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Suppressive Effect of Modified Arabinoxylan from Rice Bran (MGN-3) on D-Galactosamine-Induced IL-18 Expression and Hepatitis in Rats

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We investigated in this study the effect of modified arabinoxylan from rice bran (MGN-3) and its fractions on D-galactosamine (D-GalN)-induced IL-18 expression and hepatitis in rats. Male Wistar rats were pretreated with MGN-3 or fractions of the MGN-3 hydrolysate, or with saline 1 h before administering D-GalN (400 mg/kg B.W.). The serum transaminase activities, IL-18 mRNA expression level in the liver and IL-18 concentration in the serum were determined 24 h after injecting D-GalN. Both the oral and intraperitoneal administration of MGN-3 (20 mg/kg B.W.) alleviated D-GalN-induced hepatic injury under these experimental conditions. The low-molecular-weight fraction (LMW) of MGN-3 showed the strongest protective effect on D-GalN-induced liver injury, its main sugar component being glucose. Moreover, the D-GalN-induced IL-18 expression was significantly reduced by treating with MGN-3 and LMW. The results suggest that MGN-3 and LMW could provide significant protection against D-GalN liver injury, and that IL-18 might be involved in their protective influence.

Key words: modified arabinoxylan; MGN-3; D-galactosamine; hepatitis; IL-18

MGN-3 is a modified water-soluble hemicellulose from rice bran that can be obtained by partial hydrolysis with enzymes from a basidiomycete. The main chemical constituent of MGN-3 is arabinoxylan, with a xylose in its main chain and an arabinose polymer in its side chain (Fig. 1).[1)] MGN-3 also contains β-1,3-glucan and has a variety of physiological functions. NK cell, T cell, and B cell functions are augmented by MGN-3 both *in vitro* and *in vivo*.[1–3)] In addition, when MGN-3 is administered in conjunction with conventional chemotherapeutic agents, it has been highly effective in inducing cancer remission in animal models.[4)] However, its effect on liver dysfunction in rats induced by D-galactosamine (D-GalN) has not been fully investigated.

Liver injury induced by D-GalN has been used as the animal model for liver injury, since its morphological and pathophysiological characteristics are similar to those of human hepatits B.[5,6)] Hepatitis induced by D-GalN in rats is considered to be mediated by the inhibition of protein and mRNA biosynthesis through the depletion of cellular UTP[7)] and by the enhanced absorption of endotoxins from the intestines to the blood stream.[8,9)] The precise mechanism for D-GalN-induced hepatitis has not yet been elucidated. Recent investigations have also demonstrated that D-GalN hepatotoxicity in rats involved the release of cytokines related to apoptosis and necrosis.[10–12)]

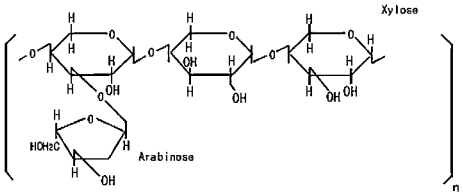

Fig. 1. Main Chemical Structure of MGN-3.

We found in our previous study that IL-18 expression was elevated in D-GalN-induced hepatitis.[13)] IL-18 is a potent inflammatory cytokine which regulates autoimmune and inflammatory diseases.

However, the effects of MGN-3 and its hydrolysate on D-GalN-induced IL-18 expression and hepatitis, as well as their mechanism, have not yet been clarified. We therefore investigated in this study the effect of MGN-3 and its hydrolysate on the development of D-GalN-induced hepatitis in rats.

Materials and Methods

Reagents. The following materials were commercially obtained: D-galactosamine hydrochloride (D-GalN) from Sigma Chemicals (St. Louis, MO, USA); the SV Total RNA isolation system from Promega (USA); the first-strand cDNA synthesis kit for RT-PCR (AMV) from Roche Diagnostics (Germany); the SYBR® Premix Ex Taq™ II kit (perfect real-time PCR) from Takara Bio (Otsu, Japan); and the reaction mixture for PCR, Absolute™ QPCR SYBR Green mixes from Abgene (United Kingdom). The rat IL-18 ELISA kit was purchased from Abnova Corporation (Jhongli, Taiwan) and MGN-3 was provided by Daiwa Pharmaceutical Co. (Tokyo, Japan).

Fractionation of MGN-3 by gel filtration. MGN-3 (1 g) was hydrolyzed in boiling water at 100 °C for 1 h in 100 mL (final volume) of 1 N hydrochloric acid. The hydrolysate was cooled under running tap water for 10 min and neutralized with 1 N sodium hydroxide. The solution was evaporated to dryness under decreased pressure by using a rotary evaporator.

The cation-exchange resin used was DOWEX 50W-X8 (Dow Chemicals). To this resin was added 1 N NaOH, and the mixture was stirred. The resin was washed with distilled water (D.W.) and then packed into a column (55 mm × 250 mm). The column was washed with D.W. after sufficient 1 N HCl had flowed through.

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The anion-exchange resin used was DOWEX 1-X2 (Dow Chemicals). To this resin was added 1 N HCl, and the mixture was stirred. The resin was washed with D.W. and packed into a column (55 mm × 250 mm). The column was washed with D.W. when sufficient 1 N NaOH had flowed through. An aqueous solution of BioBran/MGN-3 (10 mg/mL), which was hydrolyzed by HCl, was used with water as the mobile phase at a flow rate of 1 mL/min. The non-absorbed components were collected and freeze-dried.

The purified hydrolysate of MGN-3 was fractionated in a column (15 mm × 100 mm) of Sephadex-G 15 (GE Healthcare, Uppsala, Sweden), and separated into three fractions by molecular weight which was determined by using two markers (blue dextran (M.W. 2,000,000) and riboflavin (M.W. 376.4)). The total sugar content was measured by the phenol sulfuric acid method.

Animal and treatment protocols. Male Wistar rats aged 4 weeks (SLC, Japan) and weighing 60–80 g were maintained in an environmentally controlled room at 22 ± 1 °C with a 12-h light/dark cycle (light from 7:00 to 19:00). Three separate experiments were performed. All the rats were fed with a CE-2 commercial diet (Clea Japan, Japan) for 4 d and then with the standard AIN-93G diet (Galanos *et al.*, 1979) for 7 d.

The rats in the control and experimental groups (n = 6) were intraperitoneally administered with the D-GalN solution on day 7 of the standard diet at 400 mg/kg of body weight, and the standard group (the D-GalN non-treated group, n = 3) was injected with saline solution in the same manner. At 1 h before the D-GalN administration, the rats in the experimental groups were pretreated with MGN-3 or the fractionated samples of MGN-3. In experiment 1, the rats were intraperitoneally or orally given MGN-3 (n = 6). In experiment 2, the rats in experimental groups (n = 6) were intraperitoneally pretreated with MGN-3 at 0.5, 5 and 20 mg/kg of body weight. In experiment 3, the rats were administered with the high-molecular-weight fraction (HMW), medium-weight fraction (MMW) and low-molecular-weight fraction (LMW) of MGN-3 at respective doses equivalent to 20 mg/kg of body weight (n = 5).

All the rats were fasted for 4 h before and after the D-GalN treatment (8 h total). The rats were anesthetized with pentobarbital 24 h after the D-GalN or saline treatment. Blood was taken from the rat heart, and the liver was carefully removed and immediately frozen in liquid nitrogen. Serum was obtained from the blood by centrifugation at 3,000 × *g* for 20 min at room temperature. The care and treatment of the rats were carried out according to "The Ethical Guideline for Laboratory Animals" prescribed by Chiba University.

Transaminase activities. The alanine amino transferase (ALT, C.E.2.6.1.2) and aspartate amino transferase (AST, C.E.2.6.1.1) activities were analyzed by a Transaminase CII-test Wako kit in accordance with the manufacturer's instructions.

Real-time quantitative PCR. Total RNA was isolated from the liver by using the SV Total RNA Isolation system. cDNA was then synthesized from 1 μg of RNA by using the First-strand cDNA Synthesis kit for RT-PCR (AMV). Real-time PCR was performed for IL-18 and the housekeeping gene, encoding glyceladehyde-3-phosphate-dehydrogenase (GAPDH) by using the ABI PRISM 7000 sequence detection system (Applied Biosystems, USA). The reaction mixture was composed of Absolute™ QPCR SYBR Green Mixes (12.5 μL), the forward and reverse primers (5 μM, 1 μL each), nuclease-free water (8 μL), and a cDNA sample (2.5 μL). The primers used for IL-18 (GenBank accession no. NM_019165) were as follow: forward, 5′-GACTGGCTGTGACCCTATCTGTGA-3′; reverse, 5′-TTGTGTCCTGGCACACGTTTC-3′. Those for GAPDH (GenBank accession no. AB017801) were as follow: forward, 5′-TGCCAAGTATGATGACATCAAGAAG-3′; reverse, 5′-AGCCCAGGATGCCCTTTAGT-3′. The following PCR conditions were used: 30 s at 95 °C for 1 cycle; 5 s at 95 °C and 31 s at 60 °C for 45 cycles; and finally 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. The results were analyzed by ABI sequence detection system software (Applied Biosystems, USA).

ELISA for rat serum IL-18. The rat serum samples were analyzed for the IL-18 level by using the Rat IL-18 ELISA kit in accordance with the manufacturer's instructions.

Analytical methodology for the sugar composition of LMW from MGN-3. Acid hydrolysis was performed before measuring LMW of MGN-3. The hydrolysis process involved stirring for 1 h at room temperature in 72% sulfuric acid and then autoclaving (121 °C) for 1 h in 4% sulfuric acid.

The glucose, arabinose, mannose and xylose concentrations were determined by high-performance liquid chromatography (HPLC) with an LC-20AD chromatograph (Shimadzu, Japan). Sugar was determined with a refractive index detector (340 nm excitation wavelength and 430 nm fluorescence wavelength) and a TSKgel SUGAR AXI column (150 mm × 4.6 mm) at 60 °C. A 0.5 M boric acid buffer (pH 8.7) was used as the eluent at a flow rate of 0.4 mL/min. Sugar phosphates were analyzed by the arginine-reaction method, the effluent from the UV detector being mixed with an arginine solution (1%, w/v) and used at a flow rate of 0.7 mL/min at 150 °C.

The galactose concentration was determined by HPLC, using the DX500 sugar analysis system with a CarboPac PAI pulsed amperometric detector (Dionex Corporation) and an ion chromatograph (Dionex). A CarboPac PA1 column (4 × 250 mm; Dionex) was used for sugar separation. A 25-μL sample was automatically injected into the column which was eluted by an NaOH/Na acetate gradient with the following composition: eluent A, 75 mM NaOH/0 mM Na acetate, and eluent B, 75 mM NaOH/500 mM Na acetate. The flow rate of the mobile phase was set at 1 mL/min, and detection was carried out by pulsed amperometry with a Dionex ED40 electrochemical detector.

ESIMS analysis of LMW. Mass was analyzed by an Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray ionization (ESI) probe.

Statistical analysis. All the values in the figures and text are expressed as the mean ± SEM. Scheffe's multiple-comparison test was applied when significant differences were obtained by one-way analysis of variance. The level of significance was $p < 0.05$.

Results

Hepato-protective dose and administration method

The rats intoxicated with D-GalN alone developed hepatocellular damage, as was evident from a significant elevation in the serum transaminase activity. Pretreating with MGN-3 by oral administration afforded significant protection against D-GalN-induced liver injury. The pretreatment with MGN-3 by intraperitoneal injection also showed the same effect (Table 1). Intraperitoneal administration was used in further studies, considering the lack of significant differences between the orally and intraperitoneally administered MGN-3 groups.

MGN-3 was administered to the rats at three different doses to quantitatively study the protective effects of MGN-3 against D-GalN-induced liver injury. The AST

Table 1. Effect of MGN-3 Administration Methods on Serum Transaminase Activity in Rats at 24 h after D-GalN Treatment

	AST (IU/L)	ALT (IU/L)
Standard	93.4 ± 5.4 a	57.4 ± 0.7 a
Control	1448 ± 498 b	145 ± 24 b
MGN-3 i.p.	219 ± 109 a	73.2 ± 13.1 a
MGN-3 p.o.	379 ± 125 a	76.5 ± 12.5 a

MGN-3 was administered i.p. at 20 mg/kg of body weight (MGN-3 i.p.) or p.o. at 60 mg/kg of body weight (MGN-3 p.o.). An equal amount of saline was administered i.p. to the control group instead of the MGN-3 treatment. All the groups except the standard group received an i.p. injection of D-GalN (400 mg/kg body of weight). 24 h after the D-GalN treatment, serum was collected for the measurement of ALT and AST activities. Values are means ± SEM (standard n = 3, other groups n = 6). Values with different letters are significantly different at $p < 0.05$.

Table 2. Effect of MGN-3 on Serum Transaminase Activity in Rats at 24 h after D-GalN Treatment

	AST (IU/L)	ALT (IU/L)
Standard	39.6 ± 6.7 a	14.1 ± 1.1 a
Control	1074 ± 76 b	239 ± 35 b
MGN-3 0.5 mg/kg B.W.	603 ± 140 b	161 ± 35 b
MGN-3 5 mg/kg B.W.	712 ± 73 b	230 ± 4 b
MGN-3 20 mg/kg B.W.	197 ± 73 a	14.1 ± 0.7 a

MGN-3 (0, 0.5, 5 or 20 mg/kg B.W.) was administered i.p., and D-GalN (400 mg/kg B.W.) was administered 1 h after the MGN-3 treatment of the rats. 24 h after D-GalN treatment, serum was collected for the measurement of ALT and AST. Values are means ± SEM. (n = 6). Values with different letters are significantly different at $p < 0.05$.

Table 3. Effects of the Molecular Weight Fractions of MGN-3 Hydrolysate on Serum Transaminase Activity in Rats at 24 h after D-GalN Administration

	AST (IU/L)	ALT (IU/L)
Control	1878 ± 626 a	537 ± 261 a
Hydrolysate	734 ± 96 b	303 ± 38 b
High-molecular-weight fraction	1443 ± 310 a	441 ± 101 a
Medium-molecular-weight fraction	541 ± 83 b	94.5 ± 16.1 b
Low-molecular-weight fraction	185 ± 29 b	77.8 ± 8.8 b

MGN-3 was hydrolyzed with 1 N HCl at 100 °C for 1 h (hydrolysate, 20 mg/kg B.W.), and then the hydrolysate treated with cation or anion exchange resin was fractioned by molecular weight (high, medium, and low molecular weight). The MGN-3 hydrolysate and three fractionated samples were administered i.p. and an equal amount of saline was administered i.p. to the control group. All the groups received an i.p. injection of D-GalN (400 mg/kg B.W.) 1 h after the treatment with the MGN-3 samples. 24 h after the D-GalN injection, serum was collected for the measurement of ALT and AST activities. Values are means ± SEM (n = 5). Values with different letters are significantly different at $p < 0.05$.

and ALT activities of the MGN-3-treated groups injected at a dose of 20 mg/kg were significantly lower than those of the standard groups (Table 2).

Effect on hepato-protective action of different molecular weight fractions from MGN-3

The hydrolysate of MGN-3 significantly inhibited-D-GalN-induced liver injury when compared with the control. There was no significant decrease in the AST or ALT activity of the rats treated with the high-molecular-weight fraction (HMW; ≥2,000,000 Da) of MGN-3. On the other hand, compounds with the medium-weight fraction (MMW; 2,000,000 Da–400 Da) and low-weight fraction (LMW; ≤400 Da) of MGN-3 showed significant protection against D-GalN-induced liver injury; in particular, the LMW compounds had the strongest inhibitory effect on D-GalN-induced liver injury in all the groups (Table 3).

Sugar composition of LMW from MGN-3

Table 4 shows the sugar composition of LMW from MGN-3. The main constituent was glucose (22.8%), and the others were mannose (1.5%), galactose (0.5%) and arabinose (0.3%). No xylose was detected in LMW.

ESIMS analysis of LMW

An intense peak at m/z 409 was observed by ESIMS for LMW of MGN-3.

Table 4. Chemical Composition of LMW

Chemical component	Composition (%)
Glucose	22.8
Arabinose	0.3
Xylose	Not detected
Mannose	1.5
Galactose	0.5
Uronic acid	Not detected
Protein	2.85

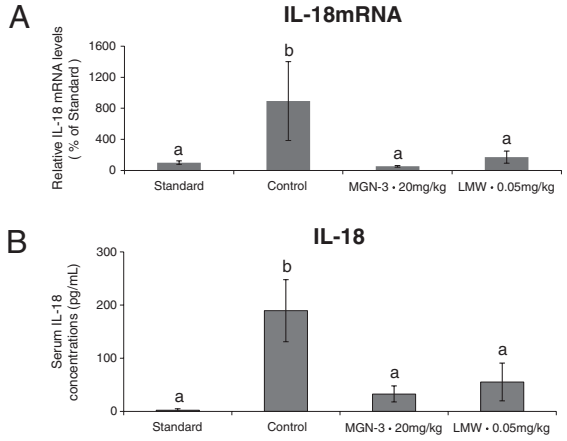

Fig. 2. Effects of MGN-3 and LMW of the MGN-3 Hydrolysate on the Liver IL-18 mRNA Expression Level (% of standard) (A) and the Serum IL-18 Concentration (B) in D-GalN-Injected Rats.

MGN-3 (20 mg/kg), LMW (0.05 mg/kg) or an equal amount of saline was administered i.p., and-D-GalN (400 mg/kg) was injected i.p. 1 h after treatment by the MGN-3 samples. 24 h after the D-GalN treatment, serum was collected and the liver was removed for IL-18 mRNA measurement by real-time PCR. Values are presented as the mean ± SEM, values with different letters being significantly different at $p < 0.05$.

Effect on liver IL-18 mRNA expression and the serum IL-18 level and concentration

This study also showed that the liver IL-18 mRNA expression level and serum IL-18 concentration of the D-GalN-treated group were both significantly higher than those of the normal (standard group) 24 h after the D-GalN treatment. There were significant effects when the rats were pretreated with MGN-3 or LMW. They showed a significantly lower IL-18 mRNA expression level in the liver and serum IL-18 concentration than the control values (Fig. 2A and B).

Discussion

The biological functions of MGN-3 for its anti-HIV activity and enhanced NK cell activity in cancer patients[14] have been reported. In this present study, the serum AST and ALT activities of the rats treated with MGN-3 1 h before the D-GalN injection were significantly decreased with both intraperitoneal injection and oral administration (Table 1). MGN-3 showed a liver protective effect both p.o. and i.p. under these experimental conditions. However, more than 30 mg/kg of B.W./d of MGN-3 intake increases the NK cell activity, so it is important to select the most suitable amount of MGN-3 for its anti-inflammatory effect. A preliminary experiment with a large amount of MGN-3 did not show a liver protective effect in D-GalN treated rats.

MGN-3 is an arabinoxylan extracted from rice bran enzymatically treated with an extract from shiitake mushroom.[15] We hydrolyzed MGN-3 at 100 °C for 1 h with 1 N HCl to study its active components. The results show that the MGN-3 hydrolysate had a protective effect on D-GalN-induced hepatitis and that its active components were stable against heating to 100 °C and against hydrolysis with 1 N HCl for 1 h. The active ingredients of MGN-3 could not be adsorbed to anion or cation exchange resin. LMW showed the strongest protective effect on D-GalN-induced liver injury in the rats (Table 3). The effective compounds in MGN-3 seemed to have low molecular weights. We measured the molecular weight of LMW by ESIMS. An intense peak at m/z 409 was observed by ESIMS for the LMW fraction of MGN-3. This result suggests that neutral oligosaccharides and monosaccharides in the LMW were candidates for the effective ingredients for treating D-GalN-induced liver injury.

We speculate that LMW was a mixture of monosaccharide and oligosaccharides, constituted by glucose as the main component. Our previous study has shown that the administration of arabinose and xylose, the main components of MGN-3, to rats had no inhibitory effect on D-GalN-induced liver injury (data not shown). The liver-protective components of MGN-3 were therefore different from these. It has also been reported that galactose had a noticeable inhibitory effect on D-GalN-induced liver injury.[16] However, the galactose content of LMW was only 0.5%. This relatively low galactose content would have played no major role in the protective effect of LMW on D-GalN-induced hepatitis. Zanobbio *et al.*[17] have reported that glucose administration prevented LPS/D-GalN-induced liver injury, so that glucose may be a candidate for the hepatoprotective ingredient of MGN-3.

LMW is considered to have contained oligosaccharide compounds from the result of ESIMS. It has recently been reported that oligosaccharides had various protective functions in organisms.[18,19] Our previous study has shown that the intake of some oligosaccharides inhibited the increase in plasma transaminase activity in rats injected with D-GalN,[16] and the oligosaccharide fragments of corn bran hemicelluloses have also shown a hepato-protective effect on D-GalN treated rats.[20]

IL-18 is an inflammatory cytokine belonging to the IL-1 family, and is clinically associated with such liver diseases as fulminant hepatitis, viral liver cirrhosis and primary biliary cirrhosis. The serum IL-18 level was correlated with the disease severity[21] in patients suffering from these diseases, although IL-18 itself was not cytotoxic to hepatocytes.[22,23] IL-18 is related to various immunological and inflammatory events,[24] and the over-expression of IL-18 has caused substantial liver injury in mice.[25] In addition, the plasma IL-18 concentration was significantly elevated in patients with liver injury caused by the hepatitis C virus.[26–29] We have recently reported that the serum IL-18 concentration was elevated in rats with D-GalN-induced liver injury.[13] The present results show that the IL-18 mRNA expression level was decreased by the LMW treatment. IL-18 is synthesized from a biologically inactive precursor and cleaved by IL-1-converting enzyme caspase-1.[30] The activation of the caspase-1 pathway to promote IL-18 activation in Kupffer cells has reportedly caused a large quantitative release of TNF-α into the blood by increasing the INF-γ activity, eventually resulting in inflammatory damage and aggravated liver injury.[21] In this present study, both IL-18 and TNF-α were suppressed by MGN-3 or LMW in D-GalN-induced liver injury (data not shown). MGN-3 and LMW may both protect D-GalN-induced hepatitis by reducing the IL-18 concentration and its mRNA expression level.

In conclusion, MGN-3 and its hydrolysate, LMW, significantly alleviated D-GalN-induced hepatitis in rats, and IL-18 might be associated with the protective effects of MGN-3 and the LMW fraction.

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