Knockdown of N-Acetylglucosaminyl Transferase V Ameliorates Hepatotoxin-Induced Liver Fibrosis in Mice

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Aberrant N-glycosylation caused by altered N-acetylglucosaminyltransferase V (GnT-V) expression is known to regulate tumor invasion and metastasis by modulating multiple cytokine signaling pathways. However, the exact role of GnT-V in the development of liver fibrosis has not been clearly defined. Here, we induced mouse liver fibrosis by ip injections of carbon tetrachloride (CCL4) or thioacetamide (TAA) and observed significant increase of hepatic GnT-V during the processes of liver fibrogenesis. Meanwhile, upregulations of GnT-V were detected in the activated hepatic stellate cells (HSCs) and injured hepatocytes. To knock down hepatic GnT-V expression, adenovirus that expressed the GnT-V siRNA was injected via the tail vein. Adenovirus-mediated delivery of GnT-V siRNA dramatically reduced the GnT-V expression in fibrotic liver in vivo and consequently alleviated CCL4- or TAA-induced liver fibrosis as assessed through collagen deposition and profiles of profibrogenic markers. Furthermore, knockdown of GnT-V in HSCs reduced transforming growth factor beta (TGF-β)/Smad signaling and blunted the activated HSC phenotype. The suppression of TGF-β/Smad signaling in HSCs correlated with the decrease of GnT-V-modified β1,6-branched N-glycan on TGF-β receptors. Knockdown of GnT-V also suppressed platelet-derived growth factor (PDGF)-induced HSC proliferation and migration through inhibiting PDGF/Erk signaling. Finally, we demonstrated that knockdown of GnT-V profoundly suppressed TGF-β1-induced epithelial-mesenchymal transition (EMT) in hepatocytes by morphological assessment and reversal of EMT markers. In conclusion, this study demonstrates that GnT-V is implicated in hepatotoxic-inuced liver fibrosis, and targeting GnT-V may be a feasible and promising approach for treating liver fibrosis.

Key Words: liver fibrosis; N-acetylglucosaminyltransferase V; adenovirus; knockdown; hepatic stellate cells; epithelial-mesenchymal transition.

The liver responds to different chronic insults by developing fibrosis characterized by the accumulation of extracellular matrix (ECM) materials. Hepatic stellate cells (HSCs), which encircle the sinusoids as pericytes, have been identified as the main producers of ECM in the fibrotic liver (Bataller and Brenner, 2005; Kinoshita et al., 2007). Following liver injury, quiescent HSCs transdifferentiate into myofibroblast-like cells that are characterized by enhanced expression of smooth muscle α-actin (α-SMA) and ECM. Once activated, HSCs become proliferative, proinflammatory, and profibrogenic through increased responsiveness to soluble mediators (Friedman, 2000). Transforming growth factor beta 1 (TGF-β1) has been identified as the key cytokine promoter of HSC activation and stimulates the synthesis of ECM proteins in the activated HSCs mainly through the Smad2/3 pathway (Bataller and Brenner, 2005; Kinoshita et al., 2007). Additionally, activated HSCs can migrate and/or proliferate in response to platelet-derived growth factor (PDGF), which is markedly upregulated in the fibrotic liver and is one of the most potent mitogens for HSCs (Pinzani et al., 1996).

Although HSCs have been identified as the dominant source of myofibroblasts, the other sources of fibroblasts remain controversial (Choi and Diehl, 2009). Recent studies suggest that myofibroblasts can develop from cholangiocytes and hepatocytes by epithelial-mesenchymal transition (EMT) programs (Kaimori et al., 2007; Zeisberg et al., 2007). EMT describes a transdifferentiation process whereby epithelial cells acquire the structural and functional characteristics of mesenchymal cells and is known to be a major driver of morphogenesis and tumor progression (Thiery and Sleeman, 2006; Thiery et al., 2009). The main hallmark of EMT is downregulation of the adherent junction protein E-cadherin due to transcriptional repression. In both mature mouse hepatocytes and cultured mouse hepatocyte cell lines, TGF-β1 induces the EMT program through activation of Smad2/3 pathway, following downregulation of E-cadherin and upregulation of EMT-associated factors including Snail, Twist, and N-cadherin (Caja et al., 2007; Peinado et al. 2007)
Glycosylation is one of the most common posttranslational modifications in eukaryotic cells. Aberrant glycosylation, most often arising from changes in the expression levels of glycosyltransferases in the Golgi compartment, occurs in nearly all types of cancers and correlates with disease progression (Zhao et al., 2008). Among these glycosyltransferases, N-acetylglucosaminyltransferase V (GnT-V, also known as Mga5) has been identified as one of the most relevant glycosyltransferases for tumor invasion and metastasis (Lau et al., 2007; Mendelsohn et al., 2007). GnT-V catalyzes the addition of β1,6-linked GlcNAc units to the α1,6-linked mannose of the trimannosyl core of N-linked glycans to form tri- or tetra-antennary branches. This branched structure can be further extended with poly-N-acetylactosamine chain, which is the preferred ligand for the β-galactoside-binding animal lectin, Galectin-3 (Hirabayashi et al., 2002). Of note, recent studies have demonstrated that Galectin-3 expression is upregulated in fibrotic liver. Disruption of the galectin-3 blocked myofibroblast activation and markedly attenuated liver fibrosis, suggesting that GnT-V-modified N-glycosylation plays an important role in hepatic fibrogenesis (Henderson et al., 2006). Moreover, several cytokine signaling pathways, including the TGF-β1, PDGF, and epidermal growth factor (EGF), can be modulated by changes in GnT-V-modified N-glycans on their receptors (Guo et al., 2007, 2012; Partridge et al., 2004). For example, knockdown of GnT-V in human breast carcinoma cells resulted in an inhibition of EGF-stimulated SHP-2 activation and a consequent reduction in tumor cell invasion (Guo et al., 2007). Whereas GnT-V overexpression in mouse keratinocytes promoted EMT and keratinocyte migration through enhanced EGF receptor signaling (Terao et al., 2011).

Carbon tetrachloride (CCL4) and thioacetamide (TAA) are the most commonly used hepatotoxic reagents to induce liver fibrosis. CCL4 is metabolized to highly reactive trichloromethyl free radicals by CYP2E1 and thus induces an acute centrolobular necrosis, which triggers a wound-healing response (Jiménez et al., 1992). Administration of TAA increases the activation of HSCs during hepatic fibrogenesis and closely resembles alcoholic liver fibrogenesis (Müller et al., 1988; Salguero Palacios et al., 2008). Here, using a recombinant adenovirus that can express the GnT-V siRNA in vivo, we demonstrate that knockdown of hepatic GnT-V significantly ameliorates CCL4- or TAA-induced mouse liver fibrosis by inhibiting HSC activation, proliferation, and migration and by suppressing the EMT process in hepatocyte.

MATERIALS AND METHODS

Antibodies and reagents. The primary and secondary antibodies used in this study are listed in Supplementary Materials and Methods. Recombinant human TGF-β1 and recombinant murine PDGF-BB were purchased from Peprotech (Rocky Hill, NJ). Other reagents were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise indicated.

Construction of recombinant adenovirus. Construction of recombinant adenovirus can be found in the Supplementary Materials and Methods.

Animals. Five-week-old male ICR mice, weighing 20±2 g, were obtained from the Animal Center of Yangzhou University (Yangzhou, China). For 1 week prior to experimentation, animals were acclimatized in a ventilated, temperature-controlled room (23°C) with a 12-h light: 12-h dark cycle. All animals had free access to rodent chow and water. The experiments were conducted according to the use and care guidelines of the experimental animals of Jiangsu Province, P.R. China. For CCl4-induced mouse liver fibrosis, mice were ip injected with a 20% solution of CCl4 in sterile mineral oil at a dose of 2.5 ml CCl4 per kilogram body weight twice per week for 4 weeks. Mice were given a iv injection of 1 × 106 plaque-forming units (PFU) AdMS1 or AdNC via tail vein after 1 week CCl4 treatment and were sacrificed 20 days after adenovirus administration. TAA-induced mouse liver fibrosis was induced by ip injection of TAA (200 mg/kg body weight) twice per week for 8 weeks. Mice were given a iv injection of 1 × 106 PFU AdMS1 or AdNC via the tail vein after 4 weeks of injections of TAA.

Cell culture. Primary mouse HSCs and hepatocytes were isolated by pronase/collagenase perfusion digestion followed by subsequent density gradient centrifugation as previously described (Schrum et al., 2000; Weiskirchen and Gressner 2005). The isolated HSCs were identified by their intrinsic vitamin A autofluorescence and staining for desmin. Isolated HSCs were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Camarillo, CA) supplemented with 10% fetal bovine serum (FBS). The rat hepatic stellate cell line HSC-T6 and the human embryonic kidney cell line 293A were cultured in high-glucose DMEM supplemented with 10% FBS. For TGF-β1 treatment, primary HSC or HSC-T6 cells were replaced with serum-free medium for 24 h. Afterwards, TGF-β1 was added at a final concentration of 5 ng/ml, and the cells were continued to incubate for indicated times.

The nontumorigenic mouse hepatocyte cell line AML12 was maintained in DMEM/F12 medium containing 10% FBS, 1x ITS (Insulin-transferrin-sodium selenite media supplement, Sigma-Aldrich), dexamethasone (40 ng/ml), penicillin (100 units/ml), and streptomycin (100 µg/ml). To induce EMT, AML12 cells were seeded at about 60% confluence and changed to serum-free medium 18 h later. TGF-β1 was added at a final concentration of 5 ng/ml. The cells were examined under a phase-contrast microscope (×200) at 24, 36, and 48 h after TGF-β1 treatment. To knockdown GnT-V expression, AML12 or HSC-T6 cells were infected with 50 multiplicity of infection adenovirus for 1 h and then incubated in serum-free medium for the indicated time.

Histological examination and immunofluorescence staining. Excised liver tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. The tissue was then stained with hematoxylin and eosin (H&E) or Sirius red. For semiquantitative analysis of liver fibrosis, 10 randomly selected fields from each slide were recorded, and the Sirius red-stained areas were quantified using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD). For immunofluorescence staining, paraffin-embedded liver sections were incubated with the primary antibody at 4°C overnight according to the manufacturer’s recommendation. After the sections were washed with Tris-buffered saline, they were subsequently stained with Fluorescein Isothiocyanate–conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and imaged by fluorescent microscopy.

Immunoprecipitation and lectin blot. HSC-T6 cells were lysed in lysis buffer for 10 min at 4°C. For immunoprecipitation of the TGF-β1 receptors, the cell lysates were incubated with primary antibody and protein A/G plus-agarose immunoprecipitation reagent (Santa Cruz) according to the manufacturer’s instructions. For the lectin blot, the immunocomplexes were separated by 10% SDS-PAGE, transferred onto a PVDF membrane, and probed with biotinylated L-PHA. To detect the binding of L-PHA, the membrane was then incubated with HRP-Streptavidin (Beiyotime, Nantong, China). A Western blot analysis, using antibodies against the immunoprecipitated receptor, was performed using the same amount of immunocomplexes as the internal control.

RNA isolation and quantitative real-time PCR. Total RNA was prepared from cells or liver tissues by homogenization and purification using TRIzol reagent (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed
using the SYBR PrimeScript RT-PCR Kit (Takara, Dalian, China) in an ABI 7300 Fast Realtime PCR System (Life Technologies Corporation, Carlsbad, CA) according to the manufacturer’s instructions. Individual gene expression was quantified using a standard curve and normalized to β-actin mRNA expression, and results are shown as fold change compared with control. The PCR conditions were as follows: 95°C for 30 s and then 40 cycles of amplification for 5 s at 95°C and 30 s at 60°C. Primers are listed in Supplementary Materials and Methods.

**Western blot.** Cell pellets or liver tissue were lysed in lysis buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, NaVO₃, and leupeptin) containing 1mM PMSF. After heat denaturation at 100°C for 5 min, equal amounts of lysate (20 μl) were separated by SDS-PAGE and then transferred onto PVDF membranes. The membranes were incubated with primary antibody at appropriate dilutions for 1 h. After washing with PBST, the membrane was then probed with horseradish peroxidase–conjugated anti-rabbit or anti-goat IgG for 1 h at room temperature. The protein bands were visualized by fluorography using an enhanced chemiluminescence system (Cell Signaling Technology, Beverly, MA).

**Statistical analysis.** Results are expressed as the means ± SEM. Differences between multiple groups were compared using one-way ANOVA with post hoc Bonferroni correction. Differences between two groups were evaluated using a two-tailed unpaired Student’s t-test. The significance level was set at p < 0.05, and p < 0.01 indicated a strongly significant difference.

## RESULTS

### Liver Fibrosis Is Associated With Increased Expression of GnT-V

To determine the dynamics of GnT-V regulation in hepatic fibrogenesis, we first conducted a time-course experiment in the mouse model of CCl₄-induced liver fibrosis, which was achieved by eight injections of CCl₄ within 4 weeks. qRT-PCR results demonstrated that hepatic GnT-V increased after CCl₄ treatment and peaked at 3 weeks later (Fig. 1A). The highest hepatic GnT-V level in CCl₄-treated mouse was about 2.7 times higher than those of control. Meanwhile, hepatic Galectin-3 expression significantly increased as expected (Fig. 1A). Consistent with our observations in CCl₄-induced liver fibrosis, hepatic GnT-V and Galectin-3 mRNA levels were significantly upregulated in the process of TAA-induced liver fibrosis (Fig. 1B).

To further characterize GnT-V expression in different hepatic cell populations during hepatic fibrogenesis, we isolated HSCs and hepatocytes weekly from CCl₄-induced fibrotic liver and found that GnT-V expression in HSCs started to increase after 1-week injection of CCl₄ and peaked at the third week (Fig. 1C). An obvious decrease of GnT-V was observed in hepatocytes during the first 2 weeks of CCl₄ treatment. Nevertheless, the GnT-V expression in hepatocytes was significantly upregulated after 2 weeks of CCl₄ treatment (Fig. 1C). Finally, we investigated the regulation of GnT-V in response to TGF-β1, which has been identified as the key cytokine promoter of HSC activation and hepatic fibrogenesis. In mouse hepatocyte cell line AML12, rat HSC cell line HSC-T6 (Figs. 1D and E), and primary mouse HSCs (Fig. 1F), TGF-β1 treatment led to significant upregulation of GnT-V as indicated by qRT-PCR (Fig. 1D) and Western blot results (Figs. 1E and F).

To investigate the functional importance of GnT-V in hepatic fibrosis, we constructed the recombinant adenovirus AdMS1 and AdNC, which can stably express GnT-V siRNA and scrambled siRNA, respectively, in vivo. Because the homology of mouse and rat GnT-V mRNA is higher than 95%, and the designed GnT-V siRNA target sequence is identical in mouse and rat, AdMS1 can efficiently knock down GnT-V expression in both mouse and rat. As shown in Figures 1F and 1G, the GnT-V protein level in AdMS1-infected primary mouse HSCs (Fig. 1F) or cultured HSC-T6 cells (Fig. 1G) was significantly lower than cells infected with AdNC.

### Knockdown of Hepatic GnT-V Expression Ameliorates CCl₄ or TAA-Induced Liver Fibrosis

To characterize the role of GnT-V in modulating liver fibrosis, AdMS1 or AdNC was iv injected into CCl₄-treated mice as described in Materials and Methods. As shown in Figure 2A, AdNC-infected mice developed significant hepatic fibrosis as demonstrated by H&E, Sirius red, and α-SMA staining. However, a single administration of AdMS1 greatly reduced CCl₄-induced liver fibrosis. The Sirius red positive area (Fig. 2B) and hepatic hydroxyproline content (Fig. 2C) significantly decreased in AdMS1-infected mice. Additionally, administration of AdMS1 resulted in a significant reduction of profibrogenic markers Col1a1 (Fig. 2D), TGF-β1, and tissue inhibitor of metalloproteinase-1 (TIMP-1) (Supplementary fig. S1). As expected, Galectin-3 was greatly upregulated in CCl₄-induced fibrotic liver, whereas AdMS1-infected mice displayed a significant reduction in Galectin-3 level (Fig. 2E). Western blot further demonstrated that hepatic GnT-V and α-SMA expressions were significantly reduced in AdMS1-infected mice, indicating that HSC activation was suppressed when GnT-V expression was knockdown (Fig. 2F). To confirm that AdMS1 infection sufficiently suppressed GnT-V expression in the activated HSCs of fibrotic liver, primary HSCs were isolated from AdMS1- and AdNC-infected mice. Western blot results revealed that GnT-V and α-SMA expressions were significantly reduced in the HSCs from AdMS1-infected mice (Fig. 2G). Serum alanine aminotransferase (ALT) levels were determined at 5 and 20 days after adenovirus injection. We did not find significant differences in serum ALT levels between AdMS1- and AdNC-infected mice, suggesting that similar hepatocellular injuries occurred in both groups (Supplementary fig. 2).

We also assess the antifibrotic potential of AdMS1 in TAA-induced liver fibrosis by one administration of adenovirus after 4-week treatment of TAA. The effect of AdMS1 was essentially the same as in CCl₄-treated mice with respect to H&E and Sirius red staining (Figs. 3A and B) and reduction of hepatic hydroxyproline (Fig. 3C). The suppression of fibrosis was further confirmed by qRT-PCR, showing that...
the mRNA levels of α-SMA (Fig. 3D), Col1a1 (Fig. 3E), GnT-V (Fig. 3F), and Galectin-3 (Fig. 3G) were significantly reduced in AdMS1-infected mice. Serum ALT level markedly increased in TAA-treated mice; however, there was no significant difference between AdMS1- and AdNC-infected mice (data not shown).

Knockdown of GnT-V Expression Causes Aberrant N-Glycosylation of TGF-β Receptors and Inhibits TGF-β/Smad Signaling

TGF-β1 is the most powerful inducer of HSC activation and fibrogenesis. To address the crucial role of GnT-V in regulating HSC activation, we first investigated whether GnT-V was
implicated in modulating TGF-β/Smad signaling in primary HSCs. As shown in Figure 4A, infection of AdMS1 markedly reduced phosphorylation of Smad3 in the TGF-β1-treated primary HSCs. Consistent with the results of primary HSC, Western blot analysis in TGF-β1-treated HSC-T6 cells also exhibited similar results (Fig. 4B).

The plant lectin leukoagglutinin (L-PHA) binds specifically to mature product of GnT-V activity and has therefore been used as a probe for GnT-V-modified glycans (Cummings and Kornfeld, 1982). To further explore mechanisms underlying the reduction of TGFβ/Smad signaling in the GnT-V knockdown HSCs, type I and type II TGF-β receptors (TβRI and TβRII)
were immunoprecipitated with their antibodies, respectively, and lectin-blotted with biotinylated L-PHA. As shown in Figures 4C and 4D, the binding of L-PHA on TβRI and TβRII of AdMS1-infected HSC-T6 cells was significantly lower than those of AdNC-infected cells, indicating that knockdown of GnT-V decreased GnT-V-modified β1,6-branched N-glycans on the TGF-β receptors and consequently inhibited TGF-β signaling.

Knockdown of GnT-V Deactivates the Myofibroblasts

HSC activation is accompanied by the upregulation of mesenchymal markers and the downregulation of E-cadherin (Yue et al., 2010). HSC-T6 cells are immortalized cells exhibiting the typical features of myofibroblasts. To investigate whether GnT-V expression in HSC-T6 cells affect their myofibroblastic status, HSC-T6 cells were infected with AdMS1 or AdNC and incubated for 48–96 h. qRT-PCR results demonstrated that the expression of fibrotic and mesenchymal markers including collagen I (Fig. 5A), α-SMA (Fig. 5B), TIMP-1 (Fig. 5C), and Vimentin (Fig. 5D) significantly decreased in AdMS1-infected HSC-T6 cells from 48 to 72 h after AdMS1 infection. Meanwhile, the expression of E-cadherin increased at 96 h after AdMS1 infection (Fig. 5E), whereas its transcriptional repressor Snail1 was downregulated at 48 h after AdMS1 infection (Fig. 5F), indicating that the upregulation of
E-cadherin occurred in parallel with the decreasing of Snail1 after GnT-V knockdown in HSC-T6 cells.

**Knockdown of GnT-V Inhibits PDGF-Induced HSC Proliferation and Migration**

To investigate whether GnT-V promotes fibrogenesis through other effects on HSCs, we sought to explore the potentials of GnT-V in modulating PDGF-induced HSC proliferation and migration. Our results showed that the viability and proliferation rates of HSC-T6 cells greatly increased in the presence of PDGF. However, PDGF-induced proliferation was significantly suppressed in AdMS1-infected HSC-T6 cells (Fig. 6A). Additionally, the motility of HSC-T6 cells was determined by the wound scratch assay (Fig. 6B) and the cell migration assay.
Knockdown of GnT-V prevents hepatic fibrosis using a two-compartment Boyden chamber (Fig. 6C). The results indicated that knockdown of GnT-V profoundly inhibited PDGF-induced HSC migration.

To confirm whether GnT-V was involved in modulating PDGF signaling, we tested the effects of GnT-V knockdown on the activation of Erk pathway, which is critical for PDGF-dependent HSC proliferation and migration. Following treatment with PDGF, the phosphorylation of Erk1/2 significantly increased. Nevertheless, phosphorylation of Erk1/2 was greatly inhibited in AdMS1-infected HSC-T6 cells compared with AdNC-infected cells (Fig. 6D).

Knockdown of GnT-V inhibits TGF-β1-induced EMT in Hepatocyte

To explore the functional importance of GnT-V in promoting fibrogenesis through mechanisms other than HSC activation, we investigated whether GnT-V was involved in modulating EMT in hepatocytes. TGF-β1-induced EMT was performed in the cultured mouse hepatocyte cell line AML12 according to a previously described method (Pan et al., 2009). Following TGF-β1 treatment, AML12 cells lost their epithelial honeycomb-like shape and acquired a spindle-like cell morphology. Interestingly, infection of AML12 cells with AdMS1 significantly inhibited TGF-β1-induced EMT, as indicated by reversals of cell morphology changes (Fig. 7A). qRT-PCR and Western blot results demonstrated that infection of AdMS1 significantly reduced GnT-V expression (Fig. 7B) and suppressed phosphorylation of Smad3, thereby confirming that effective GnT-V knockdown correlated with suppression of the TGF-β1/Smad signaling in AML12 cells (Fig. 7C). The shift from E-cadherin expression to upregulation of N-cadherin has been defined as a hallmark of EMT. qRT-PCR results revealed that infection of AdMS1 greatly inhibited the downregulation of E-cadherin in the process of TGF-β1-induced EMT (Fig. 7D), whereas the upregulation of N-cadherin was suppressed simultaneously (Supplementary fig. 3A). Moreover, infection of AdMS1 significantly suppressed the upregulation of the other two fibroblast markers Vimentin and FSP1 (Supplementary figs. 3B and C).

Migration is a hallmark feature of activated fibroblasts, whereas hepatocytes are immobile and tightly integrated into
the epithelial cell layer. The wound scratch assay results demonstrated that treatment with TGF-β1 for 48 h significantly increased the migratory capacity of AML12 cells. However, TGF-β1-induced cell migration was greatly inhibited in AdMS1-infected AML12 cells (Fig. 7E).

**DISCUSSION**

The enhanced β1,6 GlcNAc branching of N-linked glycans, catalyzed by increased GnT-V expression, is a common change in glycosylation that can induce malignancy and has been strongly linked to tumor metastasis (Demetriou et al., 2001; Zhao et al., 2008). This study provides the first evidence that GnT-V plays a functional role in hepatotoxin-induced liver fibrosis. Expression of GnT-V in normal liver is quite low, but it is significantly upregulated in regenerating liver after partial heptatectomy (Miyoshi et al., 1995). Our results reveal that liver fibrogenesis is accompanied by a significant upregulation of hepatic GnT-V. Of note, significant upregulation of GnT-V has also been observed in activated HSCs of fibrotic liver, thereby indicating that GnT-V participates in modulating HSC trans-activation and hepatic fibrosis and represents a potential target for antifibrotic therapies. According to the time-course results, GnT-V upregulation mainly occurs from the second to the third week after CCl₄ treatment. Therefore, suppression of GnT-V in this period will be expected to improve the hepatic fibrogenesis prominently.

Acute and chronic liver injuries are accompanied by prominently increased expression of proinflammatory and profibrogenic mediators and their receptors. These cytokine receptors are generally N-glycosylated transmembrane proteins containing GnT-V-catalyzed β1,6 GlcNAc-branched N-glycan. Previous studies have demonstrated that expression of GnT-V sensitized mouse cells to multiple cytokines (Guo et al., 2007; Partridge et al., 2004). HSC activation represents the final
Execution step in hepatotoxin-induced liver fibrosis. However, there has been no study on the actual contribution of GnT-V and GnT-V-modified β1,6-branched N-glycan to HSC activation. TGF-β1 transactivates HSC and stimulates the synthesis of ECM proteins mainly through TGF-β1/Smad pathway. In this pathway, TGF-β1 binds to the TβRII, subsequently activating the TβRI and propagating the signal inside the cell through the phosphorylation of the intracellular mediators Smad2/3. Phosphorylated Smad2 and/or Smad3 complex with Smad4 translocate to the nucleus, where they regulate the target genes (Gordon and Blobe, 2008). Here, we provide compelling evidence that administration of AdMS1 significantly reduce
GnT-V expression in the HSCs of fibrotic liver. Furthermore, suppression of TGF-β/Smad signaling in GnT-V knockdown HSCs correlates with decreased β1,6-branched N-glycosylation on TβRI and TβRII, suggesting that the inhibitory effects of GnT-V knockdown on HSC activation are mediated by decreasing β1,6 N-glycan branching on TGF-β receptors.

The role of Galectin-3 in hepatic fibrogenesis has been considered to be an immediate early gene upregulated rapidly in response to tissue injury (Chiariotti et al., 2004; Kadrofske et al., 1998). In the experimental models of CCl₄ or TAA-induced liver fibrosis, there was a close relationship between the regulation of GnT-V and Galectin-3. Knockdown of GnT-V efficiently reduces CCl₄- or TAA-induced liver fibrosis and simultaneously downregulates the expression of GnT-V and Galectin-3, suggesting that the roles of GnT-V and Galectin-3 in regulating hepatic fibrogenesis were closely related. Although Galectin-3 has been suggested to bind the N-glycans on cytokine receptors to form a molecular lattice that limits receptor internalization and maintains downstream signaling sensitivity (Partridge et al., 2004), there are still conflicting data on this hypothesis (Guo et al., 2007). Thus, the precise mechanisms, by which GnT-V-modified β1,6 GlcNAc branching and Galectin-3 regulate the intracellular processes independently or synergistically, still remain to be defined.

Gain of motility is an important feature of HSC activation in liver fibrosis, where activated HSCs migrate in response to PDGF and chemokines and accumulate in areas of injury. Cell migration is a complicated process requiring precise regulation and integration of multiple signaling pathways. The ECM serves as the molecular scaffold for cell adhesion and migration, whereas the cell surface proteins including integrins and cadherins modulate cell adhesion to the ECM. Previous studies have demonstrated that the presence of β1, 6-branched complex type N-glycan is positively correlated with increased cancer cell motility and malignancy (Guo et al., 2007; Lau et al., 2004). Additionally, GnT-V participates in regulating cell-matrix and cell-cell adhesion by affecting the N-glycosylation of cell surface proteins including cytokine receptors, integrins, and cadherins (Guo et al., 2002, 2003; Partridge et al., 2004). In this study, we found that knockdown of GnT-V inhibited PDGF-induced HSC-T6 cell migration by inhibiting PDGF/Erk signaling. However, further mechanistic studies are needed to investigate whether variant glycosylation of cell surface proteins, like integrin, contributes to regulating HSC cell motility in liver fibrosis.

EMT has previously been identified as an alternative mechanism for the deposition of ECM in models of renal and pulmonary fibrosis (Kasai et al., 2005; Li et al., 2003). Dooley et al. (2008) reported that hepatocytes undergoing TGF-β-dependent EMT actively participated in fibrogenesis, and ablation of TGF-β signaling by hepatocyte-specific Smad7 overexpression was sufficient to blunt the liver fibrogenesis. A recent study using GnT-V transgenic mouse showed that GnT-V overexpression in keratinocyte promoted EMT and induced the early phase of malignant transformation partly through aberrant glycosylation of EGF receptor and enhanced EGF signaling (Terao et al., 2011). However, the role of GnT-V in hepatocyte EMT has not yet been investigated. Our results demonstrated that TGF-β1 treatment markedly upregulated GnT-V expression in cultured hepatocyte. Although GnT-V expression in hepatocytes of fibrotic liver was obviously lower than control during the first 2 weeks of CCl₄ injection, probably due to the acute injury to hepatocyte, there was a significant upregulation of GnT-V in the hepatocyte after 2 weeks of CCl₄ administration. Considering that TGF-β1 promotes the EMT process in hepatocytes mainly through activating the Smad2/3 pathway (Gordon and Blobé, 2008; Vega et al., 2004), we investigated the effects of GnT-V on phosphorylation of Smad3 and demonstrated that knockdown of GnT-V led to inhibition of the TGF-β1-Smad signaling during TGF-β1-induced EMT in hepatocyte.

In conclusion, we identify the upregulation of GnT-V in the process of CCl₄- or TAA-induced liver fibrosis and demonstrate that knockdown of hepatic GnT-V by adenovirus-delivered siRNA efficiently reduces hepatic fibrogenesis. Our further investigations reveal that GnT-V and GnT-V-modified N-glycan appear to be responsible for modulating HSC activation, proliferation, and migration, as well as for inhibiting TGF-β1-induced EMT in hepatocytes. The hypothetical roles of GnT-V in hepatic fibrogenesis are schematically summarized in Supplementary figure 4. Thus, GnT-V may provide a feasible and promising therapeutic target in preventing liver fibrosis.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES

KNOCKDOWN OF GnT-V PREVENTS HEPATIC FIBROSIS


