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Glycoantigen Biosyntheses of Human Hepatoma and Colon Cancer Cells are Dependent on Different *N*-Acetylglucosaminyltransferase-III and -V Activities

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Abstract UDP-*N*-Acetylglucosamine(GlcNAc):β1,4-D-mannosideβ-1,4*N*-acetylglucosaminyltransferase-III (GnT-III) and UDP-*N*-GlcNAc:α-6-D-mannosideβ-1,6*N*-acetylglucosaminyltransferase-V (GnT-V) activities were determined in human hepatoma cell lines and metastatic colon cancer cells, and their activities were compared with those of normal liver cells and fetal hepatocytes. GnT-III activities were higher than those of GnT-V in hepatic carcinoma cells. When the two enzyme activities were assayed in highly metastatic colon cancer cells, GnT-V activities were much higher than those of GnT-III. When GlcN,GlcN-biant-PA and UDP-GlcNAc were used as substrates, the enzymes displayed different kinetic properties between hepatic and colon cancer cells, depending on their metastatic potentials. Normal cells of two origins had characteristically very low levels of GnT-III and -V activities, whereas hepatoma and colon cancer cells contained high levels of activities. These data were supported by RT-PCR and Northern blot analyses, showing that the expression of GnT-III and -V mRNAs were increased in proportion to the enzymatic activities. The increased GnT-III and -V activities were also correlated with increased glycosylation of the cellular glycoproteins in hepatoma and colon cancer cells, as examined by lectin blotting analysis by using wheat germ glutinin (WGA), erythroagglutinating phytohemagglutinin (E-PHA), leukoagglutinating phytohemagglutinin (L-PHA), and concanavalin A (Con A). Treatment with retinoic acid, a differentiation agent, resulted in decreases of both GnT-III and -V activities of HepG2 and HepG3 cells. In colon carcinoma cells, however, treatment with retinoic acid resulted in a reduction of GnT-V activity, but not with GnT-III activity. Although the mechanism underlying the induction of these enzymes is unclear, oligosaccharides in many glycoproteins have been observed of cancer cells.

Key words: GnT-III, GnT-V, hepatoma, colon cancer, metastasis, glycosylation, lectin, retinoic acid

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Carbohydrate structures of glycoproteins and glycolipids on the cell surface are associated with development, differentiation, and transformation [13], and they are mainly determined by glycosyltransferases and glycosidases. Since the synthesis of oligosaccharides requires one enzyme for one glycosidic linkage, more than one hundred kinds of glycosyltransferases seem to exist. Until now, we have cloned several glycosyltransferases such as *N*-acetylglucosaminyltransferase (GnT)-III [27], Galβ1,3GalNAcα2,3sialyltransferase (hST3GalII) [25], mouse Galβ1,3(4)GalNAcα2,3sialyltransferase (mST3GalIII) [19], Galβ1,4(3)GalNAcα2,3sialyltransferase (mST3Gal IV) [26], human α2,8sialyltransferase (hST8Sia V) [26], human Siaα2,3Galβ1,4GalNA:α2,8Sialyltransferase (hST8SialII) [32], GnT-V (GnT-V) [39], and human GM3 synthase (hST3Gal V) [21].

Human hepatoma and carcinoma cells can be induced to undergo metastatic differentiation by retinoic acid [10]. The asparagine-*N*-linked oligosaccharides found on the cell surface of hepatoma tissues differ in size and structure from those found on the cell surface of normal liver cell [43], and it has been suggested that these changes are due to changed expression of GnTs, involved in the biosynthesis of tri- and tetra-antennary *N*-linked chains, bisected structures, and elongated chains. The formation of the branches is governed by the activities of a set of GnT-I-VII [41]. To obtain more insight into the enzymatic basis for the tumor-dependent structural alterations, we assayed the activities of GnT-III and GnT-V (Fig. 1) in human hepatoma cell lines of Hep3B and HepG2, and also in human colon cancer cells of SW620 and HTB39.

GnT-III produces a bisecting *N*-acetylglucosamine (GlcNAc) residue in the *N*-linked mannose of the trimannosyl core of the oligosaccharides. GnT-III activity was observed in liver hepatocarcinogenesis [37, 44], serum from hepatoma human patients [43], rat kidney, human B lymphocytes, HL60 cells, Novikoff ascites tumor cell [29], CaCO-2

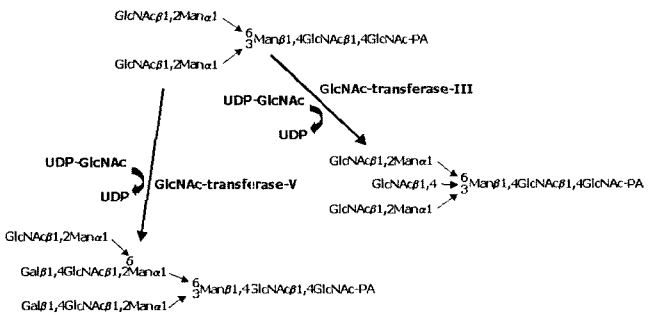

Fig. 1. The oligosaccharide chain structures of GlcN,GlcN-biant-PA, GlcN,(GlcN),GlcN-biant-PA, and GlcN,GlcN,GlcN-biant-PA.
GlcN,(GlcN),GlcN-biant-PA and GlcN,GlcN,GlcN-biant-PA are the reaction products of GnT-III and GnT-V, respectively, when GlcN,GlcN-biant-PA as acceptor substrate and UDP-GlcNAc as donor substrate are used.

cells [3], and HuH-6 cells [38]. Increased expression of the enzyme was highly associated with malignant transformation or oncofetal changes. Therefore, it would appear that an elevated GnT-III activity might cause uncontrolled expression of GnT-III by altering the glycosylation pattern of proteins and lipids in hepatocellular carcinoma and GnT-III transgenic mice [31, 45]. The biological significance of increased GnT-III expression is not clearly understood.

It has also been suggested that increased β-(1,6)-branching is associated with an increased metastatic potential [8]. Decreased branching has been associated with a differentiation of cells [36]. Therefore, study of these enzymes may yield an enzymatic basis for the changes in branching patterns upon malignant transformation and differentiation of cells. The increase of such material in malignant cells is due to an increased activity of GnT-V, which catalyzes the transfer of GlcNAc from UDP-GlcNAc to a β-D-mannoside [2]. Furthermore, GnT-V activity during rat hepatocarcinogenesis increased in correlation with the amount of GnT-V mRNA [16, 34]. The β-(1,6)-branching may contribute to the malignant or metastatic properties of tumor cells, since blocking the glycosylation pathway prior to the formation of the β-(1,6)-linked antenna leads to a loss of metastatic potential and inhibits organ colonization [8, 7, 12]. Because of its extreme instability when solubilized from membrane fractions, it is not easy to assay GnT-V activity. Little is known about the increased expression in hepatoma cells as well as the molecular mechanisms by which GnT-V activity is specifically increased in liver disease.

In order to investigate the regulatory mechanism of GnT-III and GnT-V activities in human hepatoma cells and colon cancer cells, we examined the activities of membrane forms of enzyme from HepG2, Hep3B, normal primary hepatocytes, hepatoma tissues, and SW620 and HTB39 cells.

MATERIALS AND METHODS

Materials

Cell pellets were homogenized at 0°C in 10 mM sodium cacodylate, (pH 7.0). UDP-GlcNAc and UDP-hexanolamine were obtained from Sigma. Pyridylamino (PA) fluorescence-labeled biantennary oligosaccharides such as GlcN,GlcN-biant-PA and GlcN,(GlcN)GlcN-biant-PA were obtained from bovine fetuin, following treatment with anhydrous hydrazine. Fluorescence-labeled oligosaccharides were prepared from the corresponding oligosaccharides by pyridylamination [14, 39], followed by sequential digestion with exoglycosidases. PA fluorescence-labeled oligosaccharide, GlcN,GlcN,GlcN-biant-PA, was obtained from Takara Shuzo Co. (Kyoto, Japan). The structures of the sugar chains used in this study are shown in Fig. 1. All other reagents, including retinoic acid, were purchased from Wako Pure Chemical Co. (Tokyo, Japan) and Sigma (St. Louis, MO, U.S.A.) as analytical grade.

Assays of GnT-III and GnT-V

Pyridylaminated biantennary sugar of GlcN,GlcN-biant-PA was used as the substrate in assays of GnT-III and GnT-V [44]. Incubation mixtures contained 120 mM Mes buffer (pH 7.0), 300 mM GlcNAc, 15 mM $MnCl_2$, 0.5% (0.5%) Triton X-100, 50 mM oligosaccharide GlcN,GlcN-biant-PA, 200 mM UDP-GlcNAc, and cell lysate (0.5–1.5 mg protein) in a 100 μl volume. The mixture was incubated for 18 h at 37°C. The reaction was stopped by adding 1 ml of H_2O and passing the mixture through a column of 1 ml Dowex 1-X8, Ac^--form. The column was washed twice with $NaBH_4$ for 1 h as described [41], lyophilized, and analyzed by HPLC. Sometimes, the reaction was stopped by boiling at 100°C for 5 min and passed through a 0.22 μm Millipore filter, and then lyophilized. The lyophilized material was dissolved in 200 μl of H_2O. Thirty μl of each sample was analyzed by HPLC on a column (4×220 mm) of Lichrosorb-NH_2 (Merck, Darmstadt), using a Shimadzu HPLC with 0.1 M acetate buffer (pH 4.0) containing 0.2% n-butanol, at a flow rate of 1.0 ml/min. Elution was also carried out as indicated in the legend to Fig. 3. The specific activity of the enzyme is expressed as pmol of GlcNAc transferred/h/mg.

Preparation of Cell-Free Extracts from Hepatoma and Colon Cancer Cells

The hepatoma cells (Hep3B and HepG2) and colon cancer cells (SW620 and HTB39) were separately cultivated for 1 week. Cells (5 l) were grown to confluency in DMEM media containing 5% fetal calf serum (Gibco, MO, U.S.A.), supplemented with penicillin (100 units/ml) and streptomycin (100 mg/ml), at 37°C under 5% CO_2 in air for 1 week. Remaining cells were stored at -70°C in 10% DMSO/45% FCS/45% DMEM. After harvesting by filtration through a

glass funnel, cells were stored at -20°C. Approximately 10 g of cells (wet weight) were obtained from five liters of culture. Frozen cells were suspended in 250 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM PMSF, 10 μg/ml aprotinin, and 0.5% Triton X-100, and were broken by a cell homogenizer to obtain cell extract, and the cell extract was then centrifuged (15,000 ×*g*, 30 min) at 4°C. After centrifugation, the supernatant was used as crude cell extract.

Preparation of Cell-Free Extracts from Normal Liver Tissues and Primary Cultured Cells

Tissues were homogenized in 2 volumes of 10 mM Tris-HCl buffer (pH 7.2), containing 0.25 M sucrose and 0.5% Triton X-100, and centrifuged at 900 ×*g* for 10 min [30]. The supernatants were collected and used as crude enzyme preparations [6]. For the primary culture, human hepatocytes were obtained from the livers of organ donors and fetal livers at 13-15 weeks of gestation. The liver tissues were cut with scissors into small pieces and transferred to a 50-ml falcon tube. The larger fragments were allowed to settle, and the supernatant was saved. The supernatant containing hepatocytes was used to seed culture dishes. After seeding, cells were incubated in 10% FCS/DMEM, supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml), at 37°C under 5% CO_2 in air. The cells were harvested for all experiments at the indicated times and stored at -70°C in 10% DMSO/45% FCS/45% DMEM. Preparations of cell extracts from liver tissues and primary culture were the same as described above.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Aliquots of the infected cells were washed twice with PBS. Total RNA was extracted from the cells by using RNA Isolation Kit, RIK 2.11 (Iowa Biotechnology Corp.) as described in the manufacturer's manual. RT-PCR was performed, using RNA LA PCR kit Ver. 1.1 (TaKaRa Co., Kyoto, Japan). For reverse transcription, total RNA was dissolved in 9.5 μl of RNase-free distilled water, and 4 μl of 25 mM $MgCl_2$, 2 μl of 10×RNA PCR buffer [100 mM Tris HCl (pH 8.3), and 500 mM KCl], 2 μl of 10 mM dNTP mixture, 0.5 μl of RNase inhibitor (40 units/μl), 1 μl of AMV reverse transcriptase XL (5 units/μl), and 1 μl of random 9 mers (50 pmol/μl) were added. The total reaction volume of 20 μl was incubated at 30°C for 10 min and 42°C for 30 min. The reverse transcriptase activity was destroyed by heating to 99°C for 5 min. P1 5', 5'GGATGAAGATGAGACGCTACAAG3' and P2 3', 5'GGAACTTGAGCGGCCGCGGCT3', corresponding to the N-terminal region of GnT-III, were used to amplify the cDNA [2]. Also, GNN1 5', 5'GTTGAAAATTGGTGTCCT3' and GNN2 3', 5'GAGCAAGTCCACAATATCG3', corresponding to the N-terminal region of GnT-V gene [39], were used to amplify the cDNA. DNA amplifications were done in 20 μl of reaction volumes containing 2 μl of 10× reaction buffer, 1.2 μl of 25 mM $MgCl_2$, 1 μl of 200 μM dNTP mixture, 1 μl each of primer (10 pmol/l), 0.1 μl of Taq polymerase enzyme (TaKaRa Co., Kyoto, Japan), 3.7 μl of purified water, and 10 μl of sample. The reaction mixtures were covered with 20 μl of mineral oil and placed in a TaKaRa PCR Thermal Cycler 480 (TaKaRa Co.). The amplification was performed by heating the samples for 5 min at 94°C, then by using fifty cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, and extension for 1 min at 72°C. The amplified product was analyzed by electrophoresis in 1% (w/v) agarose gels.

Northern Blot Analysis

To detect the expression of GnT-III and GnT-V genes, mRNA levels were determined, using Northern blot analysis. Total RNA was prepared from cells by using the RNAzol B reagent (Tel-test, U.S.A.), according to the manufacturer's instruction. For Northern blot analysis, total RNA was separated by electrophoresis in a 1.5% formaldehyde agarose gel. After electrophoresis, the gel was blotted onto Hybond-C membrane (Amersham, U.K.). RNA was fixed to the membrane by crosslinking for 3 min by Ultra-Violet irradiation. Hybridization was performed in Quickhyb (Stratagene, U.S.A.) to a random prime (Stratagene, U.S.A.) ^{32}P-labeled probe that encompassed the entire and partial GnT-III and GnT-V genes. The filter was washed and developed as described [18]. The membrane was dehybridized at 90°C in 0.1× standard saline citrate, containing 0.1% SDS, for 30 min twice and then hybridized with β-actin cDNA.

Lectin Blot Analysis of Hepatoma, Colon Cancer, and Normal Cells

Five micrograms of total cellular proteins of hepatoma cells and hepatocytes were subjected to 10% sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). After resolution, the gels were blotted onto a nitrocellulose filter, blocked with 2% skim milk overnight, and then incubated with 1 μg/ml of digoxygenin-labeled lectins of wheat germ glutinin (WGA), erythroagglutinating phytohemagglutinin (E-PHA), leukoagglutinating phytohemagglutinin (L-PHA), and concanavalin A (Con A) for 4 h. After washing for 10 min three times with Tris-buffered saline (TBS), containing 0.05% Tween 20, the filter was stained with 1/5,000 diluted phosphatase for 30 min [15]. To exclude any nonspecific staining, 0.5 mol/l GlcNAc (Sigma) was used as an inhibitor for L-PHA blot.

Electrophoresis and Other Analytical Methods

Discontinuous sodium dodecyl sulfate (SDS)-PAGE was done essentially by the general method [16]. The bands of protein were stained with Coomassie Blue R250 dye and destained with a solution of 5% methanol-7.5% acetic

acid (v/v). Molecular mass markers (Bio-Rad) used were phosphorylase b (94 kDa), bovine serum albumin (BSA, 67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and cytochrome c (12.5 kDa). Proteins were measured by the method of Lowry *et al.* [33] with bovine serum albumin as the standard. Absorbance at 280 nm was used to monitor protein in column eluates.

RESULTS AND DISCUSSION

Kinetics of GnT-III and -V in Hepatoma Cells

Preliminary examination of the GnT-III and -V activities from human hepatoma cell lines (Hep3B and HepG2) and from human colon cancer cells (SW620 and HTP39 cells) showed high activities of GnT-III and -V in the membrane fraction, but not in culture supernatant. This result was in contrast with that of GnT-V from human small lung cancer cell QG [38]: GnT-V from the QG cells was found in culture supernatants as a soluble form and was not needed to solubilize it from bound particles. Since the GnT-III and -V in the present study were found in the membrane fraction of the cells, we used cell extracts as the sources of the enzymes and confirmed the presence of GnT-III and -V which remained associated with cell debris by quantitative measurement of GnT-III and -V activities. Therefore, to prevent uncontrolled modifications during the preparation of cellular fractions, cells were subjected to a fast shift down of temperature prior to washing. Cells were treated with sonicator and separated into membrane fraction as insoluble form and the fraction containing cytosolic fraction as soluble form, and the enzyme activities were quantitatively analyzed. Approximately 85% of cellular enzyme were located in the insoluble fraction, while about 15% were detected in the soluble fraction (Table 1).

Table 1. Distribution of GnT-III and -V from human hepatoma and colon carcinoma cells of SW620 and HTB39.

Cell lines	Transferase	Activity (pmol/h/mg)		
		Supernatant fraction	Membrane fraction	Cytoplasmic fraction
Hep3B	GnT-III	N.D.[a]	1,423±65	254.5±23.3
	GnT-V	N.D.	1,821±125	342.7±53.6
HepG2	GnT-III	N.D.	1,643±48	276.3±26.3
	GnT-V	N.D.	1,333±78	187.2±32.4
SW620	GnT-III	N.D.	165±3.1	4.2±0.2
	GnT-V	N.D.	2,232±231	345.2±75.1
HTB39	GnT-III	N.D.	222±10	5.4±0.6
	GnT-V	N.D.	2,011±134	302.2±32.1

[a]N.D., Not determined.

Typically, the reaction products of GnT-III and -V were detected at 15.4 min and 18 min after injection, respectively, whereas the substrate GlcN,GlcN-biant-PA acceptor was eluted at 7.2 min after injection. The amount of the product was determined by a fluorescence intensity, using pyridylaminated *N*-GlcNAc as a standard. The detectable limit of the product was found to be below 0.1 pmol/assay. The kinetic properties, such as optimum pH, effect of metal ion concentration, and K_m values for UDP-GlcNAc and the acceptor substrate, are summarized in Table 2.

Colon Cancer Cells Express Higher GnT Activity than Hepatoma Cells

Activities of GnT-III and -V were assayed. As listed in Table 3, GnTs were not well detected in primary human

Table 2. Comparison of GnT-III and -V from hepatoma and colon carcinoma cells.

A) Hepatomas

Kinetics	Hep3B		HepG2	
	GnT-III	GnT-V	GnT-III	GnT-V
Optimum temperature (°C)	43	50	40	50
Optimum pH	6.5	6.5	6.0	6.5
Optimum Mn^{2+}concentration (mM)	15	2	12	1.0
K_m for UDP-GlcNAc (mM)	4.7	2.1	6.8	4.5
K_m for GlcN,GlcN-biant-PA (mM)	1.1	0.43	3.4	2.4

B) Colon carcinomas

Kinetics	SW620		HTB39	
	GnT-III	GnT-V	GnT-III	GnT-V
Optimum temperature (°C)	45	50	40	50
Optimum pH	6.0	6.0	6.0	6.0
Optimum Mn^{2+}concentration (mM)	10	2	10	2.0
K_m for UDP-GlcNAc (mM)	11.1	2.2	10.2	3.6
K_m for GlcN,GlcN-biant-PA (mM)	4.5	0.40	5.7	1.8

Table 3. Comparison of GnT-III and -V in various normal and cancer cells.

Origin	Activity (pmol/h/mg)	
	GnT-III	GnT-V
SW620	165±3	2,232±231
HTB39	222±10	2,011±134
HepG2	867±43	1,165±123
Hep3B	1,423±65	1,821±125
Normal liver	7.6±0.4	7.3±0.5
HCC liver	745±61	677±98
Fetal human liver primary cells	576±78	778±87
Adult human liver primary cells	8.1±0.5	8.9±1.4
Chang liver cells	8.1±0.5	8.9±1.2

adult hepatocytes and normal liver tissues. However, the GnTs were detected in fetal hepatocytes, hepatoma cell lines, and colon cancer cells, and continued to produce during incubation time, thus indicating that the GnTs are not expressed in human adult hepatocytes. The GnT-III activity was markedly elevated in human hepatoma tissues, especially in Hep3B and HepG2, as compared to normal adult liver. On the other hand, GnT-V activity was also expressed in hepatoma cells and hepatocarcinoma tissues. Interestingly, GnT-V activities in those cells and tissues were much higher than those of GnT-III in metastatic colon cancer cells.

Changes in the GnT-III and -V Activities Associated with the Differentiation of Hepatoma Cells (HepG2 and Hep3B) and Colon Carcinoma Cells (SW620 and HTB39)

Hepatoma cells appeared to contain relatively high GnT-III and -V activities, while colon carcinoma cells contained a relatively high GnT-V activity and relatively low activity of GnT-III (Table 4). The cells were also grown in the presence of retinoic acid. Upon induction of differentiation

Table 4. GnT-III and -V activities in hepatoma and colon carcinoma cells by retinoic acid.

Cells	Retinoic acid	GnT activity (pmol/h/mg)	
		III	V
Hep3B	-	1,615±71	2,335±76
	+	634±23	623±84
HepG2	-	1,892±122	1,497±87
	+	765±54	575±44
SW620	-	168±12	2,543±65
	+	157±21	765±43
HTB39	-	232±22	2,278±67
	+	243±25	924±22

Cells were grown for 4 days in the presence of 10^{-7} M retinoic acid [9]. Cell lysates were assayed for the GnT-III and -V activities, using oligosaccharide b as an acceptor. Enzyme activities were assayed, as described in Methods and analyzed by HPLC, as described in the legend to Fig. 1.

of HepG2 and Hep3B cells with retinoic acid, dramatic decreases of GnT-III and -V activities were encountered. However, treatment of colon carcinoma cells with retinoic acid resulted in a reduction of GnT-V activity, whereas the activity of GnT-III remained essentially unchanged (Table 4).

Earlier, bisected structures have been reported to occur in hepatomas [28]. In accordance with this structural evidence, expression of GnT-III was seen in a pre-cancerous stage of liver carcinogenesis [43, 34]. This is yet an another example to describe association of malignant transformation with this key glycosyltransferase. In the present study, induction of colon carcinoma cells by retinoic acid did not result in gross change in GnT-III, suggesting that the oligosaccharide chains of colon carcinoma cells were not different before and after induction with retinoic acid. This result seems to conflict with the decrease of GnT-V activity upon induction of colon cancer cells. However, it is known that retinoic acid acts on more mature cells, and morphological changes accompanying the differentiation process occur much faster than other differentiation factors such as DMSO [16]. Therefore, the pronounced changes of GnTs level, and consequently of cell surface oligosaccharides structures, may be expected when induced with retinoic acid.

At present, it is unclear why primary human adult and fetal hepatocytes differently express GnTs. It has recently been reported that glycosylases have some crucial role during cell differentiation of immature to mature cells [35]. This differentiation potential of fetal cells is correlated with increase of glycosylation of cell surface proteins. Therefore, it has been suggested that adult and fetal hepatocytes may have different degrees of glycosylation in glycoproteins. This hypothesis was partly supported by the study that GnT-III, a key enzyme in the biosynthesis of the core region of *N*-glycans, was highly expressed in human fetal tissue [37]. Recently, it was reported that changes in GnT-III expression correlated with hepatoma and leukemia in rodents and humans [45]. Also, our recent study [31] clearly showed that overexpression of a cDNA encoding GnT-III alters growth control and cell-cell interactions in cultured cells and in transgenic mice. Although mice lacking GlcNAc-TIII are viable and fertile, they exhibit retarded progression of diethylnitrosamine (DEN)-induced liver tumors [45], and this hypothesis was also confirmed in the hepatoma cell line, Hep3B, transfected by GnT-III in our recent work [18]: The transfected Hep3B did not produce viral antigens in the medium, being disturbed viral secretion. Hep3B carries the hepatitis B virus (HBV) genome and is closely associated with the incidence of human liver cancer [4, 42]. Therefore, proteins originated from the HBV genome may affect the expression of GnTs. HBx is a multifunctional regulatory protein and has been reported to be associated with transcriptional activation via its interaction with nuclear transcription factors and the cytoplasmic modulation of signal transduction pathways, as reported in our earlier

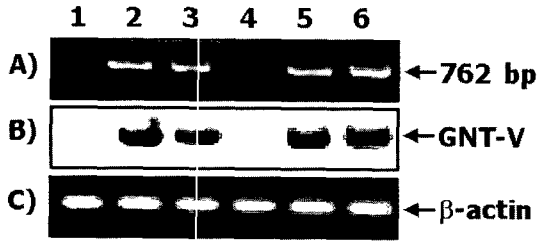

Fig. 2. Expression of the GnT-V gene in hepatoma and colon carcinomas.
Two-three micrograms of total RNA from each cell were subjected to RT-PCR. Lanes 1, human fetal liver primary cultures (16 weeks old); 2, human fetal liver primary cultures (26 weeks old); 3, Hep3B; 4, normal adult liver cells; 5, SW620; 6, HTB39. Arrow indicates PCR product of the GnT-V gene (762 bp). A) RT-PCR. B) Northern blot analysis. C) β-Actin as a control.

report [5]. Therefore, although it remains unclear, HBx is thought to be associated with the expression of GnT-III and -V genes. Therefore, we are in a process to focus our future work on the study of the correlation between HBV infection and GnT-III.

Increased Expression of GnTs mRNA in Cancer Cells

To investigate the expression of GnTs in adult, fetal hepatocytes, hepatoma cells, and colon cancer cells, RT-PCR was performed. Total RNAs were isolated from hepatocytes and cancer cells harvested at different times of culture and then used for cDNA synthesis and PCR. GnT-III DNA was not detected in adult hepatocytes, while a 0.5-kb DNA fragment originated from GnT-III DNA was detected in fetal hepatocytes, hepatoma cells, and colon cancer cells (data not shown). On the other hand, as shown in Fig. 2, GnT-V DNA was detected in hepatoma cells and colon cancer cells. These data indicate that human fetal and adult hepatocytes have different expression of GnTs at the transcriptional level.

In the case of human colon cancer cells, it should be mentioned that increased β-(1,6)-branching mediated by GnT-V is associated with an increased metastatic potential of colon cancer cells [8], and decreased branching has been associated with the differentiation of cells [36]. Therefore, study of these enzymes might yield an enzymatic basis for the changes in branching patterns upon malignant transformation and differentiation of cells. The increased amounts of such materials in malignant cells are due to an increased activity of GnT-V, which catalyzes the transfer of GlcNAc from UDP-GlcNAc to an β-D-mannoside [2]. For example, it was reported that GnT-V activity increased during rat hepatocarcinogenesis in correlation with the amount of GnT-V mRNA [34]. The β-(1,6)-branching may contribute to the malignant or metastatic properties of tumor cells, since blocking the glycosylation pathway prior to the formation of the β-(1,6)-linked antenna leads to a loss of metastatic potential and inhibits organ colonization [2, 7, 8].

Alteration in Glycosylation of Hepatoma and Colon Cancer Cells as Elucidated by Lectin Blot Analysis

To determine the effects of activated GnT-III and -V on the glycoprotein structures in hepatoma cells and colon cancer cells and to evaluate qualitative differences in protein glycosylation, a lectin blot analysis with WGA and L-PHA was carried out on crude cell membrane protein and cytosolic protein. Prior to lectin blotting, membrane and cytosolic proteins were incubated either in the presence or absence of neuraminidase. Since WGA binds to both GlcNAc and N-acetylneuraminic acid (sialic acid), neuraminidase treatment was performed to ascertain the contribution of sialic acid to WGA binding. As shown in Figs. 3A and 3B, during treatment of membrane protein with neuraminidase, WGA binding persisted after standard treatment.

It is reported that E-PHA binds to the bisecting structures of seven sugar chains, which is a product of GnT-III [46], and that Con A binds with high affinity to a high mannose-type biantennary complex or hybrid type of asparagine-linked oligosaccharide, but does not bind to a bisecting structure [6]. As seen in Fig. 4A, E-PHA blotting showed that the intensity of the bands between 100–200 kDa was uniformly dominant in fetal hepatocytes, HepG2 and Hep3B cells, and approximately 55, 45, 30, and 15 kDa bands were also detected in Hep3B cells. As expected, the binding of E-PHA with hepatoma cells of Hep3B and HepG2 was much higher than adult hepatocytes and normal cells, and slightly higher than that of fetal

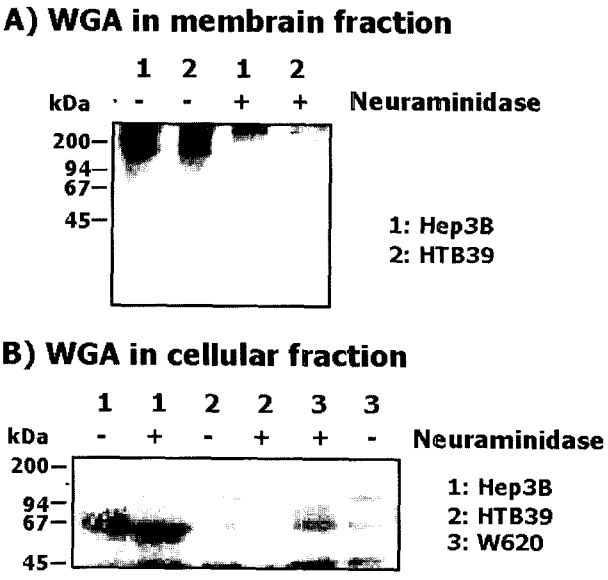

Fig. 3. WGA lectin blot analysis of fractions from normal liver, hepatoma, and colon carcinoma cells.
Ten micrograms of protein of each fraction were electrophoresed on a 10% SDS-PAGE. A) WGA blot analysis of membrane fractions of Hep3B and HTB39 with (+) or without (-) neuraminidase. Lane 1, Hep3B; 2, HTB39. B) WGA blot analysis of cellular fractions of Hep3B and HTB39 with (+) or without (-) neuraminidase. Lane 1, Hep3B; 2, HTB39; 3, W620.

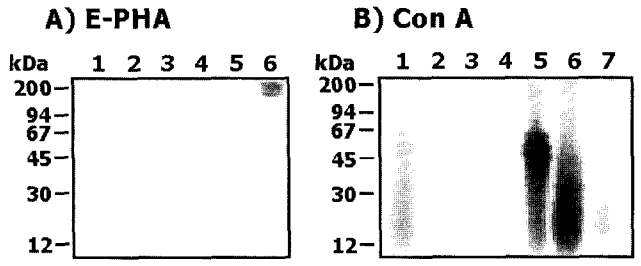

Fig. 4. E-PHA and Con-A lectin blot analysis of fractions from human fetal, normal liver, and hepatoma cells.
Ten micrograms of protein of each fraction were electrophoresed on a 10% SDS-PAGE. A) E-PHA blot analysis of total cellular fractions of Hep3B, HepG2, normal adult liver, and fetal liver hepatocytes. Lane 1, without lectin as a negative control; 2, normal hepatocyte; 3, fetal hepatocyte; 4, fetal hepatocyte with *N*-acetylglucosamineβ1,4-mannose as E-PHA inhibitor; 5, HepG2; 6, Hep3B. B) Con A blot analysis of total cellular fractions. Lanes 1, fetal hepatocyte; 2, fetal hepatocyte with 0.1 mol/l *N*-acetylgalactosamine as Con A inhibitor; 3, fetal hepatocyte with 0.5 mol/l *N*-acetylgalactosamine; 4, without Con A lectin; 5, Hep3B; 6, HepG2; 7, HepG2 with 0.5 mol/l *N*-acetylgalactosamine.

hepatocyte (Figs. 4A and 4B). Thus, the E-PHA binding appeared to be correlated with the GnT-III activity, implying that the GnT-III activity catalyzed the addition of the bisecting GlcNAc structure to the glycoproteins on whole cells. Lectin blotting of Con A showed that some bands with approximately 45–67 kDa were dominant in fetal hepatocytes and Hep3B cells; however, bands with less than 30 kDa molecular weights were observed in HepG2 cells (Fig. 4B).

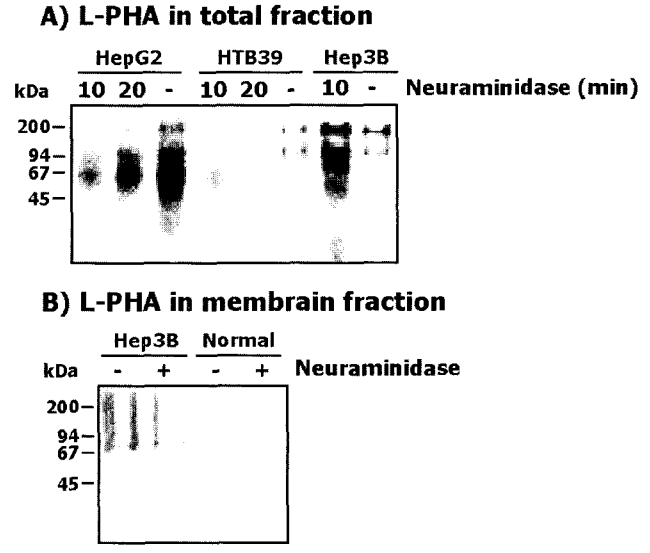

Fig. 5. L-PHA lectin blot analysis of total and membrane fractions from normal liver, hepatoma, and colon carcinoma cells.
Ten micrograms of protein of each fraction were electrophoresed on a 10% SDS-PAGE. A) L-PHA blot analysis of total cell extracts of HepG2, Hep3B, and HTB39 with (+) or without (-) neuraminidase for 10 to 20 min. B) L-PHA blot analysis of membrane fractions of Hep3B and normal hepatocytes with (+) or without (-) neuraminidase.

On the other hand, L-PHA is a lectin, which recognizes the β1-6 branch at the trimannosyl core of *N*-linked carbohydrate [16]. As expected, L-PHA binding was also increased in HTB39, HepG2, and Hep3B cells (Fig. 5A), and L-PHA reactivity was independent of sialic acid, therefore, unaffected by neuraminidase treatment. There was a slight decrease of L-PHA reactivity in all of the membrane preparations of Hep3B, following neuraminidase treatment (Fig. 5B). This probably reflects the unmasking of additional L-PHA reactive sites by cleavage of sialic acid. The enzymatic biosynthesis of these carbohydrate antigens is closely associated with the development and differentiation as reported previously [17]. Thus, the inhibition of these glycosyltransferases is a way to regulate and control the glyco-antigen biosynthesis, eventually ameliorating the glyco-mediated disease [20].

The author described herein a highly sensitive and simple assay method for glycosyltransferase GnT-III and -V activities, which involved the use of fluorescence-labeled pyrimidylaminated sugar chain and HPLC to separate and quantitate the enzyme products. The GnT-III and -V enzymes were shown to be markedly different from each other. Their pH optimum and K_m values for acceptor and donor substrates were different. GnT-III and -V activities were very high in various human hepatomas and also in the precancerous stage, as reported previously in rat [37]. The GlcNAc transferred by GnT-III is called "bisecting GlcNAc," and this residue has been found in the carbohydrate moieties of several glycoproteins, such as γ-glutamyltranspeptidase from ascites of hepatoma AH-66 cell [47]. The carbohydrate structures related to GlcNAc, derivatives as mediated by GnT-V, have been reported for α1-acid glycoprotein [36] and some transformed rodent cells [9, 40]. A relationship between their high expression and metastatic potentials was suggested by Dennis *et al.* [8]. In the present study, we found that GnT-III and GnT-V enzymes of hepatoma and colon cancer cells were elevated, as compared to those of normal liver cells. Thus, it is of interest to investigate whether GnT-III and -V activities are synergically associated with the metastatic potentiality of this and other sarcomas. To explain the biological functions of these genes, we have recently developed transgenic mice with the overexpressed genes [31].

Recently, we have cloned several human sialyltransferase genes such as the human NeuAc alpha2,3Gal beta1,3GalNAc alpha2,6-sialyltransferase (hST6GalNAc IV) gene [23] and human GM3 synthase (hST3Gal V) [22, 24], which transfers CMP-NeuAc with an alpha2,3-linkage to a galactose residue of lactosylceramide and plays a key role in the biosynthesis of all complex gangliosides. The gene expressions were also highly restricted in human fetal tissues with high transcriptional activity, and in both HepG2 cells and Hep3B cells, but not in SK-N-MC cells, suggesting cell type-specific regulations of the basal hST6GalNAc IV and hST3Gal V

promoter activities. It was reported that GlcNAc-transferase-V expression is important for normal cell proliferation and function [1]. The overexpression of *H-ras* or *v-sis* upregulates the activities of GlcNAc-transferase-V to various degrees in the transfected cells. The enhancement of GlcNAc-transferase-V mRNA expression was also observed in *H-ras*- and *v-sis*-overexpressing cells, indicating that *H-ras* and *v-sis* regulated GlcNAc-transferase-V via transcription of GlcNAc-transferase-V mRNA [11]. Thus, the results presented in this study led us to suggest that the glycosyltransferases are specifically related to hepatic cell differentiation through the biosynthesis of glycoproteins and glycolipids on the hepatic cell surface. The enzymatic biosynthesis of these carbohydrate antigens is closely associated with development and differentiation, as reported previously [16]. We are now in progress to examine whether both positive and negative regulatory elements are present in the promoter region of the GnT genes.

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