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Down-regulation of liver Galβ1, 4GlcNAc α2, 6-sialyltransferase gene by ethanol significantly correlates with alcoholic steatosis in humans

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Abstract

Hepatic steatosis and steatohepatitis are frequent results of long-term ethanol exposure. We have previously demonstrated that long-term ethanol down-regulates Galβ1, 4GlcNAc α2, 6-sialyltransferase (ST6Gal1), leading to defective glycosylation of a number of proteins including apolipoprotein (apo) E and apo J and the appearance of asialoconjugates in the blood of continuously alcohol-fed animals as well as in human alcoholics. In the current study, we have explored the possibility of whether ethanol-induced down-regulation of ST6Gal1 could contribute toward alcoholic steatosis in human alcoholics presumably because of impaired lipid and lipoprotein transport caused by this down-regulation. Real-time quantitative polymerase chain reaction analyses of liver samples from nondrinkers, moderate drinkers, and heavy drinkers as well as from subjects with and without alcoholic liver disease revealed direct evidence that the down-regulation of ST6Gal1 may be due to ethanol per se. The ST6Gal1 messenger RNA level was reduced by as much as 70% in moderate and heavy drinkers as well as in patients with alcoholic liver disease, but was not changed in subjects with liver disease due to causes other than alcohol exposure. Biochemical and histopathologic analysis demonstrated that the liver total cholesterol was increased by more than 30% ($P < .05$) and 75% ($P < .01$), respectively, in moderate and heavy drinkers compared with nondrinkers, with even more dramatic changes in triglyceride levels. Significantly, there was a strong inverse correlation between ST6Gal1 messenger RNA level and liver lipid deposit ($F = 8.68$, $P < .001$) by statistical analysis. Thus, it is suggested that alcohol-mediated down-regulation of hepatic ST6Gal1 gene leads to defective glycosylation of lipid-carrying apolipoproteins such as apo E and apo J, resulting in defective intracellular lipid and lipoprotein transport, which in turn may contribute to alcoholic steatosis.

1. Introduction

Alcoholic steatosis (AS) is a liver disease characterized by hepatic accumulation of lipids (triacylglycerol and cholesterol). Alcoholic steatosis afflicts millions of subjects worldwide and is one of the major causes of death in developed countries [1]. Essentially, AS leads to inflammation, necrosis, fibrosis, and finally cirrhosis that eventually results in death [2]. The pathogenesis of AS is a complex multifactorial process that is manifested through several mechanisms [3] involving a number of fat-metabolizing genes such as the activation of fat-synthesizing genes via sterol regulatory element–binding proteins [4] and the down-regulation of fat-oxidizing genes via peroxisome proliferator–activated receptor α [5]. Apart from these abnormalities, we propose the following mechanism that may contribute to the pathogenesis of AS. Our ongoing studies have shown impairment in the glycosylation [6,7] and trafficking [8] of apolipoprotein (apo) E and apo J, the 2 important lipid-carrying apolipoproteins. As a result, high-density lipoproteins (HDLs) are depleted of apo E, whereas very low-density lipoproteins (VLDLs) are enriched with asialo apo E [9], resulting in their impaired intracellular trafficking. This leads to a profound accumulation of lipids in the liver [10] and impairment in reverse cholesterol transport (RCT) function of

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HDL [11,12]. We have also shown that the RCT function of HDL from human alcoholics is also defective [12].

The key enzyme that is responsible for the terminal sialylation of various glycoproteins is Galβ1, 4GlcNAc α2, 6-sialyltransferase (ST6Gal1), which mediates the addition of α2, 6-linked sialic acid to glycoproteins and glycolipids in the Golgi compartment. In our ongoing studies in both rats and humans, we have shown that ST6Gal1 messenger RNA (mRNA) expression is markedly reduced in the long-term alcohol group compared with the control group [13-15]. We have shown that the concomitant decreased hepatic ST6Gal1 activity is due to its decreased synthetic rate [6], whereas the down-regulation of ST6Gal1 mRNA is due to its decreased stability [14]. Whereas our previous study confirmed the down-regulation of ST6Gal1 as a possible cause for the appearance of asialoconjugates in alcoholics [15], in the present study, we have attempted to evaluate whether impaired expression of ST6Gal1 gene may contribute to AS by correlating ST6Gal1 expression with hepatic lipids and histopathology of lipid deposits in the liver specimens of nondrinkers, moderate drinkers, and heavy drinkers. We provide direct experimental evidence that down-regulation of ST6Gal1 gene is inversely correlated with and could potentially contribute to the onset of AS.

Table 2
Patient profiles for biopsy liver specimens

Patients	Age (y)	Etiology of liver disease	Histology grading
1	51	Hepatitis B	G1, S1
2	53	Hepatitis C	G2, S1
3	52	Hepatitis C	G3, S3
4	51	Hepatitis C	G2, S1
5	46	Hepatitis C	G2, S1
6	56	Hepatitis C	G3, S2
7	60	Granulomatous hepatitis	Could not be scored based on Knodell; histology showed moderate steatosis, noncaseating granulomas
8	69	Hepatitis B	G1, S1
9	51	Hepatitis C/liver transplant	G3, S3
10	23	Nonalcohol steatosis	G1, S0
11	51	Inflammatory bowel disease	G1, S1
12	50	Hepatitis C	G2, S2

Grading and staging of histology are described herein with the Knodell score, which assigns a value to the degree of inflammation (grade; scale of 0 [none] to 4 [maximal]) and a value to the amount of fibrosis (stage; also 0 [low] to 4 [high]).

2. Experimental procedures

2.1. Chemical and reagents

Unless specifically stated, all chemicals and reagents used in this study are the highest grade from Sigma (St Louis, MO).

2.2. Ethical guideline and alcoholic specimen criteria

This research was approved by the institutional review board and the research and development committee of this medical center as well as those of the participating medical center from Australia. An informed consent was obtained from each patient before taking samples. Postmortem human liver specimens (12 samples in each group; all sample identities were kept anonymous) were purchased from Tissue Transformation Technologies (Edison, NJ) according to the following criteria:

1. Non–alcohol-drinking group (ND): less than 1 alcoholic beverage per day (<14 g ethanol per day) in the past 10 years before death.
2. Moderate alcohol drinkers (MD): 1 to 3 alcoholic beverage(s) per day (14-42 g ethanol per day) in the past 10 years before death.
3. Heavy alcohol drinkers (HD): more than 6 alcoholic beverages per day (≥84 g ethanol per day).

Four explant liver samples with advanced alcoholic liver disease (ALD) (end-stage cirrhosis without viral hepatitis or other defined liver disease) (Table 1) and 5 normal subjects (partial donation; controls without liver disease) were also used to study the expression of ST6Gal1. Twelve liver biopsy samples taken from patients with chronic liver disease of nonalcoholic etiology (Table 2) were used as the nonalcoholic liver disease (NALD) group.

Table 1
Patient profiles for explant human liver specimens

Patients	Age (y)	Histopathology
1	57	Micronodular cirrhosis, mild steatosis, focal Mallory hyaline, moderate chronic inflammation in fibrous septa
2	33	Micronodular cirrhosis, diffuse steatosis, bile duct proliferation, scattered Mallory hyaline, florid ductular proliferation, marked parenchymal iron deposition
3	60	Micronodular cirrhosis, minimal steatosis, chronic inflammation in fibrous septa, florid bile duct proliferation, focal piecemeal necrosis, focal hepatocyte dysplasia
4	51	Micro- and macronodular cirrhosis, increased hepatocyte iron staining, mild intracanalicular cholestasis, prominent bile duct proliferation

2.3. Liver total RNA extraction and real-time quantitative reverse transcription–polymerase chain reaction

Total RNA isolation and quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) were performed as described previously [15].

2.4. Liver cholesterol and triglyceride measurements

Extraction of liver lipids was performed according to the method described by Folch et al [16]. Briefly, 1 g of liver from each frozen liver sample was homogenized with 20 mL chloroform-methanol (2:1 by volume). The homogenates were centrifuged at 800*g* for 10 minutes with the Beckman J-6 centrifuge (Beckman Coulter, Fullerton, CA). The supernatants were transferred to a new glass tube and washed with 4 mL 0.25× phosphate-buffered saline twice. Liver cholesterol and triglyceride were assayed in accordance with the manuals supplied with the TECO Diagnostics (Anaheim, CA) kits.

2.5. Histology of liver specimens and lipid deposits measurement

Frozen samples of human liver from the groups labeled *ND*, *MD*, and *HD* were relabeled (primary code) to mask the drinking group of origin and processed for quantitative histochemistry by conventional methods. An orientation set was stained with hematoxylin and eosin using ready-to-use solutions from Surgipath Medical Industries, Richmond, IL. A second set, for histometric analysis of lipid accumulation, was stained with oil red O [17], counterstained with hematoxylin, and coverslipped using Crystal Mount aqueous mounting medium (Biomedia, Foster, CA). Initially, it was thought that the variability in the number, density, and size of the lipid accumulations precluded conventional histometric techniques. Therefore, a nonbiased analysis was conducted as follows. Two representative medium-power (200×) photomicrographs were taken of each sample for viewing and scoring by a panel of 3 investigators, who were blinded to the experimental group each specimen belonged to (experimental set). Essentially, each of these photomicrographs was given a new code (secondary code), so that the scorers could not know which sample *pair* it belonged to. Six additional photomicrographs were taken as examples of the amount and distribution of lipid to be scored as 1 (least) to 5 (most) in increments of 1. The panel then scored an additional set of 12 photomicrographs as a test of the degree to which they were calibrated. As the correlation coefficient (r = 0.91) was excellent, the experimental set then was scored. The secondary code was broken, and the sum of the 6 scores for each sample divided by 6 was considered the best estimate of the amount and distribution of lipid in that sample. The primary code then was broken for analysis of the data belonging to different drinking groups. Image-Pro Plus 6.1 (Media Cybernetics, Bethesda, MD), an automated histometric system, was then applied to the experimental set of digital photomicrographs adjusted to the same optimal condition throughout all samples.

2.6. Statistical analyses

Several statistical methods were used to examine relationships between alcohol history, gene expression, and liver lipid deposition. To examine the univariate relationship between liver lipid deposition and alcohol intake history, we first examined the strength of the linear relationship using the Pearson correlation with each variable coded as ordinal. We then examined mean fat deposit scores and their confidence intervals for each level of alcohol intake history. A general linear model (Proc GLM in SAS [Cary, NC] version 9.1) was used to test the significance of relationships between alcohol histories, various measures of gene expression, and lipid deposition. This is equivalent to an analysis of variance when a single categorical independent variable is used or to multiple regressions when several predictors are combined in a single model. Univariate relationships between drinking history and ST6Gal1 mRNA expression were examined using GLM with a categorical drinking history independent variable (with 3 levels: none, moderate, heavy). To examine the combined impact of ST6Gal1 mRNA expression and drinking history on liver lipid deposition, we tested a model with both a categorical drinking history variable and a continuous measure of relative ST6Gal1 mRNA expression as predictors. Again, we did this 3 times using mRNA and drinking history. Statistical significance was also determined by 1-way analysis of variance test followed by the Tukey test (SAS Software).

3. Results

3.1. Ethanol per se, not other liver pathologic conditions, mediates the down-regulation of ST6Gal1 gene

Our previous study [15] showed that ST6Gal1 mRNA was significantly down-regulated in liver samples from long-term alcoholics without liver disease. To rule out that other liver pathologic conditions may also affect the expression of ST6Gal1, we repeated our real-time quantitative RT-PCR analyses on liver samples from nondrinkers, moderate

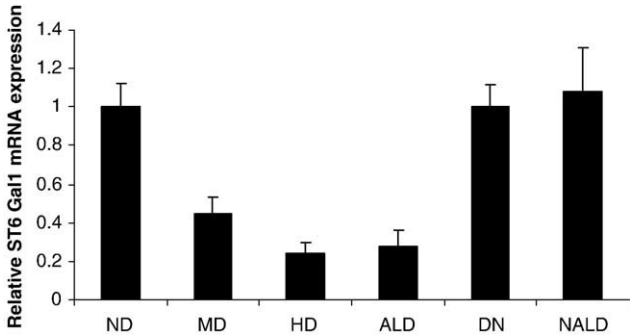


Fig. 1. Real-time RT- PCR analyses of ST6Gal1 mRNA in human liver specimens in the study groups. Total RNA from the ND group (n = 12), the MD group (n = 12), the HD group (n = 12), the end-stage ALD group (n = 4), the normal liver donors (n = 5), and the NALD group (n = 12) was reverse transcribed and used in the real-time PCR. The RNA levels were normalized to the level of β-actin. Each sample analysis was performed in triplicate independently; and each bar graph represents the mean ± SEM of 12 samples in each group except for the ALD and DN groups, in which bar graph represents the mean ± SEM of 4 and 5 samples, respectively. DN indicates normal liver donors.

drinkers, and heavy drinkers together with liver explant samples from alcoholic liver disease subjects as well as liver samples from hepatitis subjects without alcohol exposure (NALD group). The results are shown in Fig. 1. Again, compared with the nonalcohol group, ST6Gal1 mRNA level was decreased on average by 50% ($P < .01$) and by 70% ($P < .01$) in moderate– and heavy–alcohol-drinking groups, respectively, just as we reported previously [15]. Similar results were also found in liver explants from advanced ALD patients compared with normal subjects (ND). The ST6Gal1 mRNA level was decreased by as much as 65% ($P < .01$) in end-stage alcoholic liver disease subjects, whereas ST6Gal1 mRNA level was not changed or slightly increased in the NALD, compared with normal controls. These results indicate that ethanol per se may be the major cause for the down-regulation of ST6Gal1 gene in alcoholics.

3.2. Liver lipid deposition directly correlates with the amount of alcohol consumed

Liver cholesterol and triglyceride were measured separately using kits from TECO Diagnostics designed specifically for each lipid form. The results are shown in Fig. 2. Liver total cholesterol level was increased by more than 30% ($P < .05$) and 75% ($P < .01$) in the MD and HD groups, respectively, compared with the ND group (Fig. 2A).

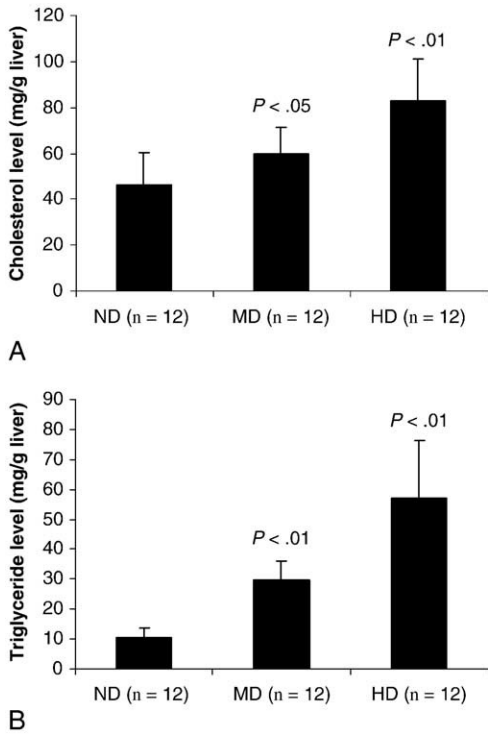


Fig. 2. Liver cholesterol and triglyceride levels in the study groups. Aliquots of each liver lipid extract were analyzed for cholesterol and triglycerides as described in the methods section. Each sample analysis was performed in duplicate and repeated at least 3 times on different dates. The concentration for cholesterol and triglyceride is expressed as milligram per gram of liver. Bar graph represents the mean ± SEM of 12 specimens in each group.

Triglyceride was increased by more than 100% ($P < .01$) in the MD group and by more than 300% ($P < .01$) in the HD group compared with the ND group (Fig. 2B).

3.3. Alcohol consumption correlates positively with hepatic steatosis

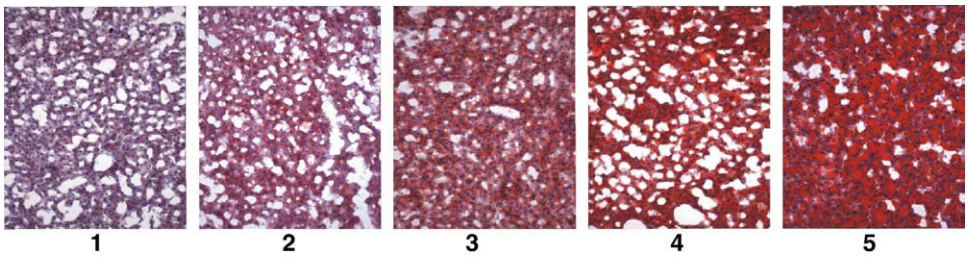
To investigate how alcohol consumption affects liver pathologically, we performed histopathology of liver tissues from the ND (n = 9), MD (n = 6), and HD (n = 9) groups. In hematoxylin and eosin–stained sections, discrete, blank vacuoles were uncommon in the livers from the ND and MD groups and prominent in the livers of the HD group (data not shown). In oil red O–stained liver sections, bright red–stained lipid droplets were scored as described in the methods section. One calibrator image for every score is shown in Fig. 3A. The number of cases put into each score as determined by the blinded scoring panel in the ND, MD, and HD groups is listed in Fig. 3B. As the amount of alcohol consumption increased, there was a clear shifting from lower score scale to higher score scale. The mean density of lipid accumulations determined with the Image-Pro Plus version 6.1 software on the digital photomicrographs of the various experimental groups is shown in Fig. 3C. There was greater than 70% more lipid deposition in the drinking groups (MD + HD combined) compared with the ND group.

3.4. Strong multivariate relationships exist between alcohol-drinking history, ST6Gal1 gene expression, and liver lipid deposits

There was a strong relationship between alcohol-drinking history, ST6Gal1 mRNA expression, and liver lipid score ($F = 8.68$, $P < .001$). Alcohol-drinking history and ST6Gal1 mRNA expression together accounted for 56.6% of the lipid score variance, and both predictors made a significant correlation to prediction accuracy (for ST6Gal1, $P < .001$; for drinking history, $P < .01$) in a multivariate model. When both alcohol history and lipid deposit score were coded as ordinal, the Pearson correlation was 0.35 ($P = .09$). However, by examining the 95% confidence intervals around the mean liver lipid deposit scores for each of the 3 levels of drinking history, we found that subjects with a history of heavy or moderate drinking had significantly more lipid deposits (mean = 3.11, confidence interval = 2.26-3.96 and 2.11-3.89 for heavy and moderate drinkers, respectively) than subjects with no drinking (mean = 2.11, confidence interval = 1.26-2.96, $P < .05$). The univariate relationship between ST6Gal1 mRNA expression and drinking history was very strong and significant ($F = 337.09$, $P < .0001$, $R^2 = .97$). Mean expression levels by drinking history are shown in Fig. 4.

4. Discussion

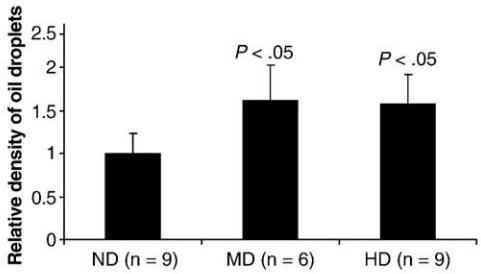
As part of our continuous efforts to better understand how ethanol affects human liver pathologically, we have previously reported that long-term ethanol causes down-regulation of



A

	Number of Cases in Each Score		
Score	ND	MD	HD
1	4	0	1
2	2	2	2
3	1	3	2
4	2	0	3
5	0	1	1
	n = 9	n = 6	n = 9

B



C

Fig. 3. Histopathology of human liver specimens and lipid deposits in the study groups. Cryosections of tissues were prepared as described in the materials and methods section. Sections were stained with oil red O and counterstained with hematoxylin. All ca ×120; bar = ca 100 μm. A, Representative photomicrographs from the standard set used to calibrate scoring of lipid accumulation. Numbers below each image frame represent the score for that frame, with 1 indicating the least and 5 indicating the most lipid accumulation. B, Number of samples from each group that fits into each score according to the standard in Fig. 3A as determined subjectively by the scoring panel. C, Quantification of oil droplets in oil red O–stained sections as determined by the Image-Pro Plus version 6.1 method. Bar graph represents the mean ± SEM of 9 specimens from the ND group, 6 specimens from the MD group, and 9 specimens from the HD group, with the ND group set at 1 for convenience.

ST6Gal1 gene [15]. In the current expanded study, we have not only confirmed our previous findings, but also have provided a direct evidence that the down-regulation of ST6Gal1 is most likely due to long-term ethanol exposure, but not due to pathologic conditions (Fig. 1).

Liver sections stained with oil red O from the various experimental groups clearly show that even moderate drinking of alcohol causes fat deposits in the liver that exacerbate after heavy drinking compared with the nondrinking group (Figs. 2 and 3). It must be pointed out that the samples belonging to metabolic and genetic obesity were excluded from the study analyses. Both histometric methods demonstrated much more lipid accumulation in the livers of the drinking groups than in the nondrinking groups and thus histologically (Fig. 3) support our biochemical findings (Fig. 2). These results, together with molecular biology evidence (Fig. 1), imply that the steatosis found in the drinking groups compared with the nondrinking groups may be more specific to ethanol consumption. Our results are consistent with other reports that ST6Gal1 gene expression is unaltered or up-regulated in nonalcoholic or neoplastic liver diseases [18-20].

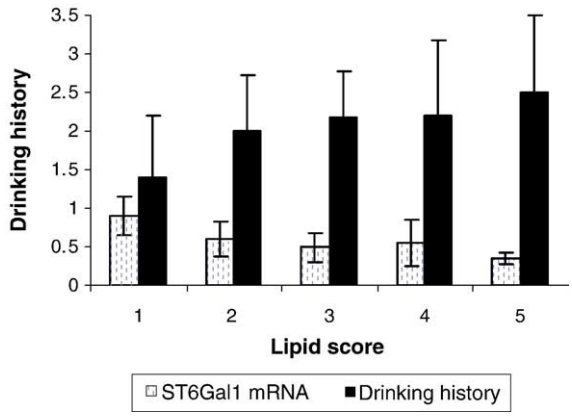


Fig. 4. Multivariate analyses of ST6Gal1 gene expression, drinking history, and liver lipid deposit in the study groups. The relative ST6Gal1 mRNA expression from all samples in the ND (n = 12), MD (n = 12), and HD (n = 12) groups was plotted together with drinking history against liver lipid score. Drinking history was coded as heavy = 3, moderate = 2, and none = 1 to obtain a mean. Error bars represent the 95% confidence interval around the mean.

When drinking history, ST6Gal1 mRNA level, and liver lipid deposit are all considered in the multivariate statistical analysis, there is a strong correlation among drinking history, ST6Gal1 gene expression, and liver lipid deposit (Fig. 4).

The functional and clinical implications of alcohol-mediated down-regulation of ST6Gal1 gene are multifarious. First, based on our findings that the sialylated apo E preferentially associates with HDL, whereas desialylated apo E associates with VLDL [9], with concomitant reciprocal changes in plasma apo E concentration in HDL and VLDL both in alcohol-fed rats [10,11] and in human alcoholics [12], we suggest the following possible action of alcohol in the onset of steatosis: In normal individuals (normolipidemic state), the lipid-deficient apo E is a good substrate for ST6Gal1 in the Golgi compartment, leading to its preferential association with HDL. On the contrary, in alcoholic subjects (hyperlipidemic

state), the lipid-rich apo E becomes a poor substrate for ST6Gal1, resulting in the sialic acid–deficient apo E that has more affinity for VLDL, which accumulates because of its impaired trafficking in the liver [8]. Second, the decreased level of apo E in HDL leads to its defective role in RCT function [11,12]. Thus, we suggest that ethanol-mediated alteration in the glycosylation of apo E alters its association with VLDL and HDL and thereby affects their lipid-carrying function and trafficking, leading to the development of AS.

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