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The Hepatitis B Virus X protein inhibits secretion of Apolipoprotein B by enhancing the expression of N-acetylglucosaminyltransferase-Ⅲ

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Abbreviations: HBx, X protein of Hepatitis B Virus; HCC, hepatocellular carcinoma; Apo B, Apolipoprotein B; VLDL, very low density lipoprotein; LDL, low density lipoproteins; GnT-Ⅲ, UDP-*N*-acetylglucosamine:β-D-mannoside-1,4-*N*-acetylglucosaminyltransferase-Ⅲ; GlcNAc, *N*-acetylglucosamine; MTP, microsomal triglyceride transfer protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; ER, endoplasmic reticulum; RT-PCR, Reverse transcriptase-polymerase chain reaction; AGR-GnT-Ⅲ, aglycosyl recombinant *N*-acetylglucosaminyltransferase-Ⅲ; CAT, chloramphenicol acetyltransferase; t-RA, all trans-retinoic acid; CE, cholesteryl ester.

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ABSTRACT

The X protein of Hepatitis B virus (HBx) plays a major role on hepatocellular carcinoma (HCC). Apolipoprotein B (Apo B), in the liver, is an important glycoprotein for transportation of very low density lipoproteins (VLDL) and low density lipoproteins (LDL). Although lipids accumulation in the liver is known as one of factors for the HCC, relationship between HBx and Apo B during the HCC development is poorly understood. To better understand the biological significance of HBx in HCC, liver Chang cells that specifically express HBx were established and characterized. In this study, we demonstrate that overexpression of HBx significantly up-regulates the expression of UDP-*N*-acetylglucosamine:β-D-mannoside-1,4-*N*-acetylglucosaminyltransferase-Ⅲ (GnT-Ⅲ), an enzyme that functions as a bisecting-*N*-acetylglucosamine (GlcNAc) transferase in Apo B, and increases GnT-Ⅲ promoter activity in CAT assay. GnT-Ⅲ expression level of HBx-transfected cells appeared to be higher than that of hepatocarcinoma cells as well as GnT-Ⅲ-transfected cells, indicating that HBx may has a strong GnT-Ⅲ promotor-enhancing activity. Intracellular levels of Apo Bs, which contained the increased bisecting-GlcNAc, were accumulated in HBx-transfected liver cells. These cells as well as GnT-Ⅲ-transfected liver cells revealed inhibition of Apo B secretion and increased accumulation of intracellular triglyceride and cholesterol, compared with vector-transfected cells. Moreover, overexpressions of GnT-Ⅲ and HBx in liver cells were shown to down-

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regulate transcriptional level of microsomal triglyceride transfer protein (MTP), which regulates the assembly and secretion of Apo B. Therefore, our study strongly suggested that HBx increase intracellular accumulation of aberrantly glycosylated Apo B resulted in secretion of Apo B as well as intracellular lipid accumulation by elevating the expression of GnT-Ⅲ.

INTORDUCTION

In Western countries, 75 to 90 % of hepatocellular carcinomas (HCC) are associated with chronic liver diseases (1). Hepatitis B Virus (HBV) is a major causative agent of acute and chronic hepatitis in human (2) and is closely associated with the incidence of human liver cancer. Among the four proteins that originate from the HBV genome, HBx protein is a 17 kDa multifunctional regulatory protein and has been detected with high frequency in liver cells from patients with chronic hepatitis, cirrhosis and liver cancer (3). In our previous study, HBx has an inhibitory effect on the p53-mediated transcription of the 3'-inositol phosphatase (PTEN), which is associated with tumor suppression (4). Therefore, HBx is thought to be associated with the development of HCC. However, the precise function of HBx in the tumorigenic transformation of liver cells remains unclear.

The liver is the major organism for both the production of plasma lipoproteins and their uptake from plasma and catabolism (5). The production of Apolipoprotein B (Apo B, a 500 kDa

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protein) containing lipoproteins by the liver is required for the assembly and secretion of very low density lipoproteins (VLDL) and low density lipoproteins (LDL) (6-10). The assembly of Apo B with lipid to form a secretion-competent particle is a complex process (11, 12). It is widely accepted that hepatic lipid availability is obligatory for Apo B-containing lipoprotein assembly within the liver. This finding has been supported by studies demonstrating the necessity of triglyceride (12, 13) and phospholipid (14). The microsomal triglyceride transfer protein (MTP) also plays a key role in Apo B secretion by catalyzing the transfer of lipids to the nascent Apo B molecule as it is co-translationally translocated across the endoplasmic reticulum (ER) membrane (15, 16).

GnT-Ⅲ catalyses the attachment of a GlcNAc residue to mannose in the β (1-4) configuration in the region of *N*-glycans and forms a bisecting GlcNAc (17), as shown in Scheme 1. Recent investigations revealed that the bisecting-GlcNAc residue, a product of GnT-Ⅲ activity, correlated with a number of biological events, including the suppression of metastasis of mouse melanoma cells (18) and has been reported to be significantly elevated in the serum of human subjects with hepatomas, liver cirrhosis, as well as in HCC (19-25). Therefore, GnT-Ⅲ also could be a factor for the development of HCC.

Apo B is a glycoprotein and contains high mannose *N*-glycans or biantennary type oligosaccharides in the case of human LDL (26, 27). Some investigators studied that the

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glycosylation of proteins and lipids are associated with development, differentiation, and carcinogensesis (28-30). Recently, aberrant glycosylation, as the direct result of the formation of bisecting-GlcNAc by GnT-Ⅲ, has been shown to disrupt the function of Apo B, leading to the generation of fatty liver (31, 32). On the basis of the findings reported herein, we provide evidence of the molecular mechanism underlying inhibition of Apo B secretion and intracellular accumulation of triglyceride and cholesterol *in vitro* model by HBx/GnT-Ⅲ expression.

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EXPERIMENTAL PROCEDURES

Cell culture and transfection—Chang cells (ATCC No.: CCL-13), a human liver cell line, were maintained using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) at 37 ℃ in a humidified 5 % CO_2 incubator. The cells were used for stable transfection with HBx and GnT-Ⅲ cDNA using LipofectAMINE reagent following the manufacturer's instructions. The 465 bp and 1.6 kb cDNAs encoding the open reading frames for HBx and GnT-Ⅲ were inserted into pcDNA3 expression vector at the *Hin*dⅢ/*Kpn*I and *Bam*HI/*Eco*RI sites, respectively. Transfected cells were then selected by cell culture medium containing 600 μg/ml of G418 sulfate.

Reverse transcriptase-polymerase chain reaction (RT-PCR)—Total RNA from parental Chang

cells and various transfected cells was prepared using TRIZOL (Gibco-BRL, MD, USA), and the cDNAs were synthesized by reverse transcriptase with an oligo dT-adaptor primer from RNA LA PCR Kit (Takara, Japan). In order to specifically detect the expression of endogenous human GnT-Ⅲ and MTP, PCR was performed with the selective primers for human GnT-Ⅲ and MTP in a PCR Cycler. The primers used in this study were designed to detect mRNA for human GnT-Ⅲ: 5'-ACTTCTTCAAGACCCTGTCCTATGT-3' (sense), and 5'-GAGCCGTTGGCCCC CTCAGGCTTCT-3' (antisense). MTP: 5'-TGCTGTCAGCATCTGG CGACCCT-3' (sense) and 5'-TCAAAACCATCCGCTGGAAGTACTAT-3' (antisense). β-Actin: 5'-CAAGAGATGGCCACGGCTGCT-3' (sense) and 5'-TCCTTCTGCATCCTGTCGGCA-3' (antisense). The sizes of products that were yielded by the PCR using these primers were expected to be 345, 500, and 247 bp for GnT-Ⅲ, MTP, and β-actin, respectively.

Northern blot analysis—To detect mRNA levels of HBx, GnT-Ⅲ, and MTP, total RNA was prepared using the TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. For Northern blot analysis, 10 μg of total RNA was separated by electrophoresis in a 1 % formaldehyde agarose gel. After electrophoresis, the gel was blotted on Hybond-C membrane (Amersham, UK). RNA was fixed to membrane by crosslinking for 3 minutes using

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Ultra-Violet (UV). Hybridization was performed in Expresshyb (Clonetech, USA) to a random prime (Clontech, USA) labeled probe that encompassed the partial HBx, GnT-Ⅲ, and MTP gene.

Production GnT-Ⅲ antibody—Aglycosyl recombinant *N*-acetylglucosaminyltransferase-Ⅲ (AGR-GnT-Ⅲ) protein, deficient in the first 23 amino acids was expressed in *E. coli* and purified by DEAE-Sephacel chromatography (Pharmacia, NJ, USA), followed by Sephacryl S-200 gel (Pharmacia, NJ, USA) filtration and, finally, preparative gel electrophoresis (33). The procedure employed for the production of monoclonal antibodies was based on the protocol described by Kohler and Milestein (34). Balb/c mice were immunized by an intraperitoneal injection of AGR-GnT-Ⅲ and mixed with Freund's complete adjuvant. Spleen cells from the mice were fused with the murine myeloma cell line SP2/0-Ag14. The mAb-producing hybridomas were cloned by the limiting dilution technique, and propagated by injecting them into mice. Subsequently, ascitic fluids were harvested and processed by Protein G-Sepharose 4B chromatography to obtain purified mAb as described previously (35).

Western blot and Lectin blot analysis—Liver Chang cells were lysed in RIPA buffer containing 150 mM NaCl, 20 mM Tris (pH 7.5), 1% Triton X-100, 2 mM EDTA, 10% (v/v)

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glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM Na_3VO_4, 20 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 100 kallikrein inhibitor units of aprotinin per ml. Cell lysates were clarified by centrifugation at 14,000 × *g* for 10 min at 4 °C. Protein (25 μg) was separated on SDS-polyacrylamide gel electrophoresis (PAGE) gels, and then transferred to nitrocellulose (NC) membrane. After blocking non-specific binding sites, the membranes were incubated with specific antibodies, anti-GnT-Ⅲ, anti-GAPDH (Chemicon, USA), anti-HBx (Koma biotech, Korea) and anti-Apo B (Calbiochem, Germany). After washing the membranes with PBS three times, they were further incubated with horseradish peroxidase (HRP)-conjugated antibody. For detection of bisecting-GlcNAc residues, the membrane was incubated with biotinylated E-PHA (erythroagglutinating phytohemagglutinin, 2 μg/ml, Boheringer Mannheim Korea, Seoul, Korea). Lectins were detected by using HRP-conjugated lectins (Seikagakukougyo, Kyoto, Japan).

Immunoblots were revealed by autoradiography using the enhanced chemiluminescence detection kit (Amersham Biosciences, UK).

Immunoprecipitation—The cell lysate (0.5 mg/ml) was pre-cleared with 50 μl of protein A-Sepharose beads at 4 °C for 1 h, and clarified by centrifugation at 14,000 rpm for 10 min. The pre-cleared lysate was incubated with an anti-Apo B antibody for 1 hr, then 50 μl of protein A-

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Sepharose beads were added and the mixture was incubated for 1 h. After extensive washing with RIPA buffer, the immunoprecipitated Apo B were eluted from beads with 50 μl of SDS sample buffer and subjected to 6 % SDS-PAGE under reducing condition. Western blot and lectin blot were performed with anti-Apo B and E-PHA, respectively, as described above.

Construction of plasmids and trasnsfections of Chang cells with GnT-Ⅲ-promoter-CAT-gene fusion vector—pSV0-CAT which expresses chloramphenicol acetyltransferase (CAT) was from the laboratory of Molecular Glycobiology, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejon, Korea. pGNT-CAT plasmid was constructed by ligating DNA fragment ranging from -1 to -1058 bp of GnT-Ⅲ upstream region of promoter in pSV0-CAT. Cells were co-transfected with 10 μg of pGNT-CAT plasmid and different concentration of pCDNA-HBx gene by LipofectAMINE based transfection method. Transfected cells were cultured at 37 °C in 3 % CO_2 for 24 h, followed by 5 % CO_2 for 24 h, and used for the CAT assay.

Chloramphenicol Acetyltransferase (CAT) Assay—The procedure previously developed by Bullock and Gorman (36) was employed. Transfected cells from one plate were harvested and resuspended in 100 μl of 0.25 M Tris-HCl (pH 7.8), underwent 3 cycles of freezing in dry ice-

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ethanol bath and thawing in 37 °C incubator. The cell debris was removed by centrifugation, and the supernatant was saved in a clean tube. Total cell lysates were assayed for CAT activity were mixed with the following reagents; 4 μl [^{14}C]chloramphenicol (54 mCi/mmole), 70 μl of 1.0 M Tris-HCl (pH 7.8), 20 μl of 4 mM acetyl CoA, and distilled water to a final volume of 150 μl. Following incubation at 37 °C for 1 h, chloramphenicol was redissolved in 30 μl of ethyl acetate and applied to a TLC plate (Merck, USA). The plate was incubated for 15 min in chloroform:methanol (95:5) and was air dried. Following autoradiography, the radioactive spots on TLC plate were shown.

Measurement of triglyceride and cholesterol in liver cells—To determine triglyceride and cholesterol content in vector, GnT-III, and HBx transfected cells, Chang cells were starved for 24 h and then incubated with DMEM supplemented with 1 μM of all trans-retinoic acid (t-RA) for 48 h. Triglyceride and cholesterol mass content from the cells was measured enzymatically (Sigma, USA).

Statistics—Results are expressed as the mean ± S.D. and averages of three to five experiments. Means were compared by *t*-tests to determine statistical significance. A *P* value of <0.05 was considered significant.

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RESULTS

HBx enhances the expression of GnT-Ⅲ by the promoter activity of GnT-Ⅲ gene—Chang cells were transfected with the HBx cDNA using pcDNA expression vector. The expression of both HBx mRNA and protein was verified prior to the investigation of HBx-induced effects. The expression of HBx mRNA was confirmed by Northern blot analysis and the RNA controls of corresponding blots are shown in Fig. 1A. Furthermore, the expression of HBx protein was confirmed by Western blot analysis using monoclonal anti-HBx antibodies (Fig. 1B).

While we were searching for some factors that induced GnT-Ⅲ, we found that HBx protein enhanced the mRNA and protein expression of GnT-Ⅲ in HBx-transfected cell, Chang-HBx. To elucidate whether the endogenous GnT-Ⅲ gene is activated in the Chang-HBx, the GnT-Ⅲ mRNA and protein levels were measured by Northern blot (Fig. 2A), RT-PCR (Fig. 2B), and Western blot analysis (Fig. 2C). As shown by these data, the expression of GnT-Ⅲ gene and protein was significantly elevated in Chang-HBx cells compared with Chang and Chang-pcDAN3 cells.

We here postulate whether HBx may enhance the promoter activity of the GnT-Ⅲ gene in the endogenous system. In a previous study, we have isolated and characterized the 5' flanking region of GnT-Ⅲ gene from human placental genome library (37). Schematic diagram of GnT-Ⅲ promoter from –1058 to -1 is shown in Fig. 3A. Putative promoter elements are based on

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sequence comparison to known motifs: GRE, half-palindromic glucocorticoid-response element (TGTCCT), recognition sites for CREB (CGTGACGA), AP-2 (GGCCTGGGGA) and SP1 (GGGCGG). The EMBL data library accession number is L48489. To examine the effect of HBx on the promoter activity of human GnT-Ⅲ gene, the plasmid pGNT-CAT containing GnT-Ⅲ promoter in front of the CAT reporter gene was co-transfected in human normal liver Chang cells with an increasing amount of HBx-expression vector (pcDNA3-HBx) (See Experimental Procedures). Indeed, as shown in Fig. 3B, the CAT activity of the GnT-Ⅲ promoter was gradually elevated up to 5-fold by increasing the HBx-expression vector, although detailed characterization of the promoter activity is limited due to its comprehensive properties of the GnT-Ⅲ promoter region (38, 39). Therefore, these results showed that the HBx protein transactivated GnT-Ⅲ gene transcriptionally and translationally.

Comparison of the levels of HBx and GnT-Ⅲ mRNA and protein in normal cells, hepatocarcinoma cell, and our transfected cells—To compare the expression levels of HBx and GnT-Ⅲ among the several cell lines, GnT-Ⅲ cDNA was transfected into liver Chang cells and RT-PCR and Western blot analysis were carried out using normal liver Chang cell, hepatocarcinoma cell line (HepG2), and our transfected cells as described in Experimental Procedures. As shown in Fig. 5, we could not detect the HBx level in HepG2 cells by RT-PCR

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(Fig. 5A) and Western blot (Fig 5B) because HepG2 is well known as hepatocarcinoma cell line, which is not integrated with HBV genome. The level of GnT-Ⅲ expression in HepG2 cells was similar to that of GnT-Ⅲ-transfected cells, but appeared to be lower than that in HBx-transfected cells. Moreover, in densitometry analysis (Fig. 4C) based on protein levels, GnT-Ⅲ level in HBx-transfected cells was increased up to two fold than that in GnT-Ⅲ-transfected cells.

Accumulation of Apo B with increased bisecting-GlcNAc in HBx-transfected cells—To investigate the increase of accumulation of intracellular Apo B in HBx-transfected liver cells, immunoprecipitation analysis were performed using cell lysates with anti-Apo B antibody as described in Experimental Procedures. As shown in Fig. 5A, compared with pcDNA transfected cells as control, HBx-transfected cells contained higher levels of intact Apo B bands, 150 kDa and 50 kDa Apo B species.

Next, to determine whether these Apo B species are aberrantly glycoslylated, lectin blot analysis were performed using E-PHA lectins, which is known to react preferentially with bisecting-GlcNAc (40). In Fig. 5B, immunoprecipitated Apo B species showed increased bisecting-GlcNAc, which is consistent result with Western blot analysis in HBx-transfected cells. However, although an approximately 80-kDa protein reacted with E-PHA, this band appeared to be non-specific because it could not detect in Western blot analysis (Fig. 5A). Furthermore, the

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reactivity of immunoprecipitated Apo B to E-PHA was blocked in the presence of an authentic inhibitor, GalNAc (41). Therefore, these results indicated that HBx-transfected cells significantly increased the intracellular accumulation of Apo B species, which contained increased bisecting-GlcNAc.

Enhanced expression of GnT-Ⅲ by HBx decreases the secretion of Apo B and increases accumulation of cellular triglyceride and cholesterol contents—Based on the finding that the expression level of GnT-Ⅲ in HBx-transfected cell was higher than that in GnT-Ⅲ-transfected cell as shown in Fig. 4, we hypothesized whether the aberrant glycosylation of Apo B by HBx-induced GnT-Ⅲ expression may be involved in Apo B secretion, the Apo B protein level was measured by Western blot from culture media in the control vector, GnT-Ⅲ, and HBx-transfected cells because Apo B plays an important role for delivery of triglyceride from liver to peripheral tissue. Cells were treated with t-RA for 2 days in the serum free media because secretion of Apo B induced by t-RA was increased in dose and time dependent manner (Data not shown). The same result was observed in treatment with oleic acid and there was no apoptotic fragmentation in the cell (Data not shown). The secretion of Apo B was significantly decreased by 40 % and 95 % in GnT-Ⅲ and HBx-transfected cell, respectively, compared with in vector control (Fig 6B), indicating that GnT-Ⅲ expression level is important factor for

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inhibition of Apo B secretion. This finding suggested that aberrant glycosylation of Apo B mediated by enhanced expression of HBx-induced GnT-Ⅲ inhibit the secretion of Apo B and increase accumulation of intracellular Apo B.

As a result from Western blot experiment of inhibition of Apo B secretion in HBx- and GnT-Ⅲ-transfected cells as shown in Fig. 6B, we postulate that cellular triglyceride and cholesterol mass may be increased in the HBx- and GnT-Ⅲ-transfected cells. To test this, we measured the accumulation of triglyceride and cholesterol in the cytosolic fraction in Chang cells transfected with HBx and GnT-Ⅲ. As we expected, in Fig. 6C, cellular triglyceride level in GnT-Ⅲ- and HBx-transfected cell were higher than vector-transfected cell up to 25 % ($P<0.05$) and 85 % ($P<0.01$), respectively. Cellular cholesterol level in GnT-Ⅲ- and HBx-transfected cells were slightly increased by 21 % ($P<0.05$) and 35 % ($P<0.01$), respectively. These results clearly indicated that increased accumulation of intracellular Apo Bs caused accumulation of triglyceride and cholesterol by enhanced expression of HBx-induced GnT-Ⅲ.

Expression level of microsomal triglyceride transfer protein (MTP) mRNA—Because MTP has been shown to play a critical role for Apo B assembly and secretion, to determine whether MTP expression may be affected in the GnT-Ⅲ- and HBx-transfected liver cells, MTP mRNA levels were measured by RT-PCR and Northern blot analysis (Fig. 7A and B). The expression of MTP

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was significantly decreased in GnT-Ⅲ- and HBx-transfected cells compared with in pcDNA-transfected cells after treatment of t-RA for 48 h in serum free media. When the cells were treated with t-RA, however, there was no difference in Apo B gene expression by RT-PCR (data not shown). This result suggested that the relationship between HBx and GnT-Ⅲ may regulate MTP expression for Apo B assembly and secretion.

DISCUSSION

We here provide an evidence of the relationship between HBx and GnT-Ⅲ responsible for inhibition of Apo B secretion *in vitro* model.

Many investigators have used HepG2 cell for studying Apo B secretion. However, HepG2 cell line is already a HCC and increase of the expression of GnT-Ⅲ gene was reported in human hepatocarcinoma tissues, fetal liver tissues, and hepatoma cell lines (42). In this study, we have chosen liver Chang cell line that is originally derived from normal liver tissue, but is subsequently established via HeLa cell contamination.

Miyoshi *et al.* (43) observed that during hepatocarcinogenesis, GnT-Ⅲ messenger RNA levels were increased in LEC rats and Ishibashi *et al.* (44) also reported that GnT-Ⅲ activity in human serum, and liver and hepatoma tissues were increased in liver cirrhosis and hepatoma patients. We observe that HBx increases GnT-Ⅲ expression by transcription as well as

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translation levels. In the previous study, we found out the promoter region of GnT-Ⅲ that has seven AP-2 sites by sequence homology search (37). Elevation of GnT-Ⅲ gene expression by HBx may be modulated by AP-2 activation since HBx has been shown to activate promoters through several transcription factors such as AP-1, AP-2, NF-κB, CREB, and ATF-2 (45-48), although HBx does not bind directly to DNA (49). We also compare that GnT-Ⅲ expression level in HBx-transfected cells is higher than that in hepatocarcinoma HepG2 cells as well as GnT-Ⅲ-transfected cells, indicating that HBx induces GnT-Ⅲ expression with strong GnT-Ⅲ promotor activity. Therefore this finding supports several studies that GnT-Ⅲ expression may be involved in the development of HCC (23, 25). Unfortunately, this study did not determine precise promotor region involved in activation for GnT-Ⅲ expression by HBx because of the limitation of comprehensive properties of the GnT-Ⅲ promoter region (38, 39), but this is the first report that HBx transactivates GnT-Ⅲ expression.

The hepatic production of Apo B-containing lipoproteins is regulated largely at posttranscriptional levels, with nascent Apo B molecules being secreted or degraded intracellularly (11). Based on our findings that intracellular accumulation of aberrantly glycosylated Apo B species, which exhibit strong reactivities to E-PHA, is increased and low molecular weight immunoreactive Apo B species (especially 150 kDa and 50 kDa) is detected in HBx-transfected cells, the present study supports our previous results (31) and Ihara *et. al.*'s

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report (32) that overexpression of GnT-Ⅲ in transgenic hepatocytes induced aberrant glycosylation of Apo B and distrupted Apo B secretion. They also showed that 130 kDa and 50 kDa Apo B species were immunoprecipitated and detected with lectin blot analysis. In addition, Apo B mRNA levels in liver Chang cells are not affected by HBx transfection by RT-PCR (data not shown), consistent with the concept that, under most conditions of altered Apo B secretion from HepG2 cells, Apo B mRNA levels remain unchanged (50-52).

Some investigators have suggested that hepatic triglyceride accumulation has a greater influence on Apo B secretion (53) and cholesteryl ester (CE) is the major lipid species stimulating VLDL secretion (54). However, in our study, even though there is intracellular accumulation of triglyceride and cholesterol in GnT-Ⅲ- as well as HBx-transfected liver Chang cells, inhibition of Apo B secretion occurs in these cells. Therefore, intracellular accumulation of Apo B caused by the ability of HBx to induce GnT-Ⅲ expression is the major determinant for inhibition of Apo B secretion and intracellular lipid accumulation.

With regard to the association of Apo B with tumorgenesis, lipid accumulation in the liver resulted in the development of dysplasia and carcinoma of the liver in mice expressing aberrantly truncated Apo B (55). Our study also reveals that increase of accumulation of triglyceride and cholesterol in liver cells is detected in GnT-Ⅲ- as well as HBx transfected cells, which is consistent with previous studies by Lee *et. al.* (31) and Ihara *et. al.* (32) that lipid

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accumulation in the liver in GnT-Ⅲ transgenic mice leads to the generation of liver abnormality. Thus, intracellular accumulation of triglyceride and cholesterol in liver cell caused by HBx induced GnT-Ⅲ expression may give us a new insight for HBx mediated HCC development.

In addition, microsomal triglyceride transfer protein (MTP), an intraluminal protein in the endoplasmic reticulum (ER), plays an essential role in regulating the assembly and secretion of Apo B containing lipoproteins (56-58). It is interesting to note that HBx- and GnT-Ⅲ-transfected cells showed down-expression of MTP mRNA, but not in vector transfected cell. As cells were treated with t-RA in our experiment, MTP expression level by RT-PCR and secretion of Apo B by Western blot were increased in dose and time dependent manners (data not shown). However, these data questioned about whether down regulation of MTP transcriptional level by overexpression of GnT-Ⅲ and HBx may result from the accumulation of triglyceride and cholesterol in liver cells primarily or secondarily. The contribution of down regulation of MTP expression by GnT-Ⅲ and HBx remains to be clarified.

The availability of transgenic mice aberrantly expressing the human GnT-Ⅲ in the liver may help not only to elucidate the role of protein/lipid glycosylation in the development and pathological change of the liver but also to develop therapeutic agents for human diseases caused by glycosylation abnormality. We are currently investigating whether hepatocytes from these transgenic mice show an altered sensitivity to viral infection or abnormal receptor-ligand

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interactions as the result of aberrant glycosylation of cell surface protein and lipids.

In conclusion, HBx induced GnT-Ⅲ expression may disrupt lipid metabolism by abnormal glycoslylation of Apo B and may target MTP for assembly and secretion of Apo B in the liver cells.

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Figure legends

Scheme I. A bisecting GlcNAc chain in *N*-glycans in biosynthesized by GnT-Ⅲ. Man, mannose; UDP-GlcNAc, uridine 5'-diphosphate-*N*-acetylglucosamine; Asn, asparagine; GlcNAc, *N*-acetylglucosamine. GnT-Ⅲ catalyzes the attachment of β-1,4-GlcNAc to the beta-D-mannoside of the tri-mannose core structure of an *N*-glucan.

Fig 1. Expression level of HBx mRNA and proteins in HBx-trnasfected liver cell. A, Twenty μg of total RNA extracted from Chang, Chang-pcDNA, and Chang-HBx-transfected cells were electrophoresed on 1 % agarose gel containing 2.2 M formaldehyde and then analyzed by Northern blot hybridization using ^{32}P-labeled HBx cDNA. **B,** Proteins (25 μg) extracted from these cells were subjected to 15 % SDS-polyacrylamide gel electrophoresis. Expression of HBx (17 kDa) was analyzed by Western blot using a specific antibody to HBx.

Fig 2. Detection of mRNA and protein for GnT-Ⅲ in HBx-transfected liver cell. A, Twenty μg of total RNAs extracted from HBx-transfected Chang cells were electrophoresed on a 1.0 % agarose gel containing 2.2 M formaldehyde and then analyzed by Northern blot hybridization using ^{32}P-labeled GnT-Ⅲ cDNA (upper panel). β-Actin indicates that equal amounts of RNAs were loaded in each lane (lower panel). **B,** One μg of total RNA from each

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cell was subjected to RT-PCR (upper panel). β-Actin mRNA expression was also examined as a control (lower panel). **C,** Twenty-five μg of proteins extracted from Chang cells, Chang-pcDNA3, and Chang-HBx cells were subjected to 10 % SDS-polyacrylamide gel electrophoresis. Expression of GnT-Ⅲ (68 kDa) was analyzed by Western blot using a specific antibody to GnT-Ⅲ (upper panel). GAPDH indicate that equal amounts of proteins were loaded in each lane (lower panel).

Fig 3. HBx transactivates GnT-Ⅲ promoter. A, Schematic diagram of GnT-Ⅲ promoter. The nucleotide sequence from -1058 to -1 is shown. Putative promoter elements are based on sequence comparison to known motifs: GRE, half-palindromic glucocorticoid-response element (TGTCCT), recognition sites for CREB (CGTGACGA), AP-2 (GGCCTGGGGA) and SP1 (GGGCGG). **B,** Ten micrograms of pGNT-CAT were cotransfected into Chang cells with different amounts of HBx-expression vector (pcDNA3-HBx) as indicated. Transfected cells from one plate were harvested and resuspended in 100 μl of 0.25 M Tris-HCl (pH 7.8), underwent 3 cycles of freezing in dry ice-ethanol bath and thawing in 37 °C incubator. The cell debris was removed by centrifugation, and the supernatant was saved in a clean tube. Portions of cell extracts were assayed for CAT activity were mixed with the following reagents; 4 μl [^{14}C]chloramphenicol (54 mCi/mmole), 70 μl of 1M Tris-HCl (pH 7.8), 20 μl of 4 mM acetyl

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CoA, and distilled water to a final volume of 150 µl. Following incubation at 37 °C for 1 h, chloramphenicol was redissolved in 30 µl of ethyl acetate and applied to a TLC plate. The plate was incubated for 15 min in chloroform:methanol (95:5) and was air dried prior to autoradiography.

Fig. 4. **Comparison of the levels of HBx and GnT-Ⅲ mRNA and protein in normal cells, hepatocarcinoma cell, and our transfected cells. A,** One µg of total RNA from each cell was subjected to RT-PCR (upper panel) for HBx and GnT-Ⅲ (middle panel). β-Actin mRNA expression was also examined as a control (lower panel). **B,** Proteins (25 µg) extracted from normal Chang, HepG2, Chang-GnT-Ⅲ, and Chang-HBx cells were subjected to 10 % and 12 % SDS-polyacrylamide gel electrophoresis. Expressions of HBx (upper panel) and GnT-Ⅲ (middle panel) were analyzed by Western blot using a specific antibody to HBx and GnT-Ⅲ. GAPDH indicate that equal amounts of proteins were loaded in each lane (lower panel). **C,** Protein bands were quantitated by densitometry.

Fig. 5. Characterization of Immunoprecipitated Apo B from HBx-transfected cells. Immunoprecipitation analysis in liver Chang cells was performed as described in Experimental

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Procedures. Immunoprecipitated Apo B from pcDNA- and HBx-transfected cells were subjected to 6 % SDS-PAGE followed by Western blot (**A**) and Lectin blot (**B**) with anti-Apo B antibody and E-PHA, respectively. On the Lectin blot, the membrane was incubated with E-PHA in the presence of or in the absence of 100 mM GalNAc. These results were reproducible in 3 independent experiments for each cell line.

Fig 6. GnT-Ⅲ expression level, inhibition of Apo B secretion from culture media, and changes in cellular triglyceride and cholesterol contents in GnT-Ⅲ- and HBx-transfected liver cell. A, After being permanently transfected with HBx and GnT-Ⅲ cDNAs, Chang cells were incubated with 1 μM t-RA in serum-free media for 2 days. Proteins (25 μg) from media were subjected to 6 % SDS-PAGE. Secretion of Apo B from the media was analyzed by Western blot and bands were quantitated by densitometry. **B,** Changes in cellular triglyceride and cholesterol mass were measured in Chang cells using enzymatic reagents. Values are means for 3-5 experiments.

Fig 7. Expression level of MTP mRNA in GnT-Ⅲ and HBx-transfected liver cell. A, One μg of total RNA from each cell was subjected to RT-PCR (upper panel) as described in Experimental Procedures. β-Actin mRNA expression was also examined as a control (lower

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panel). **B,** Twenty μg of total RNAs extracted from vector, GnT-Ⅲ, and HBx-transfected Chang cells were electrophoresed on a 1.0 % agarose gel containing 2.2 M formaldehyde and then analyzed by Northern blot hybridization using ^{32}P-labeled PCR product of MTP gene (upper panel). β-Actin indicate that equal amounts of RNAs were loaded in each lane (lower panel).

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Scheme. I

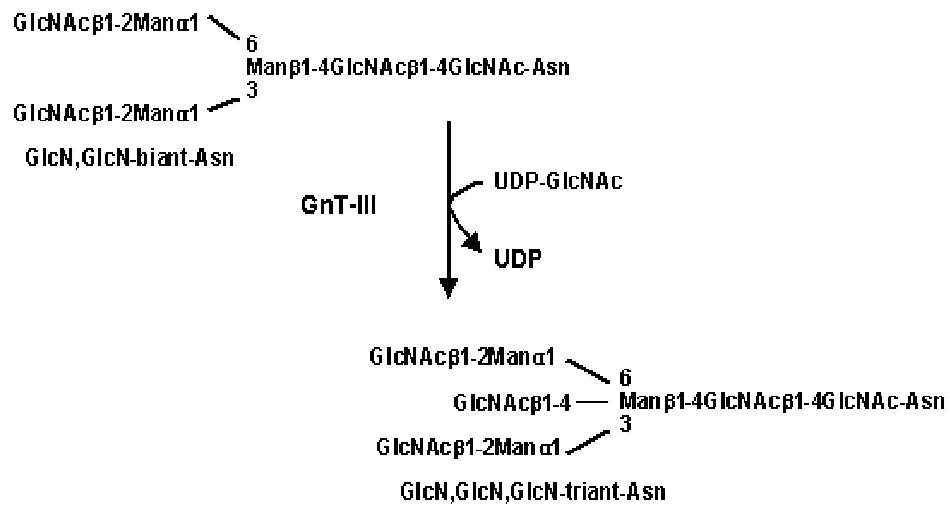

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Fig. 1

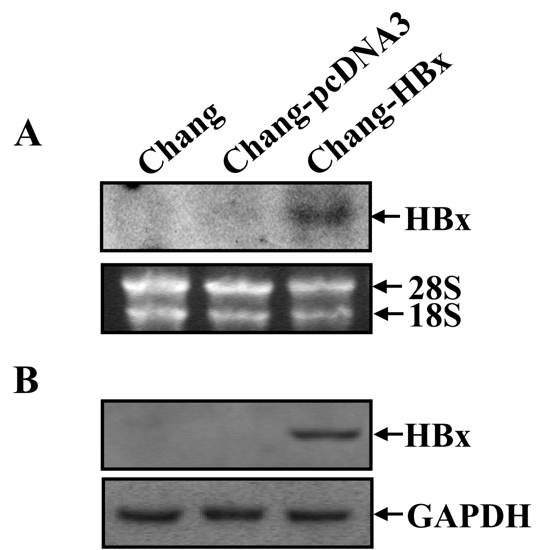

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Fig. 2

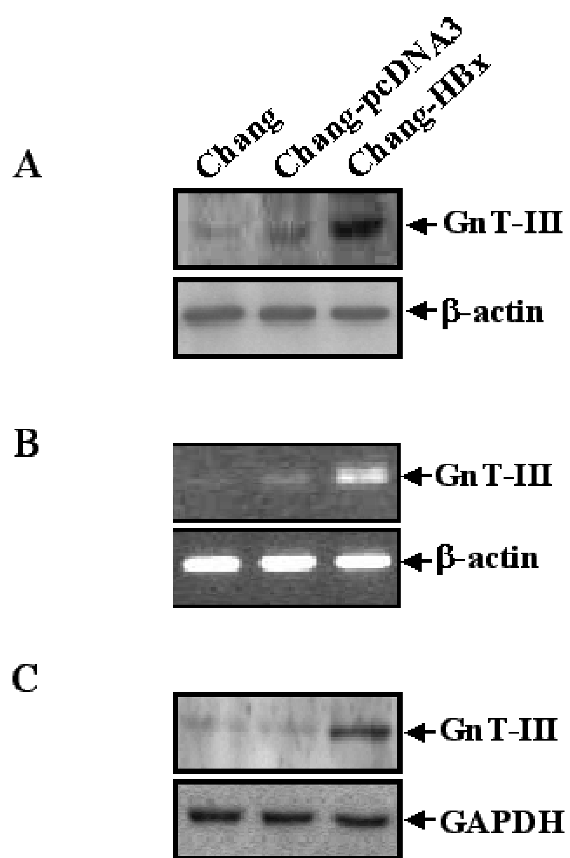

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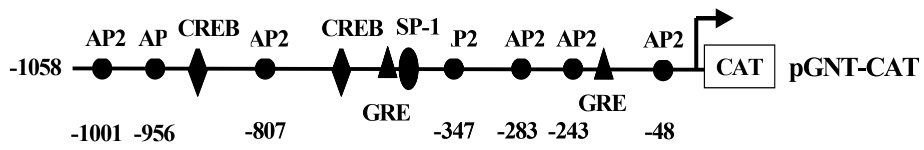
Fig. 3

A



B

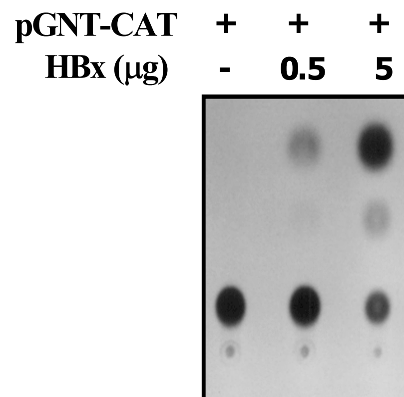


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Fig. 4

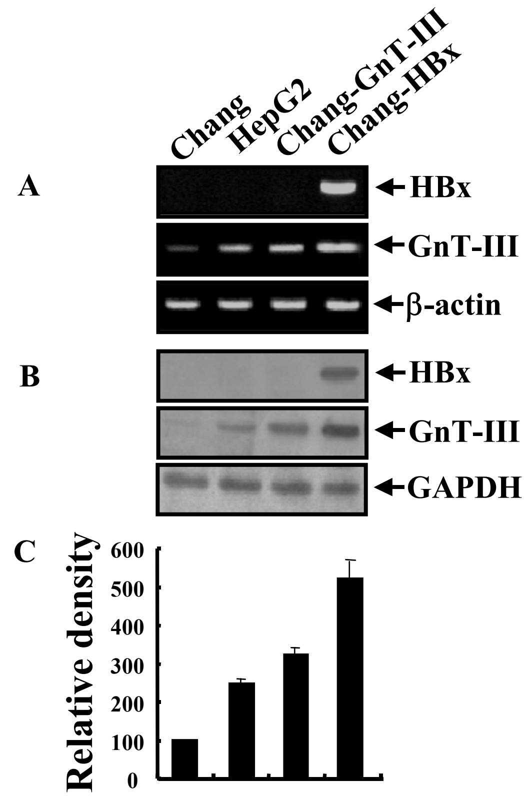

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Fig. 5

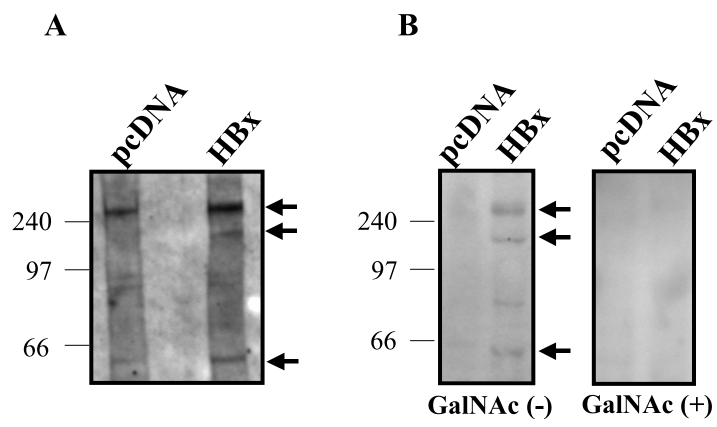

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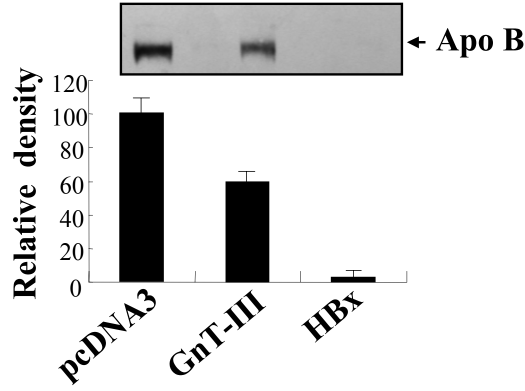
Fig. 6

A



B

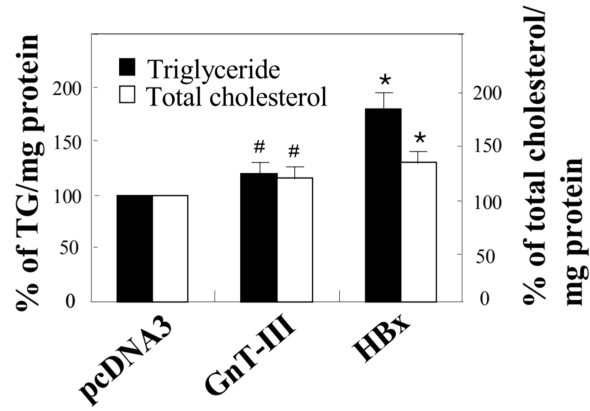


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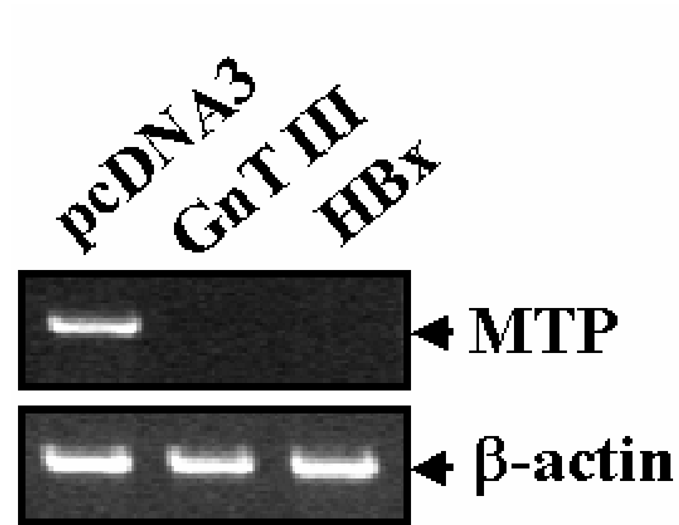
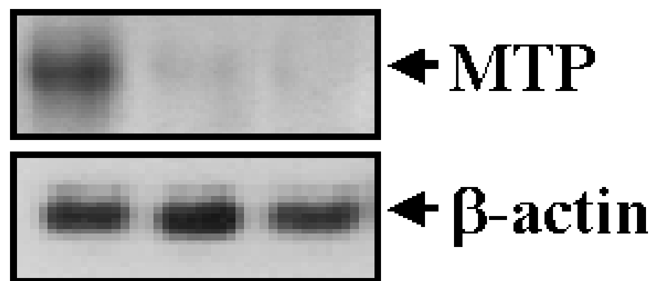
Fig.7

A



B



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