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Repeated Administration of Kupffer Cells-Targeting Nanoantioxidant Ameliorates Liver Fibrosis in an Experimental Mouse Model

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Kupffer cells are a major producer of reactive oxygen species and have been implicated in the development of liver fibrosis during chronic hepatitis in non-alcoholic steatohepatitis (NASH) and alcoholic steatohepatitis (ASH). We recently reported on the development of a polythiolated and mannosylated human serum albumin (SH-Man-HSA) that functions as a Kupffer cell-targeting nanoantioxidant. In this material, the albumin is mannosylated, which permits it to be taken up by mannose receptor C type 1 expressed on Kupffer cells, and is also polythiolated to have antioxidant activity. To clarify the anti-fibrotic property of this nanoantioxidant, we repeatedly administered SH-Man-HSA to a liver fibrosis mouse model that was induced by the repeated treatment of the concanavalin-A, which mimics the liver fibrosis observed in NASH and ASH. SH-Man-HSA dramatically improved the survival rate and suppressed liver fibrosis in the experimental model. In addition, SH-Man-HSA suppressed hepatic oxidative stress levels, thereby decreasing the numbers of apoptotic cells. In contrast, *N*-acetylcysteine, which contains the same thiol content as the SH-Man-HSA, failed to show a substantial therapeutic effect in these mice. The expression levels of inflammatory genes including epidermal growth factor module-containing mucin-like receptor (Emr-1/F4/80), Toll-like receptor-4 (TLR-4), high mobility group box-1 (HMGB-1), CC chemokine ligand-5 (CCL-5), tumor necrosis factor- α (TNF- α), CCL-2, interleukin-6 (IL-6), and IL-1 β , as well as fibrotic (α -smooth muscle actin (α -SMA), transforming growth factor- β (TGF- β), and Snail) and extracellular matrix genes (collagen, type I α 2 (Coll α 2), matrix metalloproteinase-9 (MMP-9), and tissue inhibitor of metalloproteinase 1 (TIMP-1)), showed some decreasing trends by the SH-Man-HSA administration. These findings suggest that the repeated administration of the Kupffer cell-targeting nanoantioxidant, SH-Man-HSA, ameliorates liver fibrosis in mice by suppressing the level of oxidative stress and a portion of the inflammation, and has a potential therapeutic effect against NASH and ASH.

Key words liver fibrosis; chronic hepatitis; reactive oxygen species; antioxidant

INTRODUCTION

Recent advances in the development of therapeutic drugs now permit hepatitis C virus to be treated.¹⁾ On the other hand, effective drugs against non-alcoholic steatohepatitis (NASH) and alcoholic steatohepatitis (ASH), both of which are the non-viral forms of chronic hepatitis, have not yet been developed.^{2,3)} In addition, liver fibrosis plays an important role in the long term prognosis of chronic hepatitis patients.^{4,5)} Therefore, therapeutic drugs having anti-fibrotic properties are needed for the treatment of chronic hepatitis, including NASH and ASH, however, at present, such drugs are not clinically available throughout the world.

The mainstream concept of NASH pathogenesis is the “multiple parallel hits” hypothesis,⁶⁾ which is composed of a wide range of simultaneous parallel hits such as the deposition of fat in hepatocytes, the generation of oxidative stress, and inflammatory cytokines.⁷⁾ Oxidative stress refers to an imbalance in which the production of reactive oxygen species (ROS) and/or reactive nitrogen species are increased to an extent that overrides the normal operating free radical-clearing

mechanisms.⁸⁾ The production of high levels of ROS in the liver causes lipid peroxidation, followed by inflammation, activation of hepatic stellate cells leading to fibrogenesis, necrosis, cirrhosis, and carcinoma.^{9,10)} Meanwhile, chronic ethanol intake is a crucial factor in the development of ASH¹¹⁾ and is known to depress antioxidant defenses and mitochondrial structure and function.¹²⁾ Further, ethanol increases the production of O₂^{•−} via activation or increasing the levels of CYP2E1, xanthine oxidase, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, thus resulting in the development of fibrosis, inflammation, and steatosis.¹³⁾

Kupffer cells are resident hepatic macrophages that are anti-CD68 antibody positive,¹⁴⁾ and play a central role in clearing foreign bodies clearance and the innate immune system.¹⁵⁾ However, the excessive activation of Kupffer cells results in the overproduction of inflammatory cytokines, which contributes to the development of hepatitis.¹⁶⁾ Nakashima *et al.* reported that the induction of tumor necrosis factor- α (TNF- α) by concanavalin-A (Con-A) is a trigger for the release of ROS from CD68⁺ Kupffer cells and this excess level of ROS lead extensive hepatocellular injury in mice. These mechanisms

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were corroborated by the attenuation of acute liver injury in mice, in which CD68⁺ Kupffer cells were removed or to which a TNF- α antibody was administered.¹⁷⁾ In recent years, it has been reported that ROS derived from Kupffer cells play a key role in the progression of chronic hepatitis and the subsequent liver fibrosis in NASH^{14,18)} and ASH.^{12,19)} Therefore, an antioxidant that has the ability to efficiently scavenge the ROS derived from Kupffer cells would be expected to be a candidate for a therapeutic agent for the treatment of NASH and ASH.

We recently developed a recombinant neoglyco-human serum albumin (Man-HSA) which possesses a bulky sugar chain consisting of approximately 12 mannose residues that are linked to human serum albumin (HSA) using site-directed mutagenesis and an expression system by *pichia pastoris*.²⁰⁾ Our pharmacokinetic experiment in mice showed that more than 60% of the Man-HSA migrated to the liver after its intravenous administration.²⁰⁾ In addition, we verified that Man-HSA functions as a CD68⁺ Kupffer cell-targeting drug carrier that is mediated by the mannose receptor C type 1 (CD206).²¹⁾ More recently, we developed a Kupffer cell-targeting nanoantioxidant, namely polythiolated and mannosylated human serum albumin (SH-Man-HSA), by loading Man-HSA with poly-thiol groups (7.5 mol thiol/mol Man-HSA).²¹⁾ The SH-Man-HSA exerted an excellent hepatoprotective effect by delivering thiol groups to the liver and scavenging the ROS produced by Kupffer cells in Con-A induced acute liver injury model mice.²¹⁾ These findings led us to predict that the SH-Man-HSA would exert not only a hepatoprotective effect but also an anti-fibrotic property against chronic hepatitis *via* the suppression of oxidative stress in the liver.

In this study, we repeatedly treated Con-A to the mice in order to produce an experimental model that mimics the liver fibrosis observed in NASH or ASH, and we examined the issue of whether SH-Man-HSA could improve the survival rate and suppress liver fibrosis in this experimental mice model. The findings of this study indicate that SH-Man-HSA would be expected to exert a therapeutic effect against NASH and ASH.

MATERIALS AND METHODS

Animals Seven-week-old female BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan). All experiments were performed with the prior approval of the animal experimentation committee of Kumamoto University (Kumamoto, Japan) and according to the ethical guidelines of Kumamoto University for the care and use of laboratory animals.

Induction of Liver Fibrosis Mice received Con-A (15 mg/kg; intravenously (*i.v.*)) at weekly intervals for up to 6 weeks according to previous reports.^{22,23)} Liver fibrosis was determined by pathological evaluation with hematoxylin and eosin (HE) and Masson's trichrome staining and the content of liver hydroxyproline (Fig. 1). All mice were maintained on a 12-h light/dark cycle at 24°C with free access to food and water.

SH-Man-HSA Preparation Man-HSA was expressed and purified according to previously reported procedures.^{20,21)} SH-Man-HSA was synthesized using methods developed by us, in which the moiety of thiol groups conjugated to Man-HSA was determined to be 7.5 mol thiol/mol Man-HSA.²¹⁾

Fig. 1. The Preparation of Liver Fibrosis Model in Mice Induced by the Repeated Treatment of Con-A

(A) Schematic summary of the experimental protocol concerning the preparing chronic hepatitis induced by the repeated treatment of Con-A. (B) HE and Masson's trichrome staining of mice liver were performed at 5, 6 and 7 weeks after starting the Con-A administration. Scale bars, 100 μ m. (C) Masson's trichrome positive areas were normalized by field with an area of 540 $\times$ 720 μ m (original magnification, $\times$ 200). (D) Hydroxyproline content in liver collected from healthy mice (Control) or chronic hepatitis mice at 7 weeks after starting Con-A administration. Results are the average $\pm$ standard error ($n = 5-8$). (Color figure can be accessed in the online version.)

SH-Man-HSA and N-Acetylcysteine Administrations

SH-Man-HSA or N-acetylcysteine (comparative drug) were dissolved in warm saline and administered to the mice (*i.v.*) once per week just before the injection of Con-A for up to 6 weeks. The dosage of SH-Man-HSA was set at 20 μ mol thiol/kg, a dose that showed a significant protective potential against acute liver failure in our previous study,²¹⁾ and that of N-acetylcysteine was set at an identical dose (*i.e.*, 20 μ mol thiol/kg).

Histological Analyses Sections 4 μ m thick were cut from formalin-fixed, paraffin-embedded mouse liver tissue blocks. The sections were subjected to HE and Masson's trichrome staining for the morphologic analysis and the detection of collagen fibers, respectively. Masson's trichrome positive areas were normalized by field with an area of 540 $\times$ 720 μ m (original magnification, $\times$ 200). At least five fields of liver specimens from mice were used in the analysis for fibrosis positive areas.

Measurement of Hepatic Hydroxyproline Hydroxyproline levels were measured using the same procedures as we reported previously.²⁴⁾

Immunohistochemical Analyses For immunohistochemistry, paraffin sections were heated in HistoVT One (Nacalai Tesque, Kyoto, Japan) for 40 min for antigen retrieval and

then incubated with peroxidase blocking solution (0.3% H₂O₂ in methanol) at room temperature for 30 min. The sections were incubated with anti-Nitrotyrosine (NO₂-Tyr, 1:100, Millipore, Billerica, MA, U.S.A.) primary antibodies at 4°C overnight. The primary antibodies were visualized by a universal immuno-enzyme polymer method using a Histofine Immuno-histochemistry Kit (Nichirei, Tokyo, Japan) and 3,3'-diaminobenzidine (Dojin Chemical Co., Kumamoto, Japan) according to the manufacturer's protocol. For the immunofluorescence of mouse liver specimens, paraffin sections of liver specimens were heated in HistoVT One for 40 min and then incubated with a blocking reagent (Block Ace; DS Pharma Biomedical, Osaka, Japan) for 1 h at room temperature. The mouse specimens were incubated with anti-myeloperoxidase (MPO) (1:50; Santa Cruz Biotechnology, Dallas, TX, U.S.A.), primary antibodies at 4°C overnight, followed by incubation with fluorochrome-conjugated secondary antibodies (1:200, Alexafluor anti-rabbit 546, Invitrogen, Waltham, MA, U.S.A.) at room temperature for 90 min. Fresh-frozen liver sections were fixed with 4% paraformaldehyde. The resulting sections were solubilized in Tween/Tris-HCl (0.1% (v/v) Tween20 and 50 mM Tris-HCl [pH 7.5]) followed by blocking with a blocking reagent for 1 h at room temperature. These sections were incubated with anti-F4/80 (1:200, clone BM8; eBioscience) antibodies at 4°C overnight and with fluorochrome-conjugated secondary antibodies (Alexafluor anti-rat 647, 1:200, Abcam, Cambridge, U.K.) at room temperature for 90 min. These slides were then observed using a fluorescence microscope (BZ-8000; Keyence, Osaka, Japan). NO₂-Tyr or F4/80 positive areas were normalized by field with an area of 270 × 360 μm (original magnification, ×400) or 540 × 720 μm (original magnification, ×200), respectively. At least five fields of liver specimens from mice were used in the analysis for NO₂-Tyr and F4/80 positive areas. The number of MPO positive cells were counted in the field with an area of 540 × 720 μm (original magnification, ×200). At least ten fields of liver specimens from mice were used in the analysis for MPO positive cells.

Terminal Deoxyribonucleotidyl Transferase (TdT)-Mediated dUTP Nick End Labeling (TUNEL) Staining
For detection of apoptotic cells in paraffin-embedded liver tissues, these cells were visualized by TUNEL using *In Situ*

Cell Death Detection Kit, Fluorescein (Roche, Basel, Switzerland). The numbers of TUNEL positive cells were counted in the field with an area of 270 × 360 μm (original magnification, ×400). At least ten fields of liver specimens from mice were used in the analysis for TUNEL positive cells.

Quantitative Real-Time PCR (RT-qPCR) Total RNA was extracted from liver tissues using an RNAiso Puls kit (TaKaRa Bio Inc., Shiga, Japan), and cDNA was prepared using PrimeScript® RT master mix (TaKaRa Bio Inc.), respectively. The synthesized cDNA was mixed with SYBER® Premix Ex TaqII (TaKaRa Bio Inc.) and amplified by a Real-Time PCR detection system (CFX connect™, Bio-Rad Laboratories, Hercules, CA, U.S.A.) with the primers listed in Table 1. The threshold cycle (Ct) values for each gene amplification were normalized by subtracting the Ct value calculated for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical Analyses All data are represented as the average ± standard error. The continuous variables between the groups were compared using Student's *t*-test or an ANOVA followed by Tukey's multiple comparison. The survival rate was plotted based on the Kaplan-Meier method, and comparisons between the treatment groups were carried out using the log-rank test. A *p* value <0.05 was considered to be statistically significant.

RESULTS

The Experimental Model of Liver Fibrosis in Mice Was Prepared by Repeated Treatment of Con-A We attempted to prepare an experimental mouse model of liver fibrosis by the weekly administration of Con-A based on previous studies^{22,23)} (Fig. 1A). Pathological evaluation in the liver with HE and Masson's trichrome staining at 5, 6 and 7 weeks after starting the Con-A administration showed the presence of aggravated tissue injury and an expanded fibrotic region that was weekly dependent (Fig. 1B). Furthermore, in comparison with the mice without the Con-A treatment (Control), Masson's trichrome positive area and the content of liver hydroxyproline at 7 weeks after starting the Con-A administration were increased remarkably (Figs. 1C, D). Because these results were similar to those in previous studies,^{22,23)} we concluded that a

Table 1. Sequence of Primers for RT-qPCR

Target gene	Forward primer (5'→3')	Reverse primer (5'→3')
Mouse Emr1 (F4/80)	GGATGTACAGATGGGGGATG	CATAAGCTGGGCAAGTGCTGA
Mouse TLR4	ATCCCTGCATAGAGGTAGTTCCTA	TCCAGCCACTGAAGTTCTGAAA
Mouse HMGB-1	ATCCCTGCATAGAGGTAGTTCCTA	TCCAGCCACTGAAGTTCTGAAA
Mouse CCL-5 (RANTES)	GACAGCACATGCATCTCCCA	GTGTCCGAGCCATATGGTGA
Mouse TNF-α	TAGCCACGTCGTAGCAAAC	GCAGCCTTGTCCTTGAAGA
Mouse CCL-2 (MCP-1)	GATGCAGTTAACGCCCCACT	GAGCTTGGTGACAAAACTACAGC
Mouse IL-6	TCTCTGCAAGAGACTTCCATCC	AGACAGGTCTGTTGGGAGTG
Mouse IL-1β	TGAGCTGAAAGCTCTCCACC	CTGATGTACCAGTTGGGGAA
Mouse Snail	CAACTATAGCGAGCTGCAGGA	ACTTGGGGTACCAGGAGAGAGT
Mouse TGF-β	GGATACCAACTATTGCTTCAGCTCC	AGGCTCCAAATATAGGGGCAGGGTC
Mouse α-SMA	AGCCATCTTTCATTGGGATGG	CCCCTGACAGGACGTTGTGA
Mouse Col1α2	CACCCAGCGAAGAACTCATA	GCCACCATTGATAGTCTCTCTAAC
Mouse MMP-9	CGTGTCTGGAGATTCGACTTGA	TGGAAGATGTCGTGTGAG
Mouse Timp1	AGACACACCAGAGCAGATACC	TATGACCAGGTCCGAGTTGC
Mouse GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

Primer used in this study.

liver fibrosis model could be prepared by the repeated treatment of Con-A, even under our experimental conditions.

Anti-fibrotic Property of SH-Man-HSA Was Observed in the Experimental Model of Liver Fibrosis We evaluated the hepatoprotective effect of the repeated administration of SH-Man-HSA on the experimental model of liver fibrosis (Fig. 2A). As shown in Fig. 2B, the repeated treatment of Con-A resulted in a remarkable decrease in the survival rates at 1 or 2 weeks and the rates finally reached 20%. The repeated treatment of SH-Man-HSA just before the injection of Con-A improved the survival rates up to 80%, while, in the *N*-acetylcysteine treatment group, the rates were as low as 30% after 7 weeks. To clarify whether the improvement in the survival rates by SH-Man-HSA was attributed to its anti-fibrotic property, we analyzed the degree of liver fibrosis in the mice that had survived after 7 weeks. HE and Masson's trichrome staining showed that SH-Man-HSA suppressed the expansion of the fibrotic regions (Fig. 2C). On the other hand, the anti-fibrotic property was limited in the case of the *N*-acetylcysteine treatment group (Fig. 2C). Furthermore, Masson's trichrome positive area and the content of liver hydroxyproline were significantly reduced by SH-Man-HSA, while a distinct suppression was not observed in *N*-acetylcysteine treatment group (Figs. 2D, E).

Anti-oxidant Property of SH-Man-HSA Was Observed in the Experimental Model of Liver Fibrosis To evaluate whether SH-Man-HSA ameliorates hepatic oxidative stress in the experimental model of liver fibrosis, we measured the level of hepatic peroxynitrite-mediated nitration ($\text{NO}_2\text{-Tyr}$; nitro-tyrosine) by enzyme immunostaining (Fig. 3). The $\text{NO}_2\text{-Tyr}$ -positive area were significantly reduced by the SH-Man-HSA compared with the *N*-acetylcysteine treatment group (Fig. 3). To verify the anti-oxidant property of SH-Man-HSA, we also analyzed the change in the number of apoptotic cells in liver tissue using a TUNEL assay. The number of apoptotic cells elicited by repeated treatment of Con-A was also suppressed by SH-Man-HSA treatment, while some TUNEL-positive cells were observed in *N*-acetylcysteine treatment group (Fig. 3). To confirm whether these changes caused by the SH-Man-HSA treatment affected the infiltration of inflammatory cells, such as macrophages or lymphocytes, we quantified the area of F4/80 (a typical macrophage marker) positive areas and the numbers of MPO (typical neutrophil marker) positive cells (Fig. 3). As shown in Fig. 3, no change was observed for the infiltrated macrophages or lymphocytes among the No treatment, *N*-acetyl cysteine, and SH-Man-HSA treated groups.

The Mechanism for Anti-fibrotic Property of SH-Man-HSA Hepatic fibrogenesis is known to be a consequence of inflammation- or the oxidative stress-elicited activation of hepatic stellate cells and the subsequent increase in the production of extracellular matrix components by activated hepatic stellate cells.²⁵⁾ Therefore, we examined the effect of SH-Man-HSA on genes that are associated with these reactions and processes. The expression levels of inflammatory genes including epidermal growth factor (EGF) module-containing mucin-like receptor (Emr-1/F4/80), Toll-like receptor-4 (TLR-4), high mobility group box-1 (HMGB-1), CC chemokine ligand-5 (CCL-5/RANTES), TNF- α , CCL-2 (MCP-1), interleukin-6 (IL-6), and IL-1 β were unchanged between the Control, No treatment and SH-Man-HSA groups (Fig. 4A). The expression levels of fibrotic genes (α -smooth muscle actin

Fig. 2. Anti-fibrotic Property of SH-Man-HSA in Liver Fibrosis Model Mice

(A) Experimental protocol concerning the anti-fibrotic property of SH-Man-HSA in chronic hepatitis model mice that received a repeated treatment of Con-A (15 mg/kg/week; *i.v.*) administration. (B) Impact of SH-Man-HSA on survival rate in liver fibrosis model mice received Con-A repeated administration. 20 μmol thiol/kg of SH-Man-HSA and *N*-acetylcysteine were administrated (*i.v.*) just before the treatment of Con-A once a week for up to 6 weeks ($n = 10$). The survival rates were calculated using the cumulative number of dead mice within every week. * $p < 0.05$ vs. *N*-acetylcysteine, and ** $p < 0.01$ vs. No treatment. (C) HE and Masson's trichrome staining of mice liver were carried out at 7 weeks after starting Con-A administration. Scale bars, 100 μm . (D) Masson's trichrome positive areas were normalized by field with an area of $540 \times 720 \mu\text{m}$ (original magnification, $\times 200$). (E) Hydroxyproline levels in liver were determined at 7 weeks after starting Con-A administration. Results are the average $\pm$ standard error ($n = 5-7$). (Color figure can be accessed in the online version.)

(α -SMA), transforming growth factor- β (TGF- β), and Snail) and extracellular matrix genes (collagen, type Ia2 (Col1a2), matrix metalloproteinase-9 (MMP-9), and no significant differences were found for the tissue inhibitor of metalloproteinase 1 (TIMP-1)) (Figs. 4B, C). However, there was a tendency for the mRNA levels of inflammatory and fibrotic genes to be increased by the Con-A treatment compared with Controls, and these elevations were decreased by the administration of SH-Man-HSA.

DISCUSSION

It has been verified that ROS derived from Kupffer cells play an important role in the progression of chronic hepatitis and subsequent liver fibrosis in NASH^{14,18)} and ASH.^{12,19)} To investigate the issue of whether SH-Man-HSA exerts a protective effect against liver fibrosis *via* the suppression of hepatic oxidative stress and inflammation, we first repeatedly treated

Fig. 3. Anti-oxidant Property of SH-Man-HSA in Liver Fibrosis Model Mice

NO₂-Tyr, TUNEL, F4/80 and MPO immunostaining of liver were carried out at 7 weeks after starting the Con-A administration. 10% formalin-fixed paraffin-embedded liver sections were incubated overnight with an anti-NO₂-Tyr (1:100) and an anti-MPO (1:50) antibody. Fresh frozen liver sections that were fixed in 4% paraformaldehyde were incubated overnight with an anti-F4/80 (1:200) antibody. TUNEL staining was performed for *in situ* detection of apoptotic cells using a commercial kit. Scale bars, 100 μ m. (Color figure can be accessed in the online version.)

Con-A to the mice in order to produce an experimental model which mimics the progression of liver fibrosis during chronic hepatitis in NASH or ASH. It was previously reported that a mice model of liver fibrosis can be produced by the repeated administration of Con-A, which results in an increase in the production of TGF- β ,^{22,23} which is similar to that observed in NASH or ASH patients. In the present study, SH-Man-HSA dramatically improved the survival rate and suppressed liver fibrosis in the mice that had been repeatedly treated Con-A (Fig. 2B). On the other hand, *N*-acetylcysteine, which has the same thiol content as SH-Man-HSA, failed to show a substantial therapeutic effect (Fig. 2B). Our previous study demonstrated that SH-Man-HSA exerted a better therapeutic effect than *N*-acetylcysteine when administered to Con-A induced acute liver injury model mice and that this effect can be attributed to the capacity of thiol delivery to the liver (SH-Man-

HSA >> *N*-acetylcysteine).²¹ Therefore, it is possible that the amount of thiol delivered to mouse liver explains the large difference in the therapeutic effects between the treatment groups in this study.

The development of hepatic fibrosis is attributed to an excessive accumulation of extracellular matrix components, which are formed from the increased synthesis of various collagens through the inflammation- or oxidative stress-mediated activation of hepatic stellate cells. In our previous study, we reported that SH-Man-HSA remarkably suppressed ROS derived from Kupffer cells and attenuated liver injury without decreasing the expression levels of TNF- α and interferon- γ (IFN- γ) in Con-A induced acute liver injury model mice.²¹ In the present study, we evaluated the anti-inflammatory and anti-fibrotic function of SH-Man-HSA at 7 weeks after starting the Con-A administration repeatedly. Although the mRNA

Fig. 4. Effects of SH-Man-HSA on Gene Expression Levels in Liver Fibrosis Model Mice

The livers were collected from healthy mice (Control) or mice at 7 weeks after starting the Con-A administration with or without 20 μ mol thiol/kg of SH-Man-HSA treatment. The expression levels of (A) inflammatory, (B) fibrotic and (C) extracellular matrix genes in the liver were determined by RT-qPCR. Results are the average $\pm$ standard error ($n = 5$). All data show statistical analysis results (p -value) between Control and No treatment, and between No treatment and SH-Man-HSA.

levels of inflammatory and fibrotic genes were not changed significantly between the Control, No treatment, and SH-Man-HSA groups (Fig. 4), the administration of SH-Man-HSA showed a tendency to decrease these mRNA levels compared

with the No treatment group.

It has been reported that inflammation plays an important role in the development of hepatitis, but also contributes to the early phase of its onset and disease progression.²⁶⁾ This indi-

Fig. 5. The Mechanism for Anti-fibrotic Property of SH-Man-HSA

The Kupffer cell is found in the hepatic microvascular, is lining the walls of the sinusoids, and is hence in proximity to sinusoidal endothelial cells. In spite of the barrier of the liver sinusoid endothelial cells, inflammatory cytokines and ROS, both of which are overproduced by Kupffer cell, activate the stellate cells. SH-Man-HSA attenuated activation of stellate cell *via* suppression of ROS derived from Kupffer cells and following oxidative stress in the liver, thereby decreasing the accumulation of extracellular matrix. (Color figure can be accessed in the online version.)

cates a possibility that SH-Man-HSA may also exert an anti-inflammatory effect at the early phase of the present hepatitis model mice. Therefore, it should be evaluated the anti-inflammatory function of SH-Man-HSA at the early phase of Con-A induced hepatitis. However, it was difficult to examine this issue because the fibrosis model prepared in this study has a high lethality rate even in the early phase of hepatitis (Fig. 2B). To clarify the anti-inflammatory effect of SH-Man-HSA, it is necessary to conduct the experiment using other chronic hepatitis model mice, such as NASH in the future.

Activated hepatic stellate cells also contribute to ROS production and play a key role in the progression of NASH²⁷⁾ and ASH.¹²⁾ We recently reported that the mannose receptor C type 2 (CD280) expressed on the activated hepatic stellate cells is a novel target molecule for Man-HSA (data not shown). Given that the mannose receptor is also expressed on neutrophils,²⁸⁾ SH-Man-HSA may deliver thiol groups directly to hepatic neutrophils and activated hepatic stellate cells *via* mannose receptors, thereby conferring anti-oxidant and anti-fibrogenic properties on these types of cells. These possibilities should be examined to further clarify the underlying mechanism(s), by which the SH-Man-HSA suppresses liver fibrosis.

Anti-drug antibodies can sometimes appear after the repeated administration of biomedicines, such as therapeutic proteins,²⁹⁾ therapeutic monoclonal antibodies³⁰⁾ and nanomedicines,³¹⁾ and may neutralize drug target binding or enhance drug clearance. In addition, anti-drug antibodies may cause hypersensitivity reactions, such as anaphylaxis, infusion reactions, and immune-complex mediated diseases.³²⁾ In the present study, there are some concerns regarding the production of anti-HSA antibodies because xenogeneic albumin, *i.e.* HSA, was administered to the mice. However, SH-Man-HSA suppressed the liver injury without any reduction in its thera-

peutic effect after the repeated administration of SH-Man-HSA for periods of up to 6 weeks (Figs. 2, 3). According to results from clinical trials,^{33,34)} it is generally thought that the administration of exogenous HSA does not influence immune responses. Taken together, it is unlikely that such antibodies against SH-Man-HSA would be produced in a clinical setting.

Liver fibrosis is a common feature in almost all chronic liver diseases including the hepatitis virus,^{35–37)} NASH,⁴⁾ ASH,⁵⁾ and autoimmune hepatitis.³⁸⁾ Because cirrhosis, which is an end-stage chronic liver disease, is accompanied by an elevated risk for the development of hepatocellular carcinoma,³⁶⁾ preventing the development of liver fibrosis is a beneficial therapeutic strategy. In fact, extensive clinical research indicates that liver fibrosis is the only determinant of the long-term prognosis in NASH patients.⁴⁾ Therefore, an anti-fibrotic property is a crucial factor in the development of a new drug candidate that would be effective in the treatment of NASH and ASH patients. The findings of this study indicate that SH-Man-HSA exerts an anti-fibrotic property without any reduction in efficacies even when it is administered repeatedly, hence it should satisfy the requirement for chronic hepatitis therapy.

Because SH-Man-HSA was found to suppress the ROS levels derived from CD68⁺ Kupffer cells in our previous study,²¹⁾ the present data serve to confirm that the ROS produced by activated CD68⁺ Kupffer cells is an essential factor in liver fibrosis during chronic hepatitis (Fig. 5). However, considering clinical applications in humans, the anti-fibrotic property of SH-Man-HSA should be examined even when SH-Man-HSA is administered at least 5 weeks after starting the Con-A treatment where liver fibrosis was developed in mice. The findings of this study warrant further investigations to verify that the ROS derived from CD68⁺ Kupffer cells are an essential ef-

factor in the development of liver fibrosis using other liver fibrosis models, such as those induced by carbon tetrachloride or bile duct ligation.

CONCLUSION

This is the first paper to report that the repeated administration of a Kupffer cell-targeting nanoantioxidant, SH-Man-HSA, suppresses the development and progression of liver fibrosis in mice. According to the findings from the current study, SH-Man-HSA is expected to have a potential therapeutic effect against NASH and ASH.

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Conflict of Interest The authors declare no conflict of interest.

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