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Reactive oxygen species modify oligosaccharides of glycoproteins in vivo: A study of a spontaneous acute hepatitis model rat (LEC rat) ☆

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Abstract

The Long–Evans Cinnamon (LEC) rat, an animal model of Wilson's disease, spontaneously develops hepatitis as the result of abnormal copper accumulation in liver. The findings of this study show that copper, hydrogen peroxide, and lipid peroxides accumulate to drastically high levels in LEC rat serum in acute hepatitis but not chronic hepatitis. The effect of these reactive oxygen species (ROS) on oligosaccharides of glycoproteins in the LEC rat serum was examined. Lectin blot and lectin ELISA analyses showed that sialic acid and galactose residues of serum glycoproteins including transferrin were decreased in acute hepatitis. Further analyses of oligosaccharide structures of transferrin demonstrated that di-sialylated and asialo-agalacto biantennary sugar chains, but not tri-sialylated sugar chain, exist on transferrin in the acute hepatitis rats. In addition, treatment of non-hepatitis rat serum with copper ions and hydrogen peroxide decreased tri-sialylated sugar chain of the normal transferrin and increased di-sialylated and asialo-agalacto biantennary sugar chains. This is the first evidence to show that ROS result in the cleavage of oligosaccharides of glycoproteins in vivo, and indicate this cleavage of oligosaccharides may contribute the development of acute hepatitis.

Keywords: Acute hepatitis; Glycoprotein; LEC rat; Oligosaccharide; Reactive oxygen species; Transferrin

The Long–Evans Cinnamon (LEC) rats, established from a closed colony of Long–Evans rats as a mutant strain displaying hereditary hepatitis accompanied by severe jaundice at about 3–4 months after birth, develop hepatic cancer at about one year after birth. Wu et al. [1] cloned cDNAs for the rat gene (*atp7b*) homologous to the human Wilson disease (WD) gene and have found that at least 900 bp of the coding region at the 3′ end, including the critical ATP binding domain, had been deleted in the LEC rat. Similar to WD, LEC rats exhibit defective incorporation of copper into the ceruloplasmin, failure in excretion of copper into bile, and, consequently, elevated hepatic copper levels [2]. Copper and hydrogen peroxide (H_2O_2) can readily generate toxic free radicals, hydroxyl radicals, via Fenton and Harbor-Weiss chemistry [3]. In fact, an accelerated generation of hydroxyl radicals was detected in LEC rats with acute hepatitis due to the accumulation of copper [4]. Li et al. [5] proposed that the abnormal accumulation of copper in the LEC rat results in the development of hepatitis, hepatic fibrosis, and subsequent hepatocarcinogenesis. In fact, D-penicillamine, a copper-chelating agent, which is used in the treatment of WD patients, has been reported to prevent the development of acute hepatitis in LEC rats [6]. Moreover, the activities of

☆ *Abbreviations:* ALT, alanine aminotransferase; AST, aspartate aminotransferase; LEC, Long–Evans cinnamon; LPO, lipid peroxide; ROS, reactive oxygen species.

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antioxidant enzymes such as Cu, Zn-superoxide dismutase, glutathione peroxidase, and catalase were markedly decreased in LEC rat liver with hepatitis, leading to enhanced levels of reactive oxygen species (ROS) [7–9]. Therefore, copper accumulation and the resulting free radical formation appear to be responsible for the acute hepatitis and hepatic carcinogenesis in LEC rats.

Glycoproteins play an important role in pathological and physiological conditions and the oligosaccharide moiety is responsible for many of their functions. For example, the *N*-linked sugar chain in human epidermal growth factor receptor controls receptor activation [10]. De-*N*-glycosylation of both the α and β subunits of the α5β1 integrin receptor induces a loss of fibronectin binding activity [11]. Moreover, several reports have shown that transferrin, a major glycoprotein in blood that is responsible for iron transport, is desialylated in alcoholic hepatitis patients [12–15]. The desialylation of transferrin resulted in a substantial increase in iron uptake by liver because the number of exposed galactose residues, which are required for iron uptake by the liver, are increased on the asialotransferrin [16]. When transferrin was deglycosylated by *N*-glycosidase F, the uptake of iron by the liver was decreased due to the loss of the exposed galactose residues [17].

We previously demonstrated that the glycosidic linkages of GlcNAc residues of *N*-linked oligosaccharides are degraded by treatment with ROS generated by copper ions and H_2O_2 [18]. We also found that the treatment of endothelial cells with ROS accelerates adhesion of neutrophils or colon cancer cells without any de novo synthesis of adhesion molecules, such as P-selectin, E-selectin, and ICAM-1, suggesting that ROS modify the endothelium glycocalyx, a network of charge-bearing glycoproteins and glycosaminoglycans that cover the luminal surface of endothelium cells, and cause this enhancement of adhesion [19]. It has also been reported that ischemia-reperfusion causes disruption of the endothelial glycocalyx [20–22] and this damage is prevented by the addition of superoxide dismutase [20]. Furthermore, ROS degrade glycosaminoglycans in the extracellular matrices, thus causing a subsequent impairment in proteoglycan functions [23,24]. These reports suggest that oligosaccharides are an important biological target for ROS attack, and that the degradation of oligosaccharides of glycoproteins alters their biological functions.

Thus, we investigated the alteration of oligosaccharides of serum glycoproteins, including transferrin, in LEC rats during the development of hepatitis, and examined the issue of whether ROS cause an alteration in these oligosaccharides. Our results suggest that ROS generated in acute hepatitis accelerate the cleavage of oligosaccharide structures of serum glycoproteins and that this modification may be implicated in the development of hepatitis in LEC rat.

Experimental procedures

Animals. LEC rats were purchased from Charles River Japan, Inc. and maintained under an artificial 12:12-h light/dark cycle as well as at a constant temperature of 20 °C. The animals had free access to food and tap water. D-penicillamine-supplemented rats had free access to 0.1% D-penicillamine (Wako Pure Chemical Industries, Ltd.) dissolved in water instead of ordinary tap water. All experiments were approved by the Institutional Animal Research Committee in Hyogo College of Medicine.

Serum preparation. Blood was obtained from LEC rats at 10–50 weeks of age by cervical vein puncture. Sera, obtained by centrifugation at 5000*g* for 20 min, were stored at −80 °C until used.

Measurement of AST, ALT, and total bilirubin. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in LEC rat serum were assayed using NescautoVL AST kit and NescautoVL ALT kit (Alfresa Phama), respectively, and total bilirubin was measured using Clinimate BIL-2 kit (Daiichi Pure Chemicals), according to the manufacturer's instructions.

Electrophoresis. SDS–PAGE was carried out according to Laemmli [25]. Twenty microgram of serum proteins was separated on 10% polyacrylamide gel under reducing conditions. Coomassie brilliant blue (CBB) R-250 was used to visualize the protein bands.

Lectin blot analysis. Following SDS–PAGE, proteins were transferred electrophoretically onto a nitrocellulose membrane (Amersham Bioscience). After blocking with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), the membrane was incubated with 1 μg/ml biotinylated sambucus sieboldiana (SSA), ricinus communis (RCA) or canavalia ensiformis (ConA) for 1 h at room temperature. After washing with PBS containing 0.05% Tween 20 (PBS-T), the blots were incubated with a horseradish peroxidase–avidin complex (Vector) and developed using an ECL detection system (Amersham Bioscience).

Lectin ELISA for rat serum transferrin. A 96-well microtiter plate was coated with 50 μl of rabbit anti-rat transferrin antibody (1 μg/ml in 50 mM sodium carbonate buffer, pH 9.6, Inter-Cell Technologies) overnight at 4 °C. After washing with PBS-T, wells were blocked with 1% BSA in PBS for 1 h at room temperature. Rat sera were diluted 1000-fold with PBS for the SSA and ConA analyses, and 100-fold for the RCA analysis then added to the wells followed by incubation for 2 h at room temperature. After washing with PBS-T three times, 50 μl of biotinylated lectin solution was added to the wells, and then incubated for 45 min at room temperature. After washing, coloring reaction was carried out using horseradish peroxidase–avidin complex and *o*-phenylenediamine. The reaction was terminated by 1 N HCl, and the absorbance was measured at 490 nm using SPECTRAmax PLUS^{384} spectrophotometer (Molecular Devices).

Determination of copper, H_2O_2, and LPO concentrations in LEC rat serum. The copper concentration in LEC rat serum was analyzed by atomic absorption spectrophotometry (HITACHI Z-5000). The concentrations of H_2O_2 and lipid peroxides (LPO) were determined using PeroXOquant Quantitive Peroxide Assay Kits (Pierce Biotechnology) and determiner LPO (Kyowa Medex), respectively, according to a manufacturer's instruction.

ROS treatment. Serum was diluted with PBS for the ELISA analysis using SSA (1000-fold), RCA (100-fold), and ConA (1000-fold). These serum samples from 12-week-old LEC rats were incubated with 40 μM copper sulfate and/or 100 μM H_2O_2 in the presence or absence of 100 μg/ml catalase for 1 h at room temperature. The reaction was terminated by the addition of catalase. Purified transferrin from D-penicillamine-supplemented rat (26-week-old) was treated with 40 μM copper sulfate and 100 μM H_2O_2 at 37 °C for 2 h.

Glycosidase digestion. For *N*-glycosidase F digestion, transferrin was incubated in 50 mM sodium phosphate buffer (pH 7.5) containing 0.75% Triton X-100, 0.1% SDS, 50 mM 2-mercaptoethanol, and 1 U *N*-glycosidase F (*Chryseobacterium meningosepticum*, Calbiochem). Sialidase digestion was carried out with 0.1 U of sialidase (*Arthrobacter ureafaclens*, Nacalai Tesque) in 50 mM sodium acetate buffer (pH 5.0). β-galactosidase digestion was carried out with 0.1 U β-galactosidase (*Escherichia coli*, TOYOBO) in 0.1 M phosphate buffer (pH 7.3) containing 1 mM $MgCl_2$. All glycosidase digestions of oligosaccharides were performed at 37 °C for 24 h.

Preparation of PA-oligosaccharides from serum transferrin. Transferrin in LEC rat serum was purified by affinity chromatography using anti-rat

transferrin IgG (Inter-cell Technologies, Inc.)-coupled HiTrap NHS column (Amersham Biosciences). The purified transferrin was desalted through PD10 column (Amersham Biosciences) and lyophilized. Oligosaccharides released from the transferrin by *N*-glycosidase F digestion were purified by cellulose cartridge column (TaKaRa) and labeled by fluorescent reagent, 2-aminopyridine (PA) [26]. The PA-oligosaccharides were further purified by a cation exchange chromatography using Dowex 50W-X8.

HPLC analysis of PA-oligosaccharides. Reversed-phase HPLC was performed using a ODS column (TSK-gel ODS-80TM, 4.6 × 150 mm) at 55 °C. PA-oligosaccharides were eluted with 20 mM ammonium acetate buffer (pH 4.0) containing 0.05% 1-butanol at a flow rate of 1 ml/min. Ion exchange chromatography was performed by MonoQ column (Mono Q HR5/5, Amersham Pharmacia) equilibrated by 10% acetonitrile (pH 9.5) adjusted by triethylamine. PA-oligosaccharides were eluted with a linear gradient of acetic acid (0–0.6%, pH 7.3), as described by Nakagawa et al. [27]. PA-oligosaccharides were detected by their fluorescence at excitation and emission wavelengths of 320 and 400 nm, respectively.

MALDI-TOF MS analysis. PA-oligosaccharide was mixed with equal volume of 2,5-dihydroxybenzoic acid (10 mg/ml) containing 30% acetonitrile and 0.1% trifluoroacetic acid, and subjected to matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) (Voyager-DE STR Biospectrometry Workstation, Applied Biosystems). The mass spectra were observed in the reflection mode under a 20-kV accelerating voltage with positive detection.

Results

We first assessed the liver condition in LEC rats by analyzing AST/ALT activities and total bilirubin in the serum. As shown in Table 1, AST, but not ALT, activity began to increase at 19–21 weeks of age, and both activities were then drastically increased at 26–31 weeks of age. Total bilirubin was also significantly increased at 26–31 weeks of age. AST and ALT activities, and total bilirubin were decreased at 44–50 weeks of age, although the values were higher than those at 10–14 weeks of age. These data indicate that LEC rats develop a serious hepatic dysfunction with jaundice at 26–31 weeks of age and the rats surviving acute hepatitis were under chronic hepatitis conditions (44–50 weeks). Therefore, we classified the rats into four groups of liver injury; a non-hepatitis group (NH, 10–14 weeks), a pre-hepatitis group (PH, 19–21 weeks), an acute hepatitis group (AH, 26–31 weeks), and a chronic hepatitis group (CH, 44–50 weeks). The serum concentrations of copper, H_2O_2 and LPO were also significantly increased in the AH group, and then decreased in the CH group, suggesting that ROS were generated in LEC rat serum during acute hepatitis but not chronic hepatitis.

We next analyzed the oligosaccharide structures of the serum glycoproteins in LEC rats at different stages of hepatitis. The lectin blot analysis showed that the binding of SSA (preferentially binds to α2,6 linked sialic acid) and RCA (preferentially binds to Galβ1,4GlcNAc) to proteins were substantially decreased in serum from the AH group rats (Figs. 1A and B), even though no remarkable alterations in the concentrations of any of the proteins were observed among the groups except for slight decrease in the proteins around 180–220 kDa in the AH group (Fig. 1D). These results suggest that sialic acid and galactose residues had been cleaved from the sugar chains of serum glycoproteins in the AH group. The binding of ConA, which preferentially binds to α-D-mannose, to 180–220 kDa proteins was decreased in the AH group, while the binding to other proteins remained unchanged (Fig. 1C).

Table 1
Biochemical parameters for LEC rat serum

	10–14 weeks (non-hepatitis)	19–21 weeks (pre-hepatitis)	26–31 weeks (acute hepatitis)	44–50 weeks (chronic hepatitis)
AST (IU/L)	115 ± 10.2	160.4 ± 37.2*2	366.7 ± 63.8*1	175.3 ± 39.5*2
ALT (IU/L)	64.8 ± 5.7	77.2 ± 13.1	442.0 ± 54.9*1	167.7 ± 74*2
Bilirubin (mg/dl)	0.06 ± 0.009	0.06 ± 0.009	0.16 ± 0.08*2	0.08 ± 0.02
Copper (μg/ml)	0.04 ± 0.005	0.07 ± 0.007*1	1.67 ± 0.21*1	0.26 ± 0.05*1
H_2O_2 (μM)	5.22 ± 2.2	3.41 ± 1.4	31.9 ± 3.9*1	3.75 ± 1.14
LPO (nmol/ml)	2.40 ± 0.52	1.79 ± 0.86	12.7 ± 3.1*2	1.05 ± 0.43

Data represent mean ± SD of three experiments.
*1 $p < 0.01$, compared with the values in non-hepatitis group.
*2 $p < 0.05$, compared with the values in non-hepatitis group.

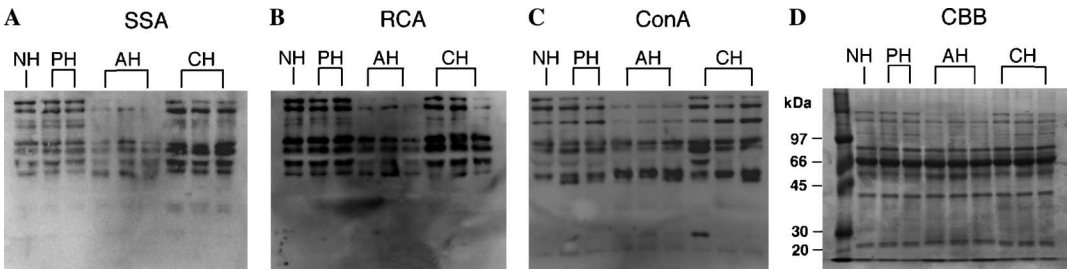

Fig. 1. Lectin blot analysis of glycoproteins in LEC rat serum. The LEC rat serum proteins were separated by electrophoresis on a 10% SDS–polyacrylamide gel and analyzed by lectin blotting using SSA (A), RCA (B), and ConA (C). CBB staining of the separated proteins is shown in (D). NH, non-hepatitis rat; PH, pre-hepatitis rat; AH, acute hepatitis rat; CH, chronic hepatitis rat.

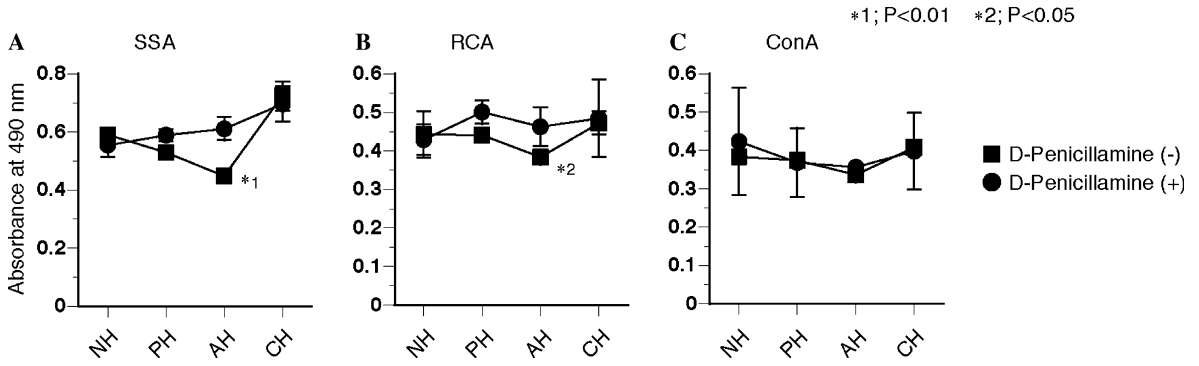

Fig. 2. Analysis of oligosaccharide structure of transferrin by ELISA using lectins. The oligosaccharide structures of transferrin were analyzed by lectin ELISA using, SSA (A), RCA (B), and ConA (C). The closed circles and closed squares in each panel indicate samples from D-penicillamine-supplemented and non-supplemented LEC rats, respectively. NH, non-hepatitis rat; PH, pre-hepatitis rat; AH, acute hepatitis rat; CH, chronic hepatitis rat. Details of the experimental conditions are described under Experimental procedures. Each value represents the mean ± SD of three experiments. $^{*1}p < 0.01$, $^{*2}p < 0.05$, compared with the corresponding group (D-penicillamine-supplemented LEC rats).

To confirm that the alteration of oligosaccharides of the glycoproteins (Fig. 1) is caused by acute hepatitis, the oligosaccharides structure of transferrin was analyzed in D-penicillamine-supplemented and non-supplemented LEC rats. An oral supplement of 0.1% D-penicillamine suppressed the accumulation of copper in the serum, and completely prevented the development of hepatitis in LEC rats at 26–31 weeks of age (AST; 101 ± 9.8 IU/L, ALT; 63 ± 4.2 IU/L, copper; 0.03 ± 0.002 μg/ml). Among the serum glycoproteins, transferrin was used to study the oligosaccharides structure in acute hepatitis, because it is known that the transferrin contains biantennary complex type *N*-glycan. As shown in Fig. 2, the binding of SSA and RCA, but not ConA, to serum transferrin was decreased only in the AH group, despite no difference in the concentrations of transferrin between age-matched D-penicillamine-supplemented and non-supplemented LEC rats (data not shown). The binding of SSA and RCA was restored in chronic hepatitis rats to the level of D-penicillamine-supplemented rats.

To further demonstrate that ROS directly affect the oligosaccharide structure of serum glycoproteins, a lectin ELISA for transferrin was performed after treatment of serum from NH group rats with H_2O_2 and copper. This ROS treatment decreased the binding of SSA and RCA to transferrin, and catalase restored the binding (Figs. 3A

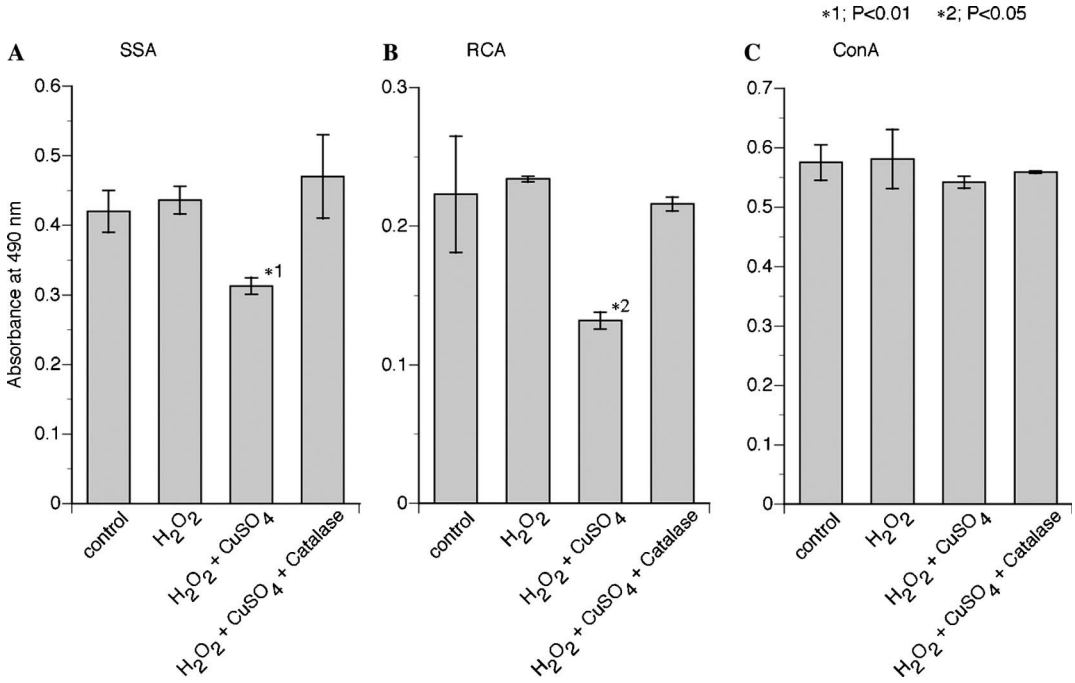

Fig. 3. Effect of ROS on the oligosaccharide structure of transferrin. LEC rat serum obtained from NH group was treated with copper and H_2O_2 in the presence or absence of 100 μg/ml catalase at 37 °C for 1 h. The reaction was terminated by the addition of 100 μg/ml catalase. After the treatment, samples were analyzed by ELISA using (A) SSA, (B) RCA, and (C) ConA as described in Experimental procedures. Each value represents means ± SD of three experiments. $^{*1}p < 0.01$, $^{*2}p < 0.05$, compared with control group.

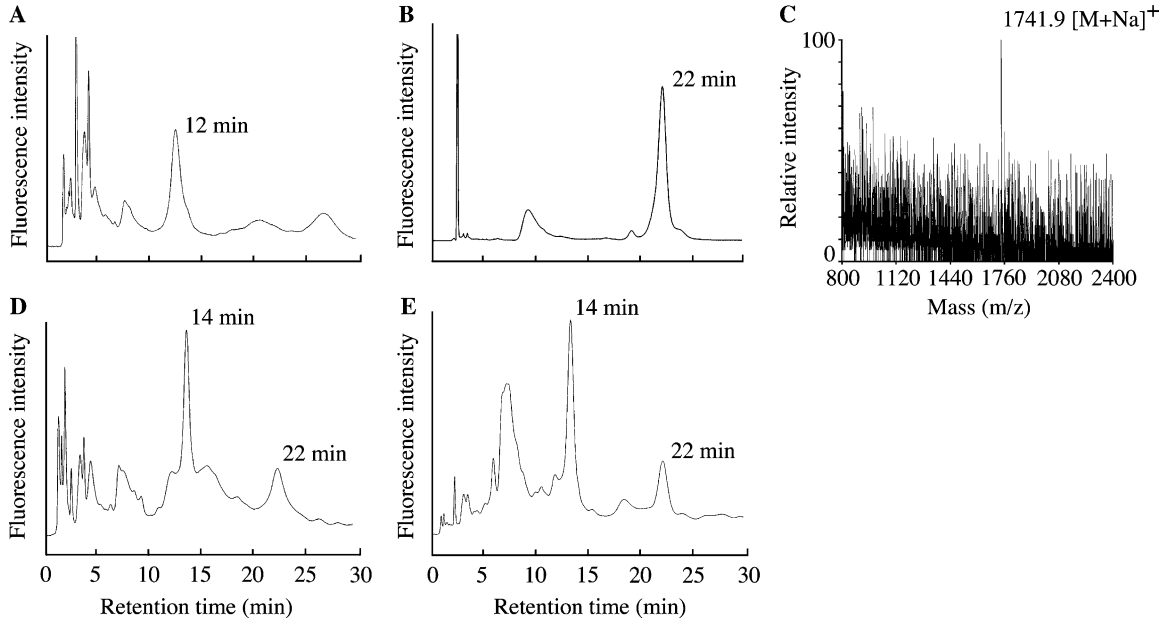

Fig. 4. Reversed-phase HPLC profiles and mass spectrum of oligosaccharides of transferrin. Oligosaccharides were released from transferrin by *N*-glycosidase F, labeled with 2-aminopyridine and then subjected to reversed-phase HPLC. (A) Elution profile of oligosaccharides of transferrin in non-hepatitis rat. (B) The oligosaccharide eluted at 12 min (A) was digested with sialidase and β-galactosidase and then analyzed by reversed-phase HPLC. (C) The oligosaccharide eluted at 12 min (A) was digested with sialidase and then analyzed by MALDI-TOF MS. (D) Elution profile of oligosaccharides of transferrin in acute-hepatitis rats. (E) Elution profile of oligosaccharides of non-hepatitis rat transferrin after treatment with H_2O_2 and $CuSO_4$.

and B), although a decrease in lectin binding was not observed by incubation with H_2O_2 alone. These data indicate that the cleavage of oligosaccharides requires hydroxyl radicals generated by Fenton chemistry. On the other hand, the binding of ConA was not affected by ROS treatment (Fig. 3C), suggesting that the mannose residues in oligosaccharide are stable, as was observed in the lectin blot (Fig. 1C) and the lectin ELISA (Fig. 2C).

We finally identified the oligosaccharides structure of serum transferrin in LEC rat in order to elucidate what alteration in the structure is caused by ROS in acute hepatitis. We first prepared PA-oligosaccharide from transferrin in D-penicillamine-supplemented LEC rat (26 weeks old) as a control oligosaccharide. As shown in Fig. 4A, the PA-oligosaccharide was eluted from reversed-phase HPLC as a main peak at the retention time of 12 min (oligosaccharide A). Analyses using a MonoQ Sepharose column chromatography clarified that the oligosaccharide A has three sialic acids (data not shown, 27). This oligosaccharide A was not digested with either β-galactosidase or *N*-acetylhexosaminidase alone (data not shown), suggesting that sialic acids locate at all the termini. Therefore, we supposed the structure of oligosaccharide A from rat transferrin to be a tri-sialylated sugar chain as shown in Fig. 5. Thus, the oligosaccharide A was digested with β-galactosidase after sialidase treatment. The digested oligosaccharide was eluted from reversed-phase HPLC at 22 min as one peak (oligosaccharide C, Fig. 4B). The structure of the oligosaccharide C was identified to be a GlcNAcβ1,2Manα1,6(GlcNAcβ1,2Manα1,3)Manβ1, 4GlcNAcβ1,4GlcNAc-PA (Fig. 5) by comparison with a standard PA-oligosaccharide. In addition, the mass of oligosaccharide A was measured by MALDI-TOF MS after digestion with sialidase. The *m*/*z* value of $(M+Na)^+$ was 1741.9 corresponding to that of asialo-biantennary oligosaccharide (Galβ1,3/β1,4GlcNAcβ1,2Manα1,6(Galβ1, 3GlcNAcβ1,2Manα1,3) Manβ1, 4GlcNAcβ1,4GlcNAc-PA, theoretical mass: 1741.3, Fig. 4C). From these results,

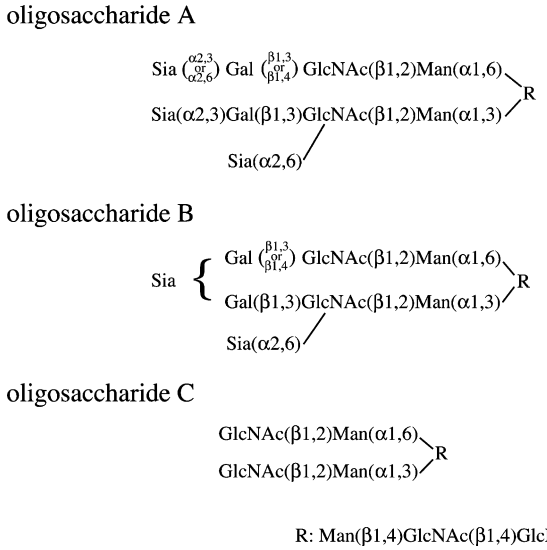

Fig. 5. Structures of oligosaccharide of transferrin. Structures of the oligosaccharides eluted from reversed-phase HPLC at 12 min (oligosaccharide A), 14 min (oligosaccharide B), and 22 min (oligosaccharide C) are presented. Brace in oligosaccharide B indicates that one sialic acid links to either of two β-galactose residues.

the structure of oligosaccharide A is determined to be a tri-sialylated oligosaccharide as shown in Fig. 5. Next, the PA-oligosaccharides from transferrin in acute hepatitis rat (27-week-old) were eluted from reversed-phase HPLC at 14 min (oligosaccharide B) and 22 min (oligosaccharide C) (Fig. 4D). A series of structure analyses as described above identified the oligosaccharide B and oligosaccharide C with di-sialylated and asialo-agalacto oligosaccharides, respectively (Fig. 5). In addition, the di-sialylated oligosaccharide (oligosaccharide B), major oligosaccharide of the transferrin in acute hepatitis rat, was digested with β-galactosidase alone (data not shown). This fact also supports that one of the sialic acids linked to two β-galactose residues is lacked and the β-galactose residue is bared (Fig. 5). To elucidate whether ROS release sialic acids and galactose residues from the tri-sialylated oligosaccharide, the normal transferrin obtained from D-penicillamine-supplemented rat (26 weeks old) was treated with 100 μM H_2O_2 and 40 μM $CuSO_4$ at 37 °C for 2 h, and then the oligosaccharides were analyzed. As shown in Fig. 4E, the tri-sialylated oligosaccharide (peak at 12 min) disappeared, and both di-sialylated oligosaccharide (peak at 14 min) and asialo-agalacto oligosaccharide (peak at 22 min) appeared, as observed in the case of acute hepatitis (Fig. 4D). These results demonstrate that ROS generated in acute hepatitis release sialic acids and galactose residues from tri-sialylated oligosaccharide of transferrin.

Discussion

The oligosaccharide structures of serum glycoproteins were investigated in the LEC rat. Lectin blot analyses clearly showed that the glycoproteins containing sialic acid and galactose are decreased in acute hepatitis (Fig. 1). To further examine this, we employed transferrin as a model glycoprotein, because it is a major serum glycoprotein that is synthesized by the liver, and contains a well characterized biantennary complex type *N*-glycan. Lectin ELISA showed that the transferrin containing sialic acid and galactose is also decreased in acute hepatitis (Fig. 2). LEC rat serum in acute hepatitis contains high concentrations of copper, H_2O_2, and LPO (Table 1). Thus, normal LEC rat serum was treated with a combination of copper ion and H_2O_2. This ROS treatment also decreased the transferrin containing sialic acid and galactose (Fig. 3). Further analyses of the oligosaccharides structure of transferrin demonstrated that ROS generated in acute hepatitis cause the release of sialic acids and galactose residues from the tri-sialylated sugar chain of transferrin (Figs. 4 and 5). These results indicate that ROS generated in acute hepatitis cleave and modify the oligosaccharide structure of various serum glycoproteins.

Several reports have indicated that the endothelial glycocalyx is degraded by ROS generated by ischemia-reperfusion treatment [20–22]. However, no evidence was presented to show that ROS are able to cleave oligosaccharides in vivo. The data reported here clearly demonstrate that ROS, probably hydroxyl radicals derived from the reaction of copper ions and H_2O_2, have the ability to cleave the oligosaccharides of glycoproteins, including transferrin, in vivo. ROS generation [28] and high copper concentrations in the liver [29] have been observed, even in alcoholic hepatitis. In addition, total sialic acid concentration in plasma was increased in certain types of cancer [30–32]. We, therefore, consider that the alteration in oligosaccharides of glycoproteins such as transferrin even in alcoholic hepatitis and cancer may be caused by ROS.

In a previous study, we demonstrated that the *N*-linked oligosaccharide is degraded at glycosidic linkages involving GlcNAc residues by treatment with copper ion and H_2O_2 [18], and that this specific cleavage may be caused by the binding of copper to GlcNAc residues. In LEC rat serum from the AH group, it is possible to consider that the accumulated copper binds to transferrin via GlcNAc residues, thus permitting the localized generation of hydroxyl radicals, thereby leading to the cleavage of the oligosaccharide. However, oligosaccharides cleaved at the reducing end site of GlcNAc were not detected in the acute hepatitis serum (data not shown). Because most of the oligosaccharides in the serum exist as glycoproteins, the degradation of oligosaccharide by copper ions and H_2O_2 may be affected by the presence of amino acid residues. There are some reports that histidine residues affect the generation and toxicity of ROS by association with copper ion via imidazole ring [33–35]. The detailed degradation mechanism of oligosaccharide of glycoproteins by ROS should be studied.

On the other hand, glycosyltransferases must be considered to be a candidate for this oligosaccharide alteration. Miyoshi et al. [36] reported that *N*-acetylglucosaminyltransferase V activity is increased in the LEC rat liver in acute hepatitis. Noda et al. [37] reported that the expression of α1,6-fucosyltransferase mRNA is enhanced in hepatoma tissues but not in hepatitis tissues in LEC rat. It has also been reported that the activities of various glycosyltransferases are altered as a result of liver injury or by the intake of alcohol [38–40]. However, we were not able to detect sialidase and β-galactosidase activities in the acute hepatitis serum (data not shown). Further studies of the glycosyltransferases in LEC rats with acute hepatitis are also needed.

Rudolph et al. [41] reported that asialotransferrin has a shorter biological lifetime by 25–30%. The desialylation of transferrin was reported to enhance its binding affinity to aluminum but not to iron [42]. The desialylation of transferrin resulted in a markedly enhanced uptake of iron by the liver [16], while the deglycosylation of transferrin by treatment with *N*-glycosidase F reduced the iron uptake by the rat liver, due to the loss of exposed galactose residues [17]. These reports indicate that the oligosaccharide structure of transferrin is important for its biological functions. In fact, LEC rats have an abnormal metabolism of iron as well as copper [7,43]. Therefore, our results suggest that alterations in the oligosaccharide structure of

transferrin may lead to an abnormal metabolism of metals and liver damage in LEC rats.

In conclusion, free radicals generated in LEC rats with acute hepatitis degraded the oligosaccharide structures of serum glycoproteins. The resulting alterations may induce hypo- or hyper-functions of glycoproteins and may contribute to the development of hepatic diseases.

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