Transfection of N-Acetylglucosaminyltransferase III Gene Suppresses Expression of Hepatitis B Virus in a Human Hepatoma Cell Line, HB611*

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Oligosaccharides in glycoproteins have various biological functions including cell adhesion, sorting, birth, differentiation, and carcinogenesis (1). It is known that many viruses have specific oligosaccharides in their structural or secreted proteins. For example, gp120 produced by the AIDS virus has 24 asparagine-linked oligosaccharides that are involved in attachment to CD4 in lymphocytes (2, 3). Mouse Moloney leukemia virus has six asparagine-linked oligosaccharides (4), and Hepatitis B virus (HBV)* has two asparagine-linked oligosaccharides in the surface antigen (HBsAg) (5). Some of the oligosaccharides on those viral proteins have functions in viral replication, transport, and secretion (4, 6–8). These studies provide evidence for the importance of oligosaccharides in certain viruses. However, they are not representative of physiological conditions, and the inhibitory effects of tunicamycin or deoxynojirimycin are not specific for the processing of sugar chains in viruses.

β-O-mannoside β-1,4-N-acetylglucosaminyltransferase III (GnT-III) catalyzes the addition of N-acetylglucosamine in β1-4 linkage to the β-linked mannose of the trimannosyl core of N-linked oligosaccharides and forms a bisecting GlcNAc structure. Although the biological meaning of the bisecting GlcNAc structure remains unclear, it is known that the attachment of a bisecting GlcNAc inhibits further processing of oligosaccharides by other glycosyltransferases. To investigate whether or not structural changes of oligosaccharides affect secretion and gene expression of hepatitis B virus (HBV), we introduced the GnT-III gene into a human hepatoma cell line, HB611, which secreted HBV-related proteins into the medium. Positive transfectants were cloned by hygromycin resistant selection. Three clones have high activities of GnT-III and secreted lower levels of HBV-related proteins into the medium in comparison with other clones. These clones showed marked suppression of HBV-related mRNAs and an increased binding with E-PHA as judged by lectin blot. Expression of β actin, α fetoprotein, albumin, and prealbumin was not correlated with GnT-III activity in all the seven clones. Treatment of these cells with tunicamycin or swainsonine resulted in enhanced expression of HBV-related mRNA. These results indicate that some glycoproteins whose oligosaccharide structures are changed by overexpression of GnT-III suppress HBV gene expression.

β-O-mannoside β-1,4-N-acetylglucosaminyltransferase III (GnT-III) is one of the glycosyltransferases that catalyzes formation of asparagine-linked oligosaccharides in glycoproteins (9). Although the activity of GnT-III is very high in rat kidney, brain, and fetal liver, it is very low in normal adult rat liver (10). However, GnT-III is strongly expressed during hepatic carcinogenesis in a rodent model (11). In human liver diseases, GnT-III activity in serum or liver is also increased with progression of the disease (12). The product of GnT-III, a bisecting GlcNAc structure of N-linked oligosaccharides, inhibits further addition of another sugar chain by other glycosyltransferases (13). Recently, we were able to demonstrate suppression of lung metastasis of melanoma cells by GnT-III gene transfection (14), suggesting that a bisecting GlcNAc structure has a biological meaning in vivo. Based on these observations, there is a possibility that GnT-III induces structural changes in viral sugar chains, thereby altering their replication.

The cell line HB611 established by transfecting the HBV genome into a human hepatoblastoma cell line Huh6 (15) produces a large amount of HBsAg, hepatitis B e antigen (HBeAg), and HBV virion into the medium. Several studies on HBV replication or expression of HBV-related proteins have been reported using the HB611 cell (16–18). Recently, we have found that expression of GnT-III was specifically suppressed by HBV (19). Although the biological meaning is at present unknown, this phenomenon may be concerned with the fact that HBsAg does not have a bisecting structure in its sugar chains (5). According to our previous studies, we proposed a hypothesis that a high activity of GnT-III in host cells influences replication or sorting of HBV. To test this hypothesis, we transfected a GnT-III gene into HB611 cells and investigated the expression of HBV gene as compared with other genes of host cells. We found that overexpression of GnT-III suppressed gene expression of HBV in the HB611 cells.

MATERIALS AND METHODS

Cell Culture and Transfection of GnT-III Gene—HB611 was established by transfecting three copies of the complete HBV genome arranged in tandem into a human hepatoblastoma cell Huh6 (15). This cell was kindly provided by Prof. K. Matsubara (Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan). GnT-III expres-

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1 The abbreviations used are: HBV, hepatitis B virus; GnT-III, β-o-mannoside β-1,4-N-acetylglucosaminyltransferase III; GnT-IV, α-3-o-mannoside β-1,4-N-acetylglucosaminyltransferase IV; GnT-V, α-6-o-mannoside β-1,6-N-acetylglucosaminyltransferase V; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; AFP, α-fetoprotein; E-PHA, erythroagglutinating phytohemagglutinin; FACS, fluorescence-activated cell sorter.
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RESULTS

Glycosyltransferase Activities of Each Transfected and Production of HBV-related Proteins—We obtained seven doses as shown in Table I. Positive transfectants were described as HB611-GNT-III, and negative transfectants were described as HB611-hygro. Positive transfectants had high activities of Gnt-III, about 8–10 times the level in the parental cell. Transfection of HBV gene into a HuH6 cell suppressed Gnt-III activity (19), and transfection of Gnt-III gene into HB611 cells recovered the Gnt-III activity to the original level in the HuH6 cell. Positive transfectants showed high expression of Gnt-III mRNA, being consistent with Gnt-III activity (Fig. 1). Enzymatic activities of other glycosyltransferases, Gnt-IV and Gnt-V, were not changed by the transfection procedure. Levels of HBSAg and HBeAg in the medium were markedly lower in positive transfectants in comparison with negative transfectants or the parental cell (Table II).

Northern Blot Analysis—To determine the cause of low production levels of HBV-related proteins in positive transfectants, Northern blot analysis was performed. HBV-related mRNAs were detected as 3.5-, 2.4-, and 2.1-kilobase bands in HB611 (15). Expression of these bands was almost negligible in HB611-GNT-III(1) and HB611-GNT-III(3) and markedly decreased in HB611-GNT-III(2) (Fig. 2). Long exposure of the filter showed the faint bands in HB611-GNT-III(1) and HB611-GNT-III(3). Because expression of HBV-related mRNA was slightly changed at the stage of cell proliferation, Northern blot analysis was independently performed at least three times, in a sparse condition, a subconfluent condition, and a confluent condition. The pattern of HBV-related mRNA expression was almost the same as shown in Fig. 2 under any conditions used (data not shown). Because transfection of certain genes into a cell sometimes changes the phenotype of the cell, mRNA expression of AFP, albumin, and prealbumin were investigated (Fig. 3). HB611-GNT-1(1) and HB611-hygro(3) showed low expression of AFP mRNA, albumin, and prealbumin (Fig. 3). HB611-GNT-III(1) and HB611-hygro(3) showed low expression of albumin mRNA. Expression of prealbumin was almost the same in eight transfectants. Expression of these mRNAs was not correlated with the level of Gnt-III activity nor the expression of HBV-related mRNA in each transfectant.

Southern Blot Analysis—Southern blot analysis was performed to confirm that the integrated HBV gene had not been altered by the transfection of the Gnt-III gene. DNAs from seven transfectants were digested with three restriction enzymes, for which there were no recognition sites in the HBV genome sequence (15). A fragment of high molecular mass bands of more than 10 kilobase pairs was observed in all seven transfected DNAs digested by EcoRI, BamHI, and HindIII (Fig. 4). Even in HB611-GNT-III(1)–(3), the integrated HBV DNA was observed, suggesting that transfection of Gnt-III gene did not directly alter the HBV gene.

Southern Blot Analysis and FACS Analysis—To investigate the structure of oligosaccharides in total cellular proteins or cell surface proteins, lectin blot analysis and FACS analysis using E-PHA were performed (Figs. 5 and 6). E-PHA blot showed that the density of some bands appearing between approximately 60 and 130 kDa was enhanced in HB611-GNT-III(1)–(3). Huh6, which had high levels of Gnt-III activity (19), also showed a similar pattern of binding capacity with E-PHA, especially consistent with that of HB611-GNT-III(1). In FACS analysis, HB611-GNT-III(1) showed higher affinity to E-PHA than HB611-hygro(1), suggesting that bisecting structures of oligo-
saccharides in cell surface proteins were increased in the positive transfectant.

Treatment of Oligosaccharide Processing Inhibitor—To test whether or not the bisecting structure of oligosaccharides produced by the GnT-III gene suppresses expression of HBV-related mRNA, HB611-GNT-III(1), HB611-GNT-III(3), and the parent cell HB611 were treated with the oligosaccharide processing inhibitors, tunicamycin and swainsonine (Fig. 7). When HB611-GNT-III (1) and HB611-GNT-III(3) were treated with these agents, expression of HBV-related mRNA was dramatically increased. The effect was more prominent in tunicamycin-treated HB611-GNT-III(1) cells than in swainsonine-treated HB611-GNT-III(1) cells. Similar but minor increases were also observed in the parent HB611 cells, but expression of prealbumin mRNA was not changed in the three clones.

DISCUSSION

Many viruses, including the AIDS virus, HBV, and mouse Moloney leukemia virus, have specific sugar chains in their structural proteins. Although their functions in vivo are little known, in vivo experiments suggest they may be important for infection or secretion (4, 6, 8). Recently, Block et al. (7) reported that secretion of HBV was inhibited in HBV-infected HepG2 cells when the imino sugar N-butyldeoxynojirimycin, a potent inhibitor of oligosaccharide processing, was present. Molecules such as this result in changes to many oligosaccharides, including glycoproteins of the host cells. It is difficult to conclude, however, that the inhibition of viral secretion results solely from changes in viral sugar chains. Transfection of a specific glycosyltransferase gene into the cell may also change the sugar chain structure, but here again an explanation for the results obtained is difficult to formulate. In this regard, site-directed mutagenesis of amino acids in viral genes is a plausible argument for the functions of sugar chains. However, up-regulation of some glycosyltransferases is observed in vivo, and GnT-III activity in the liver is changed in relation to the clinical stage of liver disease (12). This change in activity is thought to affect sugar chains of viral proteins and host cells. The levels of HBV-related proteins in serum and its distribution in patients with hepatitis are greatly changed during the different stages of hepatitis (26, 27). Although the immune system may be involved in this phenomena, the morphology of hepatocytes
change in the liver of patients with liver cirrhosis. Expression of GnT-III is very high in fetal rat liver (28) and in chronic myelogenous leukemia cells when in blast crisis (29). These data show that GnT-III is expressed in undifferentiated cells. All attempts to produce HBV particles by transfection of a cloned HBV DNA into non-liver cells have so far failed (5), indicating that viral replication and gene expression are dependent on specific factors in hepatocytes. Furthermore, differentiation of hepatocytes is thought to be related to HBV gene expression. This notion is supported by some reports that only well differentiated hepatoma cell lines can produce the viral replicative intermediates and virions after transfection of a cloned HBV DNA (30, 31). Recent work using a HBV transgenic mouse shows low expression of HBV gene in hepatoma lesion despite the high expression in the surrounding tissues (32). These data suggest that it is possible to speculate that some inhibitory factors that can express HBV gene may exist in some kinds of hepatoma cells. The present study demonstrates that bisecting structures of N-glycans are one of the likely candidates. The mechanism by which HBV gene expression is suppressed by the oligosaccharide structure is not yet clear; a bisecting GlcNAc structure produced by ectopic expression of GnT-III may lead to changes of oligosaccharides in glycoproteins such as receptor and adhesion molecules. The second possibility is that some unidentified lectins in HB611 cells, which may directly recognize a bisecting GlcNAc may control the intracellular transport of certain proteins. For these reasons, GnT-III may change differentiation status of HB611 cells.

Korczak and Dennis (33) reported that inhibition of N-linked oligosaccharide processing in tumor cells is associated with enhanced gene expression of tissue inhibitor metalloproteinase I. They argued that the activity of an autocrine factor, of its
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Nautocrine factor was possibly affected by inhibition of receptor, or of a glycoprotein involved in the response to an autocrine factor was possibly affected by inhibition of N-linked oligosaccharide processing.

In transgenic mice containing the GnT-III gene, ballooning of hepatocytes are observed (data not shown). This phenomenon suggests that GnT-III changes the character of hepatocytes. On the other hand, HBV suppressed GnT-III activity selectively among the various glycosyltransferases (19), suggesting that HBV may transform host cells in a good place for viral replication. The suppression of HBV gene expression by enhancement of GnT-III might be a unique approach to prevent HBV replication.

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