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St6gal1 knockdown alters HBV life cycle in HepAD38 cells

Surya Agung Priyambada [a], Ryo Misaki [a], Toru Okamoto [b], Takao Ohashi [a], Keiji Ueda [c], Yoshiharu Matsuura [b], Kazuhito Fujiyama [a, *]

[a] International Center for Biotechnology, Osaka University, Yamada-oka 2-1, Suita, Osaka, 565-0871, Japan
[b] Department of Molecular Virology, Research Institute for Microbial Disease, Osaka University, Yamada-oka 3-1, Suita, Osaka, 565-0871, Japan
[c] Division of Virology, Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, Yamada-oka 2-2, Suita, Osaka, 565-0871, Japan

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ABSTRACT

Complex glycans at the cell surface play important roles, and their alteration is known to modulate cellular activity. Previously, we found that HBV replication in HepAD38 altered cell-surface sialylated *N*-glycan through the upregulation of *St6gal1*, *Mgat2*, and *Mgat4a* expression. Here we studied the effects of knocking them down on HBV replication in HepAD38. Our results showed that *St6gal1* knockdown (KD) reduced intracellular HBV rcDNA level by 90%, that *Mgat2* KD did not change the intracellular HBV rcDNA level, and that *Mgat4* KD increased the intracellular HBV rcDNA level by 19 times compared to Tet(−). The changes in intracellular rcDNA level were followed by the alteration of Pol and HBc expression. Our study suggests that *St6gal1* KD contributes more to the HBV life cycle than *Mgat2* or *Mgat4a* KD through the modification of intracellular L, Pol, and HBc expression.

1. Introduction

In eukaryotes, numerous asparagine-x-serine/threonine (x ≠ proline) sequons exposed to the ER lumen are *N*-glycosylated [1]. *N*-glycan matures in the Golgi. Most *N*-glycoproteins are either secreted from the cell or presented to the plasma membrane as the molecular boundary of the cell [2] and play important biological roles [3–5].

HBV carries partially double-stranded rcDNA in a nucleocapsid [6]. The nucleocapsid is surrounded by outer viral envelope *N*-glycoproteins comprised of large (L), middle (M), and small (S) proteins [7]. Displacement of the *N*-glycan from the envelope proteins affects viral assembly and egress [8,9]. Moreover, alteration of host *N*-glycan maturation machinery was previously suggested to interfere with HBV production [8,10]. This indicates the importance of *N*-glycan in the HBV life cycle.

Glycoproteins may have many *N*-glycan addition sites, each of which has the potential to be modified by plenty of different *N*-glycan structures. This is considered tolerable in biosynthesis because *N*-glycans perform the general functions of protein folding, secretion, and the solubilization of glycoproteins [5]. However, the latest evidence suggests that a specific GlcNAc linkage of *N*-glycan matters for proper glycoprotein function [11]. Also, Neu5Ac is required for cell signaling events [12,13].

Indeed, several glycosyltransferases have been suggested to be associated with the HBV life cycle [14–16]. In our previous study, HBV replication upregulated sialylated *N*-glycan in HepAD38 cells through the upregulation of *St6gal1*, *Mgat2*, and *Mgat4a* expression [16]. Therefore, we disrupted these genes in this study and evaluated the extracellular and intracellular HBV rcDNA levels as well as the viral protein expression in order to assess viral replication. Our results suggest that the disruption of the host glycosyltransferase machinery modified HBV rcDNA levels through the alteration of intracellular L, Pol, and HBc expression.

Abbreviations: HBV, Hepatitis B virus; SVP, Sub-viral particle; WT, Wild-type; Tet(+), Tetracycline-supplemented; Tet(−), Tetracycline-free; ER, Endoplasmic reticulum; *St6gal1*, β-galactoside α2,6-sialyltransferase 1; *St3gal4*, β-galactoside α2,3-sialyltransferase 4; *Cmas*, CMP-Neu5Ac synthetase; *Mgat2*, Mannosyl (α1,6-)-glycoprotein β1,2-*N*-acetylglucosaminyltransferase-II; *Mgat4a*, Mannosyl (α1,3-)-glycoprotein β1,4-*N*-acetylglucosaminyltransferase-IV isozyme A; KD, Knockdown; Neu5Ac, Neuraminic acid; Gal, Galactose; GlcNAc, *N*-acetylglucosamine; Man, Mannose; CMP, Cytidine 5′-monophosphate; CTD, Cytidine 5′-triphosphate; CMP-Neu5Ac, Cytidine 5′-monophospho-neuraminic acid; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; rcDNA, relaxed-circular DNA; cccDNA, covalently-closed circular DNA; pgRNA, pregenomic RNA; L, hepatitis B L protein; HBx, hepatitis B X protein; Pol, hepatitis B Polymerase protein; HBc, hepatitis B Core protein.

* Corresponding author.
E-mail address: fujiyama@icb.osaka-u.ac.jp (K. Fujiyama).

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2. Methods

2.1. Cell culture

HepAD38 [17] was maintained in DMEM Ham's F12 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum, 5 μg/mL insulin, 400 ng/mL tetracycline, and 400 μg/mL G418 [Tet(+) medium] at 37 °C in 5% CO_2. Tet(−) G418(−) medium was prepared as described above without the addition of tetracycline and G418.

2.2. Development of siRNA-mediated glycosyltransferase KD HepAD38 mutants

The siRNA duplex sequences of *St6gal1* 5′-AGACAGUUUGUACAAUGAAtt-3' (sense) and 5′-UUCAUUGUACAAACUGUCUtt-3' (antisense), *Mgat2* 5′-GAAGAAUGCCGCUUUGAAAtt-3' (sense) and 5′-UUUCAAAGCGGCAUUCUUCgg-3' (antisense), and *Mgat4a* 5′-GGUCUGCACUCAUCACUAUtt-3' (sense) and 5′-AUAGUGAUGAGUGCAGACCaa-3' (antisense) were used. Additionally, we depleted *Cmas* and *St3gal4* by introducing respective siRNA duplexes: *Cmas* 5′-GAAAUGCGAGCUGAACAUAtt-3' (sense) and 5′-UAUGUUCAGCUCGCAUUUCgt-3' (antisense), and *St3gal4* 5′-GCAGACCAUUCACUACUAUtt-3' (sense) and 5′-AUAGUAGUGAAUGGUCUGCtt-3' (antisense). Tet(−)HepAD38 was transfected with Stealth RNAi™ siRNA negative control low-GC duplex as an siRNA control. All siRNA duplexes were products of Ambion® (Thermo Fisher Scientific, Waltham, MA, USA). Twenty picomoles (20 pmole) of siRNA duplex was introduced to HepAD38 by reverse-transfecting the cells onto a 6-well plate at a density of 1×10^6 cells/well. The transfected cells were maintained in Tet(−) G418(−) medium, with medium changes followed by fresh siRNA introduction every 3 d.

The extracellular fractions were collected for rcDNA analysis. The cells were collected and viable cells were counted on day 9 after siRNA transfection using a Neubauer chamber. The cells were incubated with 0.5% trypan blue at room temperature for 5 min. Cells resisting trypan blue were counted under a light microscope.

2.3. Confirmation of glycosyltransferase transcript knockdown and assessment of pgRNA level by quantitative reverse transcription (qRT)-PCR assay

RNA was extracted using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). One microgram of the RNA was reverse-transcribed using SuperScript™ VILO™ Master Mix (Thermo Fisher Scientific). Eighty nanograms of cDNA was used as the template for qRT-PCR using the Thunderbird® SYBR® qPCR Mix (Toyobo, Osaka, Japan) in the StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA, USA) with the following primers: 5′-GAATGTAGATAAGGCTGGC-3′ (*Mgat2* forward), 5′-GATTGATCTCGGTCGAC-3′ (*Mgat2* reverse), 5′-GTAGGAGCAGAAACAAATGG-3' (*Mgat4a* forward), 5′-GTTGCCAATCTGTACAGC-3' (*Mgat4a* reverse), 5′-GATTCCCAGTCTGTATCCT-3′ (*St6gal1* forward), 5′-GGTTTTTGGAAGAGCTGT-3′ (*St6gal1* reverse), 5′-AGGGTGAGGCAGAGAGCAAG-3' (*St3gal4* forward), 5′-TGGATGTTCTTGGGGATGG-3' (*St3gal4* reverse), 5′-GAAGGATATGATTCTGTTTTCT-3' (*Cmas* forward), and 5′-AAGTAACCCATCTCTATCAAA-3' (*Cmas* reverse). As for the pgRNA analysis, following primers were used: 5′-CACCTCTGCCTAATCATC-3' (pgRNA forward) and 5′-GGAAAGAAGTCAGAAGGCAA-3' (pgRNA reverse). The expression level of each transcript was determined by the comparative C_T method ($\Delta\Delta C_T$). Human GAPDH was used as the endogenous control, and Tet(−)HepAD38 cDNA was used as a reference sample.

2.4. Cell lysis and phenotypic analysis of HepAD38-treated siRNAs

The cells were harvested, washed with PBS, and then lysed by application of RIPA buffer [50 mM Tris-Cl pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1× complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)]. The cell lysate was subjected to SDS-PAGE under the reduced condition, and WT Tet(−) cells were used as the control.

Phaseolus vulgaris leucoagglutinin (PHA-L4, 1:1000), *Datura stramonium* lectin (DSA, 1:1000), and *Maackia amurensis* mitogen (MAM, 1:1000) were used for the phenotypic analysis of the *Mgat2*, *Mgat4a*, and *St3gal4* siRNA mutants, respectively. *Sambucus sieboldiana* agglutinin (SSA, 1:1000) was used for the phenotypic analysis of *St6Gal1* and *Cmas* siRNA mutants. All lectins were the product of J-Oil Mills (Tokyo, Japan). Peroxidase-linked avidin (1:5000; Millipore, Billerica, MA, USA) was used to couple the lectins.

2.5. Extraction and detection of the intracellular and extracellular rcDNA and the cccDNA

The extracellular protein was recovered by PEG precipitation. The intracellular and extracellular HBV rcDNAs were extracted as previously described [18]. The cell pellet and the extracellular protein were incubated in a lysis buffer (50 mM Tris-Cl pH 7.4, 1 mM EDTA, and 1% NP-40) for 15 min on ice. The supernatant was collected and treated with 7 mM magnesium acetate, 0.2 mg/mL DNase I (Roche), and 0.1 mg/mL RNase A (Sigma-Aldrich), then incubated at 37 °C for 3 h. Next, 10 mM EDTA and 100 mM NaCl were added, then the lysates were digested by proteinase K (0.02 mAU/μL reaction volume; Takara Bio, Shiga, Japan) and 2% SDS at 37 °C for 12 h. The DNA was purified by phenol-chloroform-isoamyl alcohol, precipitated with ethanol, and resolved in pure water.

The cccDNA was extracted according to a previously described method [19]. Solution I (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 150 mM NaCl, 1% SDS) was used to lyse the cell. KCl was then added to a final concentration of 0.5 M, mixed, and incubated on ice for 5 min. Next, the SDS-protein debris was removed and the DNA was isolated by ethanol precipitation. Linear DNA was then removed by alkaline lysis [20]. Briefly, 0.05 N NaOH was added, followed by incubation at 37 °C for 30 min, and CH_3COOK pH 5.0 was added to the final concentration of 0.6 M, followed by phenol-chloroform-isoamyl alcohol purification and ethanol precipitation.

The rcDNA and cccDNA were amplified by Thunderbird® SYBR® qPCR Mix using the following primers: 5′-GGAGGGATACATAGAGGTTCCTTGA-3′ and 5′- GTTGCCCGTTTGTCCTCTAATTC- 3′ [18], and 5′-GTGCCTTCTCATCTGCCGG-3′ and 5′-GGAAAGAAGTCAGAAGGCAA-3′ [21], respectively. The HBV DNA copy number was determined by the standard curve method using the StepOnePlus™ real-time PCR system (Applied Biosystems).

2.6. Western blotting

Anti-hepB preS1 (sc-57762, 1:1000; Santa Cruz Biotechnology, Dallas, TX, USA), anti-HBcAg (ab8637, 1:1000; Abcam, Cambridge, UK), anti-HBx (ab2741, 1:1000; Abcam), anti-hepB Pol (sc-81590, 1:200; Santa Cruz Biotechnology), and anti-β-actin (M177-3, 1:1000; Medical and Biological Laboratories, Tokyo, Japan) antibodies were used to detect the intracellular L, HBc, HBx, Pol, and actin, respectively.

2.7. Statistical analysis

An independent experiment (n) was conducted three times ($n = 3$), and each value of n was obtained by triplication of

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