan aggregation of F9 teratocarcinoma cells, and adhesion of various tumour cells based on Le^x expression. Glycoconj J 11: 238-248, 1994.
33. Handa K, Takatani-Nakase T, Larue L, Stemmler MP, Kemler R and Hakomori S: Le^x glycan mediates homotypic adhesion of embryonal cells independently from E cadherin: A preliminary note. Biochem Biophys Res Commun 358: 247-252, 2007.
34. Prokop O and Uhlenbruck G. The Lewis-System. In: Human Blood and Serum Groups (English translation). O. Prokop and G. Uhlenbruck (eds). MacLaren and Sons, London, pp276-286, 1969.
35. Langlois MR and Delanghe JR: Biological and clinical significance of haptoglobin polymorphism in humans. Clin Chem 42: 1589-1600, 1996.
36. Thompson S and Turner GA: Elevated levels of abnormally-fucosylated haptoglobins in cancer sera. Br J Cancer 56: 605-610, 1987.