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Down-regulation of Gal 3-*O*-sulfotransferase-2 (Gal3ST-2) Expression in Human Colonic Non-mucinous Adenocarcinoma

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Expression levels of sulfomucin in human colonic adenocarcinomas are lower than those in normal colonic mucosa; this should be in part caused by down-regulation of expression of sulfotransferases, but it remains unclear which Gal 3-*O*-sulfotransferase (Gal3ST) is responsible for the biosynthesis of sulfomucin. In this study, we first examined the substrate specificities of four Gal3STs cloned so far, and found that Galβ1→3GlcNAcβ1→ 3Galβ1→4Glc (LNT) can be utilized only by Gal3ST-2 as an acceptor substrate. The substrate specificity of Gal3ST-2 is closely similar to those of Gal3ST activities present in human normal mucosa and adenocarcinomas, suggesting that Gal3ST-2 is the dominant Gal3ST in colon and colonic cancer. Secondly, using LNT as a substrate, we comparatively analyzed levels of Gal3ST-2 activities in non-mucinous adenocarcinoma, mucinous adenocarcinomas, and the adjacent normal mucosa. We found that levels of Gal3ST-2 activities in non-mucinous adenocarcinoma are significantly lower than those in the adjacent normal mucosa, while those in mucinous adenocarcinomas are not significantly different from those in the adjacent normal mucosa. Moreover, we showed by a competitive RT-PCR method that expression levels of transcript for Gal3ST-2 in non-mucinous adenocarcinoma are lower than those in normal mucosa. These results suggest that Gal3ST-2 is one of the enzymes responsible for biosynthesis of sulfomucin, and that expression levels of Gal3ST-2 are down-regulated in non-mucinous adenocarcinoma.

Key words: Colon cancer — Sulfomucin — Sulfotransferase — Gal3ST — Type 1

Sulfomucin is a glycoprotein containing large amounts of *O*-linked, sulfated glycans. It is well-known that expression levels of sulfomucin in colonic cancer are markedly lower than those in the adjacent normal mucosa.[1, 2] Yamori *et al.*[3] explored a monoclonal antibody, 91.9H, recognizing sulfated glycans in sulfomucin, and Irimura *et al.*[4] and Matsushita *et al.*[5] showed that expression levels of the epitope in colonic adenocarcinomas are indeed lower than those in normal mucosa. Thereafter, Loveless *et al.*[6] showed that the minimum epitope moiety is the SO_3^-→3Galβ1→3(Fucα1→4)GlcNAcβ1→3Galβ1→ structure. These results suggest that the decrease of the epitope in colon cancer is at least partly associated with that of sulfomucin. We previously showed that levels of the enzymatic activities of β1,3-galactosyltransferase, which can synthesize the backbone structure of the 91.9H antigen, are lower in colonic adenocarcinomas than in the adjacent normal mucosa.[7] Thereafter, Salvini *et al.*[8] showed that expression of *β3GalT-V* gene is down-regulated in colonic adenocarcinomas, suggesting that *β3GalT-V* gene is involved in biosynthesis of the 91.9H antigen and its low expression in colonic adenocarcinomas. However, it remains unclear whether or not 3-*O*-sulfation at the Gal residue is also down-regulated in the course of colonic carcinogenesis.

Gal3ST transfers sulfate from PAPS to the C-3 position of Gal residues in various glycoconjugates. Four *Gal3ST* genes have so far been identified[9–13] and their genetic and biochemical characters are summarized in Table I. Honke *et al.*[9, 14] purified cerebroside 3′-sulfotransferase (Gal3ST-1) from human renal cancer cells and isolated its cDNA based on partial amino acid sequences. Gal3ST-2 has broad substrate specificity and is expressed in various tissues including colonic epithelial cells.[10] Gal3ST-3 exhibits rather limited expression.[11, 12] Gal3ST-4 was identified by us and shown to have a characteristic strict substrate specificity for core 1 and core 2.[13] The sulfation process in the

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Abbreviations: asialo GP, Galβ1→4GlcNAcβ1→2Manα1→3(Galβ1→4GlcNAcβ1→2Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc; Bn, benzyl; CEA, carcinoembryonic antigen; core 1, Galβ1→3GalNAc; core 2, Galβ1→3(GlcNAcβ1→6)GalNAc; DMSO, dimethylsulfoxide; Fuc, fucose; Gal, galactose; GalAAG, galactosyl 1-alkyl-2-acyl-*sn*-glycerol; Gal3ST, galactose 3-*O*-sulfotransferase; GalCer, galactosylceramide; GalNAc, *N*-acetylgalactosamine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Lac-*O*-pNP, Galβ1→4Glcβ1-*O*-pNP; LNT, Galβ1→3GlcNAcβ1→3Galβ1→4Glc; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; pNP, *p*-nitrophenyl; RT-PCR, reverse transcription-polymerase chain reaction; SulT, sulfotransferase; TLC, thin layer chromatography; type 1, Galβ1→3GlcNAc; type 2, Galβ1→4GlcNAc.

Table I. Characters of Four Gal3STs

	Gal3ST-1 (CST)	Gal3ST-2 (GP3ST)	Gal3ST-3 (GAL3ST2)	Gal3ST-4
cDNA cloning	Human[9] Mouse[31]	Human[10]	Human[11, 12]	Human[13]
Accession number	D88667[9] AB032939[31] AB032940[31]	AF048727[10]	AY026481[11] AB053232[12]	AF316113[13]
Chromosome localization	22q12 (Human)	2q37.3	11q13	7q22
Amino acids	423 a.a. (Human, mouse)	398 a.a.	431 a.a.	486 a.a.
Tissue distribution	Broad	Broad	Thyroid, brain, kidney, heart, spinal cord	Broad
Substrate specificity	GalCer, LacCer GalAAG	type 2, type 1, core 1	type 2, core 1, (type 1)[a]	core 1, core 2

a) Weak activity in comparison with type 2 and core 1.[12]

biosynthesis of the 91.9H antigen seems to be catalyzed by some of these Gal3STs, but there is no evidence as to which enzymes are involved in sulfomucin formation and its down-regulation in colonic adenocarcinomas.

In this study, we first comparatively examined the substrate specificities of four Gal3STs and Gal3ST activities in human normal colonic mucosa and colonic adenocarcinomas. The specificities of the Gal3ST activities in colonic mucosa and cancer are very similar to that of Gal3ST-2, and the levels of Gal3ST activities in normal mucosa are significantly higher than those in non-mucinous adenocarcinoma. Moreover, we analyzed expression levels of Gal3ST-2 transcript by competitive RT-PCR, and found that the levels in non-mucinous adenocarcinoma are lower than those in the adjacent normal mucosa.

MATERIALS AND METHODS

Preparation of normal human colonic mucosa and adenocarcinoma tissues Fresh samples of normal colonic mucosa and carcinomas from 39 patients were stored frozen at −80°C before use. Tables II and III show the Dukes' staging system, the histological character of the adenocarcinomas, and the serum CEA levels of the 32 patients before surgery. Two of the 39 patients were assessed as "A," 15 patients were "B," 13 patients were "C," and the other 9 patients were "B(D)" or "C(D)," according to Dukes' staging system. Twenty-four cases (cases 1−18 in Table II and cases 1−6 in Table III) were classified as non-mucinous adenocarcinoma, 6 (cases 22, 24−26, 30 in Table II and case 9 in Table III) were classified as mucinous adenocarcinoma, and 9 (cases 19−21, 23, 27−29 in Table II and cases 7, 8 in Table III) were classified as adenocarcinoma with a mucinous component. Nine patients had a focus of distant metastasis in the liver (cases 13−18, 28−30 in Table II). Serum CEA levels were within the normal range (less than 2.5 ng/ml) in the 12 patients (cases 2, 3, 6−8, 21, 25, 26, 30 in Table II and cases 2, 7, 9 in Table III), while elevation of serum CEA levels was seen in other patients.

Normal mucosal tissue was obtained from an area 10 cm distant from the margin of the carcinoma, and it was separated completely from the muscle layer. The carcinoma tissue was obtained from the surface portion of the carcinoma which was rich in carcinoma cells and had little interstitial tissue.

The percentage of the mucinous component in the carcinoma tissue was evaluated by periodic acid Schiff staining which was diastase-resistant, in the tissue sections taken from the areas next to the fresh samples.

The study was approved by the Kagoshima University Faculty of Medicine Human Investigation Committee (No. H13-4).

Preparation of microsomes from human colonic tissues and Gal3ST-expressed COS-7 cells The microsome preparation procedures were described previously.[7] Briefly, tissues (0.1−0.3 g) or cells were immersed in 9 volumes of 10 m*M* HEPES-NaOH buffer (pH 7.2), 0.25 *M* sucrose at 4°C, homogenized with a Potter-Elvehjem type homogenizer, and then centrifuged. The extraction was further performed twice and the three supernatant fractions were mixed and ultracentrifuged at 100 000*g* for 1 h. The precipitated microsomes were suspended in 20 m*M* HEPES-NaOH buffer (pH 7.2) and kept at −80°C until use.

Chemicals and enzymes [^{35}S]PAPS (62.5 GBq/mmol) was purchased from NEN Life Science Products (Boston, MA). Type 2, core 1, core 1-*O*-pNP, Galα1-*O*-pNP, and core 2-*O*-pNP were obtained from Funakoshi Co., Ltd. (Tokyo). Type 1, GalCer, and core 1-*O*-Bn were obtained from Sigma (St. Louis, MO). Galβ1-*O*-pNP was obtained from Nacalai Tesque (Kyoto). Lac-*O*-pNP was obtained from Seikagaku Corp. (Tokyo). AsialoGP was prepared

Table II. Major Clinicopathological Features of the 30 Patients with Colonic Non-mucinous Adenocarcinoma, Mucinous Adenocarcinoma, or Adenocarcinoma with a Mucinous Component and Gal3ST Activities Detected in Adjacent Normal Mucosa (A) and Adenocarcinoma Tissues (C)

Case	Age	Sex	Site[a)]	Stage[b)]	CEA (ng/ml)[c)]	Histological character	Mucinous component (%)	Gal3ST (pmol/min/mg protein)	
								A[d)]	C[e)]
Non-mucinous adenocarcinoma									
1	63	M	S	A	29.5	papillary	0	293	140
2	83	M	S	B	1.7	tubular	0	354	217
3	62	M	D	B	1.9	tubular	0	306	169
4	51	F	S	B	N/E[f)]	papillary	0	205	62
5	74	F	R	B	32.8	tubular	0	235	36
6	65	M	R	B	2.0	tubular	0	445	219
7	81	F	A	B	1.4	tubular	0	361	178
8	51	M	R	C	0.7	tubular	0	329	240
9	77	M	D	C	N/E	papillotubular	0	218	117
10	66	F	S	C	17.7	tubular	0	152	89
11	86	F	A	C	12.9	tubular	0	275	155
12	62	F	R	C	2.5	tubular	0	224	130
13	69	M	D	C (D)	280.4	papillary	0	166	206
14	68	F	A	C (D)	N/E	tubular	0	198	71
15	77	M	R	C (D)	2.9	tubular	0	268	128
16	74	F	S	C (D)	16.3	tubular	0	410	78
17	84	M	R	C (D)	3.2	tubular	0	292	126
18	67	F	R	C (D)	7.4	tubular	0	175	123
							Mean±SD	273±19.8	138±13.8
Mucinous adenocarcinoma or adenocarcinoma with a mucinous component									
19	70	M	S	A	N/E	tubulo-villous adenoma[g)]	80	431	395
20	85	F	R	B	32.1	tubulo-villous adenoma	40	293	157
21	80	M	A	B	1.1	tubulo-villous adenoma	80	232	260
22	76	M	S	B	N/E	mucinous/tubular	60	262	109
23	56	F	C	B	N/E	tubular/mucinous	20	158	293
24	85	F	A	B	20.3	mucinous/tubular	60	114	30
25	70	M	S	C	0.5	mucinous/tubular	50	317	279
26	69	F	A	C	2.2	mucinous	90	216	258
27	85	F	A	C	2.5	tubular/mucinous	20	135	71
28	84	F	A	B (D)	146.0	tubular/mucinous	30	60	276
29	63	M	S	C (D)	380.5	tubular/mucinous	30	380	71
30	37	F	T	C (D)	1.6	mucinous/tubular	80	189	26
							Mean±SD	232±31.8	185±35.4

a) C, cecum; A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum.
b) Dukes' system.
c) Normal range, <2.5 ng/ml.
d) Adjacent normal mucosa.
e) Carcinoma.
f) Not examined.
g) Adenocarcinoma with tubulo-villous adenoma.

from egg yolk sialoglycopeptide[15)] by mild acid hydrolysis. LNT was prepared from human milk.[16)]

Assay of sulfotransferase activity Twenty microliters of reaction mixture consisting of 0.1 *M* sodium cacodylate (pH 6.3), 10 m*M* $MnCl_2$, 0.1% (v/v) Triton X-100, 0.1 *M* NaF, 2 m*M* ATP-Na_2, 6.5 μM [^{35}S]PAPS (4.9×10^5 dpm), 1 m*M* acceptor substrate, and the membrane fraction approximately diluted, was incubated at 37°C for 1 h. The ^{35}S-labeled products were purified by paper electrophoresis (pyridine/acetic acid/water, 3:1:387, pH 5.4). After extraction with water, the radioactivity was counted. In the case of GalCer used as a substrate, the detection of the ^{35}S-

Table III. Major Clinicopathological Features of the 9 Patients with Colonic Non-mucinous Adenocarcinoma and Mucinous Adenocarcinoma or Adenocarcinoma with a Mucinous Component, and Relative Values of Transcript for Gal3ST-2

Case	Age	Sex	Site[a]	Stage[b]	CEA (ng/ml)[c]	Histological character	Mucinous component (%)	Competitive PCR[d]	
								A[e]	C[f]
								SulT/GAPDH ($\times 10^3$)	
Non-mucinous adenocarcinoma									
1	61	M	T	B	3.5	tubular	0	1.9	0.47
2	66	M	R	B	1.6	tubular+villous	0	0.68	0.46
3	63	M	R	B	4.0	tubular	0	0.57	0.068
4	74	M	R	C	4.2	tubular	0	0.32	0.44
5	49	M	R	C	7.7	tubular	0	2.6	0.069
6	67	F	S	C	N/E[g]	tubular	0	6.5	0.58
							Mean±SD	2.1±0.95	0.35±0.091
Mucinous adenocarcinoma or adenocarcinoma with a mucinous component									
7	71	M	R	B	0.9	tubular+villous/mucinous	5	0.40	0.35
8	60	M	S	C	2.8	tubular+mucinous	5	0.38	1.6
9	48	M	A-T	C	0.7	villous/mucinous	50	0.074	1.8

a) A, ascending colon; T, transverse colon; S, sigmoid colon; R, rectum.
b) Dukes' system.
c) Normal range, <2.5 ng/ml.
d) Values were ratios of the amounts of transcript for Gal3ST-2 to those for GAPDH.
e) Adjacent normal mucosa.
f) Carcinoma.
g) Not examined.

labeled product was performed according to the methods reported by Kawano *et al*.[17]

Characterization of the ^{35}S-labeled product The ^{35}S-labeled product was subjected to periodate oxidation as described previously.[13] Briefly, the labeled oligosaccharides were dissolved in 20 μl of 75 m*M* sodium metaperiodate, 75 m*M* sodium acetate (pH 5.3) and incubated at 4°C for 24 h in the dark. After destruction of excess periodate, 300 μl of 0.1 *M* sodium borate (pH 9.0) containing 0.1 *M* sodium borohydride was added, and the solution stood for 1 h at room temperature. The solution was then acidified and desalted. The ^{35}S-labeled compound was hydrolyzed in 100 μl of 0.05 *N* H_2SO_4 at 80°C for 1 h. After neutralization, the ^{35}S-labeled compound was purified by paper electrophoresis, extracted with water, applied to a thin layer plate (Kieselgel 60F_{254}, Merck, Darmstadt, Germany), and developed with solvents, 1-butanol/pyridine/water (6:4:3) or 1-butanol/ethanol/water (4:1:1). The radioactivity was monitored with a radiochromatogram scanner.

Authentic $SO_3^-\rightarrow$3Galβ1$\rightarrow$3GalNAc and 3′-*O*-sulfated core 1-*O*-Bn were synthesized by Gal3ST-4.[13] Authentic [^{3}H]Galβ1$\rightarrow$3GalNAcα1-*O*-Bn and $SO_3^-\rightarrow$6GlcNAcβ1-*O*-Bn were prepared as described previously.[13]

cDNA cloning of Gal3ST-1 The cDNA encoding for Gal3ST-1[9] was amplified from "Super Script" human testis cDNA library (Life Technologies, Inc., Rockville, MD) by PCR. Oligonucleotide primers used for the PCR were 5′-tttaagcttGTCTGAGATGCTGCCA-3′ (forward primer) and 5′-tttggatccTGGGACGTCACCACCG-3′ (reverse primer). Sequences in small letters contain appropriate restriction sites. The amplified cDNA was digested with *Hin*dIII and *Bam*HI and cloned between the respective sites of pcDNA3 (Invitrogen Corp., Carlsbad, CA). The constructed plasmid was named pcDNA3-Gal3ST-1, and sequenced using an Applied Biosystems "PRISM" 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA).

Plasmids pSV-GP3ST, which is an expression vector encoding for Gal3ST-2, was a kind gift from Dr. K. Honke (Osaka University).[10] pCXNGalST encoding for Gal3ST-3 was a kind gift from Dr. T. Muramatsu (Nagoya University).[12]

Expression of SulTs in COS-7 cells The plasmids (1 μg) were transfected into COS-7 cells on 35-mm dishes using Lipofectin Reagent (Life Technologies, Inc.) according to the manufacturer's instructions. After 48 h, the cells were washed twice with phosphate-buffered saline, scraped off the dishes in 10 m*M* HEPES-NaOH (pH 7.2) and 0.25 *M* sucrose, and homogenized. The homogenates were ultracentrifuged at 100 000*g* for 1 h. The precipitated crude membranes were suspended in 20 m*M* HEPES-NaOH (pH 7.2) and kept at −80°C until use.

Determination of protein concentrations The protein

concentrations in the preparations of crude membranes were estimated using the Bio-Rad Protein Assay dye reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

Quantitation of Gal3ST-2 transcript by the competitive PCR method The levels of Gal3ST-2 transcript were measured by competitive PCR [18] based on the amounts of their respective cDNAs, which were reverse-transcribed from RNA samples. For distinction of a target cDNA from its competitor DNA, pSVK-Gal3ST-2 was digested with *Mlu*I and ligated. The treatment generated the deletion of a 239-bp fragment within the region corresponding to the cDNA. The partial cDNA for GAPDH (nucleotides 95–701 in the sequence of accession number XM_Q033263) was generated from "SuperScript" human testis cDNA library by PCR using oligonucleotides 5′-tttctcGAGTCAACGGATTTGGT-3′ as a forward primer and 5′-tttctGCAGGGATGATGTTCTG-3′ as a reverse primer. The amplified *Xho*I-*Pst*I fragment was inserted into the *Xho*I-*Pst*I site of pBluescript II SK+/−. The competitor cDNA for GAPDH was generated by deleting a 155-bp fragment by digestion with *Nco*I and ligation.

From 5 μg of total RNA isolated by lysis of normal colon tissues or adenocarcinomas in ISOGEN (Nippon Gene, Tokyo), cDNAs were synthesized using oligo(dT) primers and SuperScript II (Life Technologies, Inc.) in a total volume of 21 μl. To check for contamination by genomic DNA in RNA samples, cDNA synthesis was performed in the absence of SuperScript II using a small aliquot of isolated total RNA. One microliter of appropriately diluted cDNAs was subjected to quantitative PCR analysis according to the methods of Sasaki *et al.*[19] After incubation at 95°C for 5 min, PCR was performed in a volume of 20 μl for 30 cycles (GAPDH) or 35 cycles (Gal3ST-2) of 95°C for 30 s, 65°C for 1 min, and 72°C for 2 min, in the presence of 1.5 m*M* $MgCl_2$ and 2% DMSO. The primers used were 5′-TCGGACTTAGAGCTGGACACACC-3′ (forward primer for Gal3ST-2), 5′-GTTGAAATGCTCGTACAGGCGCCA-3′ (reverse primer for Gal3ST-2), 5′-TCAACGGATTTGGTCGTATTGGGC-3′ (forward primer for GAPDH), and 5′-CAGGGATGATGTTCTGGAGAGCC-3′ (reverse primer for GAPDH). After amplification, 10 μl aliquots were subjected to electrophoresis in 1.0% agarose gels, followed by staining with ethidium bromide. Amplified DNA fragments were quantified by scanning with a fluoro-image analyzer FLA-2000 (Fuji Photo Film Co., Ltd., Tokyo). The amounts of target cDNAs were normalized with respect to those of GAPDH.

RESULTS

Substrate specificities of four Gal3STs First, we expressed four Gal3STs in COS-7 cells and prepared the

Table IV. Substrate Specificities of Four Gal3STs So Far Cloned and Gal3ST Activities in Human Normal Mucosa (Normal), Non-mucinous Adenocarcinoma (Non-mucinous), and Mucinous Adenocarcinoma or Adenocarcinoma with a Mucinous Component (Mucinous)

Substrate[a]	Gal3ST-1	Gal3ST-2	Gal3ST-3	Gal3ST-4[b]	Normal		Non-mucinous		Mucinous	
					Case 15[c]	Case 16	Case 15	Case 16	Case 23	Case 28
type 1	<1	55	11	<1	45	52	61	51	45	50
LNT	<1	71	<1	<1	67	73	82	74	68	75
type 2	<1	108	100[d]	1	95	99	93	95	96	108
asialo GP	<1	80	121	<1	87	79	100	73	76	84
Lac-*O*-pNP	89	58	114	<1	ND[e]	ND	ND	ND	ND	ND
core 1	<1	100[f]	14	100[f]	100[f]	100[f]	100[f]	100[f]	100[f]	100[f]
core 1-*O*-pNP	160	121	9	95	106	109	121	115	108	126
core 1-*O*-Bn	224	120	23	50	102	111	116	114	111	120
core 2-*O*-pNP	4	70	13	121	85	81	92	82	80	88
GalCer	100[g]	<1	<1	<1	ND	ND	ND	ND	ND	ND
Galα-*O*-pNP	<1	53	<1	<1	ND	ND	ND	ND	ND	ND
Galβ-*O*-pNP	175	62	67	<1	ND	ND	ND	ND	ND	ND

a) The concentrations were 1 m*M*.
b) Reported by Seko *et al.*[13]
c) See Table II.
d) Relative ratios are taken with the value of type 2 as 100.
e) Not determined.
f) Relative ratios are taken with the value of core 1 as 100.
g) Relative ratios are taken with the value of GalCer as 100.

crude membrane fractions for use as enzyme sources. Specific activities of the membrane fractions derived from Gal3ST-1, -2, -3, and -4-transfected COS-7 cells were 13.8 (using GalCer as a substrate), 867 (core 1), 14.9 (type 2), and 19.0 (core 1) pmol/min/mg of protein, respectively. These activities were exclusively derived from each SulT protein, because Gal3ST activities for core 1, type 2, or GalCer were not detected in the membrane fractions from wild-type COS-7 cells or pcDNA3-transfected COS-7 cells. The substrate specificities of Gal3ST-1, -2, -3, and -4 are summarized in Table IV. Gal3ST-1 is the only enzyme capable of acting on GalCer. Interestingly, Gal3ST-1 also acts on β-Gal residues in pNP- or Bn-conjugated oligosaccharides, suggesting that Gal3ST-1 essentially recognizes hydrophobic moieties including Cer, pNP, and Bn, as well as β-Gal residues. Gal3ST-2 can act on type 1, type 2, and core 1 structure, as shown by Honke *et al.*[10] It should be noted that the enzyme can utilize not only β-Gal residues, but also Galα-*O*-pNP, and that LNT can be sulfated only by Gal3ST-2 (Table IV). Gal3ST-3 prefers type 2 oligosaccharides to type 1 and core 1, as shown by El-Fasakhany *et al.*[12] Gal3ST-4 is strictly core 1-specific, as shown by us.[13]

Substrate specificities of Gal3ST activities in human colonic tissues SulT activities for core 1-*O*-Bn as a substrate were detected in the membrane fractions prepared from human normal colonic mucosa and colonic adenocarcinomas. To assess the linkage position of [^{35}S]sulfate in [^{35}S]sulfated core 1-*O*-Bn, the ^{35}S-labeled product was subjected to periodate oxidation and Smith degradation. The ^{35}S-labeled reaction product was applied to TLC using two solvent systems (Fig. 1). In both systems, the ^{35}S-labeled reaction product migrated to the same positions as the intact ^{35}S-labeled product and authentic 3′-*O*-sulfated core 1-*O*-Bn synthesized by Gal3ST-4.[13] Periodate oxidation treatment cleaves the C-C bond of *cis*-diol groups, suggesting that [^{35}S]sulfate is exclusively linked to the C-3 position of Gal residue. The results indicate that the SulT activities are those of a Gal3ST. Next, we examined the substrate specificities of the Gal3STs present in two specimens each of normal mucosa, non-mucinous adenocarcinoma, and mucinous adenocarcinoma or adenocarcinoma with a mucinous component (Table II). All of the Gal3ST activities present in these tissues can act on type 1, type 2, core 1, and LNT oligosaccharides, and the substrate specificities are quite similar to that of Gal3ST-2 (Table IV). These results suggest that Gal3ST activities in normal mucosa and adenocarcinomas are dominantly derived from Gal3ST-2.

Levels of Gal3ST-2 activities in normal mucosa and adenocarcinomas It has been shown that expression levels of sulfomucin are lower in colonic adenocarcinomas than in normal mucosa,[1, 2] suggesting that expression levels of SulTs acting on *O*-glycan chains are down-regulated in colonic adenocarcinomas. To assess whether or not the

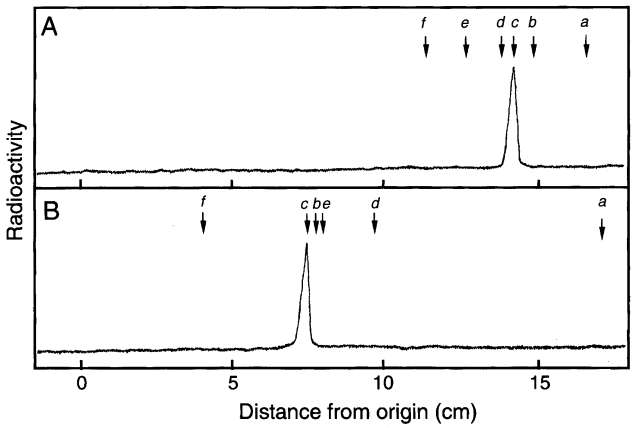

Fig. 1. Analysis of the sulfate linkages of [^{35}S]sulfated core 1-*O*-Bn synthesized by the membrane fraction of human colonic normal mucosa. Panels A and B, silica gel TLC of periodate oxidation product of [^{35}S]sulfated core 1-*O*-Bn using 1-butanol/pyridine/water (6:4:3) (panel A), or 1-butanol/ethanol/water (4:1:1) (panel B). Arrows indicate the front of the developing solvent (*a*), and the positions of standard compounds; *b*, [^{3}H]Galβ1→3GalNAcα1-*O*-Bn; *c*, 3′-*O*-[^{35}S]sulfated core 1-*O*-Bn; *d*, the periodate oxidation product of [^{3}H]Galβ1→3GalNAcα1-*O*-Bn; *e*, the periodate oxidation product of [^{35}S]SO_3^-→6GlcNAcβ1-*O*-Bn; *f*, 3′-*O*-[^{35}S]sulfated core 1.

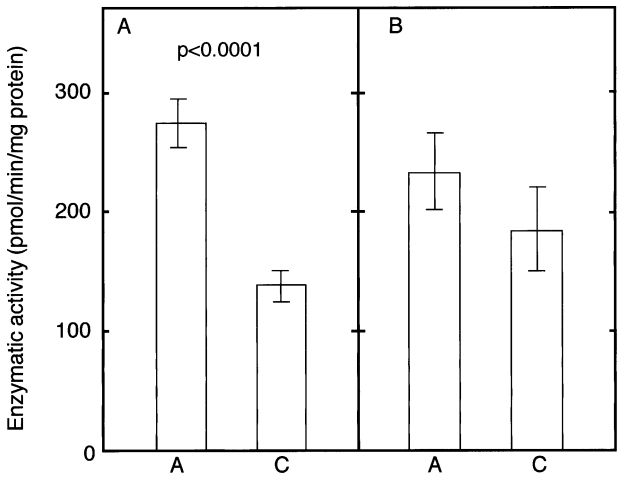

Fig. 2. Comparison of Gal3ST-2 activities in normal human colonic mucosa with those in non-mucinous adenocarcinoma (A) or mucinous adenocarcinoma or adenocarcinoma with a mucinous component (B). LNT was used as an acceptor substrate. A and C under each panel denote the adjacent normal colonic mucosa and adenocarcinomas, respectively. Columns, mean; bars, SE.

levels of Gal3ST-2 activities in colonic adenocarcinomas are lower than those in normal mucosa, we comparatively examined Gal3ST-2 activities in adenocarcinomas and the adjacent normal mucosa using LNT as a Gal3ST-2-specific substrate. The data and major clinicopathological features of 30 patients are summarized in Table II and Fig. 2. Specimens showing non-mucinous adenocarcinoma were separated from those with mucinous adenocarcinoma or adenocarcinoma with a mucinous component, because we previously found that there is a difference in expression of a GlcNAc 6-*O*-SulT, SulT-b, between mucinous and non-mucinous adenocarcinomas.[20] In the cases of non-mucinous adenocarcinoma, the levels of Gal3ST-2 activities were significantly lower in adenocarcinoma than in the adjacent normal mucosa (138±13.8 pmol/min/mg of protein in adenocarcinoma on average and 273±19.8 pmol/min/mg of protein in the adjacent mucosa, $P<0.0001$) (Fig. 2A). On the other hand, in the cases of mucinous adenocarcinoma or adenocarcinoma with a mucinous component, levels of Gal3ST-2 activity were slightly lower in adenocarcinomas than in the adjacent normal mucosa, but there was no significant difference (Fig. 2B).

Levels of transcript for Gal3ST-2 in normal mucosa and adenocarcinomas We suggested above that Gal3ST-2 is the dominant Gal3ST in normal colonic mucosa and colonic adenocarcinomas, but we can not exclude the possibility that there exists an unidentified *Gal3ST* gene which shows similar substrate specificity to that of Gal3ST-2. To assess whether or not levels of transcript for Gal3ST-2 are down-regulated in colonic adenocarcinomas, like the enzymatic activities, we performed competitive RT-PCR to quantify the amounts of the transcript. As shown in Table III, relative levels of transcript for Gal3ST-2 in normal mucosa (2.1±0.95 ($\times 10^3$) on average) are significantly higher than those in non-mucinous adenocarcinoma (0.35±0.091 ($\times 10^3$), $P<0.05$). In the cases of mucinous adenocarcinomas, levels of the transcript in the two specimens (cases 8,9) are higher in adenocarcinomas than in the adjacent normal mucosa, while in case 7, the level of the transcript is slightly lower in adenocarcinoma than in the adjacent normal mucosa. The results indicate that expression levels of Gal3ST-2 decrease in non-mucinous adenocarcinoma, in comparison with those in the adjacent normal mucosa.

DISCUSSION

In this study, we assayed the substrate specificities of four Gal3STs so far cloned, and found that LNT is a substrate utilized only by Gal3ST-2. Gal3STs detected in colonic adenocarcinomas and the adjacent normal mucosa can also act on LNT and predominantly resemble Gal3ST-2, because of their closely similar substrate specificities to that of Gal3ST-2. Levels of Gal3ST-2 activities in non-mucinous adenocarcinoma are lower than those in the adjacent normal mucosa, while there is no significant difference between those in mucinous adenocarcinoma or adenocarcinoma with a mucinous component and the adjacent normal mucosa.

It has been shown that expression levels of sulfomucin are lower in colonic adenocarcinomas than in normal mucosa.[1,2] Loveless *et al.*[6] showed that the epitope moiety of sulfomucin recognized by monoclonal antibody 91.9H is the $SO_3^-\rightarrow 3Gal\beta1\rightarrow 3(Fuc\alpha1\rightarrow 4)GlcNAc\beta1\rightarrow 3Gal\beta1\rightarrow$ structure. It has been shown that the epitope is strongly expressed in human normal colonic mucosa, while its expression in colonic adenocarcinomas is rather low.[3–5] Considering the substrate specificities of Gal3STs and α1,4-fucosyltransferase,[10–12,21–23] the epitope can be synthesized from $Gal\beta1\rightarrow 3GlcNAc\beta1\rightarrow 3Gal\beta1\rightarrow$ by 3-*O*-sulfation at the non-reducing terminal Gal followed by α1,4-fucosylation at the GlcNAc residue. Since the backbone structure, $Gal\beta1\rightarrow 3GlcNAc\beta1\rightarrow 3Gal\beta1\rightarrow$, is included in LNT, and 3-*O*-sulfation of LNT is catalyzed only by Gal3ST-2 (Table IV), the 91.9H epitope should be synthesized by Gal3ST-2. This is supported by the report of Honke *et al.*,[10] who found strong expression of Gal3ST-2 in human normal colonic mucosa, especially in epithelial cells lining the bottom to middle of the crypts. We previously showed that levels of β1,3-galactosyltransferase activities are markedly lower in colonic adenocarcinomas than in normal mucosa.[7] Thereafter, our result was supported by Salvini *et al.*,[8] who showed that expression levels of β3GalT-V are lower in colonic adenocarcinomas than in normal mucosa. On the other hand, expression levels of FucT III, which is the only enzyme catalyzing α1,4-fucosylation, do not significantly change between colonic

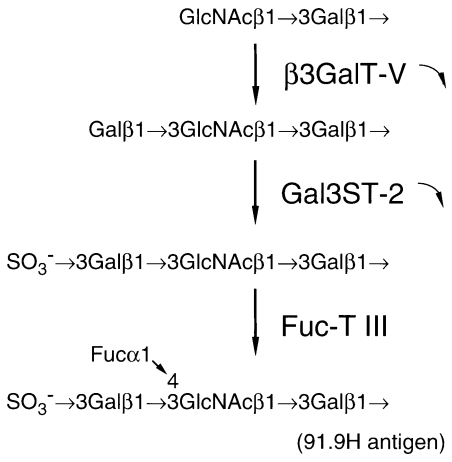

Fig. 3. Possible biosynthetic scheme of the 91.9H antigen. In human colonic non-mucinous adenocarcinoma, expression levels of *β3GalT-V* and *Gal3ST-2* genes are down-regulated (↘).

adenocarcinomas and normal mucosa.[24–26] Although the down-regulation of expression of the *Gal3ST-2* gene is evident as shown in Tables II, III, and Fig. 2, there exist substantial enzymatic activities in colonic non-mucinous adenocarcinoma. These facts and our present results suggest that the decrease of expression of the 91.9H epitope in colonic non-mucinous adenocarcinoma is caused by down-regulation of expression levels of not only Gal3ST-2, but also β3GalT-V, which synthesizes the substrate oligosaccharides for Gal3ST-2 (Fig. 3).

Decrease of expressional levels of sulfomucin in colonic adenocarcinoma might influence cell-cell adhesion in the epithelial cell layer of the colon. Recently, carbohydrate-binding proteins, especially recognizing sulfated oligosaccharides, have been identified. L-Selectin binds to 6-*O*-sulfo- or 6′-*O*-sulfo-sialyl Le^x structure on *O*-linked glycans of GlyCAM-1.[27, 28] We reported that galectin-4, mainly expressed in colonic epithelial cells, specifically recognizes 3′-*O*-sulfated core 1 structure.[29] It is possible that a sulfomucin-binding lectin is present in colonic epithelial cells and contributes to the maintenance of cellular structure of the epithelial cell layer. Identification of such a lectin protein or artificial deletion of the *Gal3ST-2* gene could assist in the elucidation of the biological significance of sulfomucin.

Levels of Gal3ST-2 activities in mucinous adenocarcinomas are not significantly different from those in normal mucosa (Table II). Colonic mucinous adenocarcinomas are defined by the WHO as those in which at least 50% of the area evaluated microscopically is covered with mucus.[30] We previously found that a GlcNAc 6-*O*-SulT, SulT-b, is expressed in mucinous adenocarcinoma or adenocarcinoma with a mucinous component, but not in normal mucosa and non-mucinous adenocarcinoma.[20] Moreover, we recently found that expression levels of galectin-4, a lectin mainly expressed in small intestine and colon, are lower in non-mucinous adenocarcinomas than in the adjacent normal mucosa, while those in mucinous adenocarcinomas are similar to those in the adjacent normal mucosa (Nagata *et al.*, manuscript in preparation). From these results, we speculate that the expression profile of glycan chains in mucinous adenocarcinomas is rather different from that in non-mucinous adenocarcinomas. It is important to elucidate how the expression level of Gal3ST-2, SulT-b, or galectin-4 is regulated in non-mucinous and mucinous adenocarcinomas.

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