筑　波　大　学

博 士 （医 学） 学 位 論 文

Protecting liver sinusoidal endothelial cells suppresses apoptosis in acute liver damage

（肝類洞内皮細胞の保護が急性肝障害における
アポトーシスを抑制する）

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Hepatology Research

Hepatology Research 2015 doi: 10.1111/hepr.12607

Original Article

Protecting liver sinusoidal endothelial cells suppresses apoptosis in acute liver damage

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Aim: Apoptosis is associated with various types of hepatic disorders. We have developed a novel cell-transfer drug delivery system (DDS) using a multifunctional envelope-type nano device that targets liver sinusoidal endothelial cells (LSECs). The purpose of this study was to determine the efficacy of the novel DDS containing siRNA at suppressing apoptosis in LSECs.

Methods: Bax siRNA was transfected into a sinusoidal endothelial cell line (M1) to suppress apoptosis induced by an anti-Fas antibody and staurosporine. C57BL/6J mice were divided into three groups: (i) a control group, only intravenous saline; (ii) a nonselective group, injections of siRNA sealed in the nonselective DDS; and (iii) an LSEC-transfer efficient group, injections of siRNA sealed in an LSEC-transfer efficient DDS. Hepatic cell apoptosis was induced by an anti-Fas antibody.

Results: Bax siRNA had an anti-apoptotic effect on M1 cells. Serum alanine aminotransferase was reduced in the LSEC-transfer efficient group, as were cleaved caspase-3 and the number of terminal deoxynucleotidyl transferase dUTP nick end labeling positive hepatocytes. Silver impregnation staining indicated that the sinusoidal space was maintained in the LSEC-transfer efficient group but not in the other groups. Electron microscopy showed that the LSECs were slightly impaired, although the sinusoidal structure was maintained in the LSEC-transfer efficient group.

Conclusion: Hepatocyte apoptosis was reduced by the efficient suppression of LSEC apoptosis with a novel DDS. Protecting the sinusoidal structure by suppressing LSEC damage will be an effective treatment for acute liver failure.

Key words: acute liver failure, apoptosis, Bax, drug delivery system, liver sinusoidal endothelial cell, siRNA

INTRODUCTION

APOPTOSIS IN THE liver is associated with various liver disorders, such as acute and chronic hepatitis and viral hepatitis.[1,2] In liver inflammation, liver sinusoidal endothelial cells (LSECs) are considered to play important roles in the migration of inflammatory cells into hepatocytes and in the altered expression of adhesion molecules.[3–5]

The suppression of apoptosis by siRNA prevents acute liver failure.[6–9] Pro-apoptotic factors, including Bax in the mitochondrial apoptosis pathway, have been targeted by siRNA.[10,11] Bax siRNA can accumulate in the liver after i.v. administration; however, the siRNA is not targeted to either hepatocytes or LSECs. The mechanisms leading to acute liver failure and the roles of hepatocytes and LSECs in apoptosis in the liver remain unknown.

In our previous study, hepatocytes were impaired following LSEC deterioration in an acute hepatitis model.[12] The study suggested that LSECs play an important role in apoptosis and that inhibiting LSEC damage diminishes acute liver failure. However, the study only indicated that LSEC damage occurred in the early phase of liver failure before hepatocyte damage. Evaluating LSECs was difficult because there was no drug delivery system (DDS) that efficiently targets LSECs.

Recently, we have developed a novel DDS, namely, a multifunctional envelope-type nano device (MEND), that accumulates effectively in LSECs.[13,14] This DDS has permitted the effective knockdown of target gene expression

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Received 2 July 2015; revision 30 September 2015; accepted 14 October 2015.

in LSECs by promoting the efficient uptake of sealed siRNA into LSECs.[14]

We hypothesized that suppressing LSEC apoptosis would diminish acute liver failure more effectively than suppressing hepatocyte apoptosis. In this study, we used Bax siRNA to suppress apoptosis in the liver and injected the novel DDS encapsulating Bax siRNA into mice with anti-Fas antibody-induced acute liver damage. Our aim was to examine the effects of suppressing LSEC apoptosis and to elucidate the mechanism responsible for acute liver damage.

METHODS

siRNA construction

IN PRELIMINARY EXPERIMENTS, we examined Bax and Bak gene expression levels in AML12 cells (CRL-2254; American Type Culture Collection, Manassas, VA, USA), which is a hepatocyte cell line in mice. Bak gene expression in AML12 cells was below the limit of detection, indicating that the expression of Bak in hepatocytes was extremely low. Additionally, we administrated an anti-Fas antibody (Jo-2; BD Pharmingen, San Diego, CA, USA) to mice and measured Bax and Bak gene expressions. Bak expression in total liver tissue did not increase after the administration compared with before the administration. In contrast, Bax expression increased. Therefore, the inhibition of Bax expression was expected to be effective to suppress apoptosis in the liver. The use of Bax siRNA to knockdown Bax gene expression has been previously reported.[8] We purchased Bax siRNA (5′-AGCGAGTGTCTCCGGCGAATT-3′) from Hokkaido System Science (Sapporo, Japan).

In vitro experiments

Cell lines

M1 is a liver sinusoidal endothelial cell line created from the isolation of non-parenchymal cells from a H-2Kb-tsA58-transgenic mouse liver transfected with the SV40 large T antigen gene.[15] M1 cells were seeded at confluence and cultured in a humidified atmosphere containing 5% CO_2 at 37°C in ASF104N medium (Ajinomoto Healthy Supply, Tokyo, Japan) containing 2% fetal bovine serum.

siRNA transfection and induction of apoptosis

Bax siRNA transfections were performed using Lipofectamine RNAiMAX (Invitrogen Life Technologies, Gaithersburg, MD, USA) and Opti-MEM (Invitrogen Life Technologies) according to the manufacturer's recommendations.[16] M1 cells were grown to 60–70% confluence in six-well plates and then transfected with 75 pmol of siRNA per well. After 24 h, the cells in each dish were treated with 2.0 μg/mL anti-Fas antibody or 0.1 μM staurosporine (STS) (Sigma-Aldrich, Tokyo, Japan). The cells were divided into two groups: (i) the Bax siRNA(−) group (medium only); and (ii) the Bax siRNA(+) group (siRNA transfection). Cell samples were taken at 0, 12 and 24 h after anti-Fas antibody administration and at 0, 2 and 4 h after STS administration. For real-time reverse transcription polymerase chain (RT–PCR) analysis, the cells were harvested using the buffer from an RNA kit (NucleoSpin RNA; Takara Bio, Otsu, Japan), which isolates total cellular RNA. For western blot analysis, the cells were washed with phosphate-buffered saline (PBS), harvested using sodium dodecylsulfate (SDS) sample buffer and mixed with β-mercaptoethanol; the samples were then centrifuged, and the supernatant was collected.

In vivo experiments

Animals

Eight- to nine-week-old female C57BL/6J mice (Charles River Laboratories International, Yokohama, Japan) weighing 16–21 g were used. All of the animal experiments were performed in a humane manner after receiving approval from the Institutional Animal Experiment Committee of the University of Tsukuba. The animal experiments were performed in accordance with the Regulations for Animal Experiments at our university and the Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Drug delivery system and fluorescent staining assay

Multifunctional envelope-type nano devices were developed for use as an siRNA DDS at the Laboratory of Innovative Nanomedicine, Faculty of Pharmaceutical Sciences, Hokkaido University.[17,18] We have developed two types of MEND: the first is a nonselective MEND, which mainly targets hepatocytes;[19,20] the second is an LSEC-transfer efficient MEND, which targets LSEC more efficiently than nonselective MEND.[13,14] Four- to five-week-old female ICR mice (Japan SLC, Shizuoka, Japan) received an i.v. injection of rhodamine-DOPE-labeled nonselective MEND or LSEC-transfer efficient MEND, and the mice were killed 25 min after treatment. The portal vein was cut, a needle was introduced into the vena cava, and 10–15 mL of a heparin-containing (40 units/mL) PBS solution was used to remove the remaining blood and cell surface-bound

MEND in the liver. The liver was then excised, washed with saline and sliced into 10–15-mm blocks with scissors. The liver sections were incubated with a 20-fold volume of a diluted solution of Hoechst 33342 (1 mg/mL) and isolectin B4 in HEPES buffer for 1 h. The specimens were placed on a 35-mm glass base dish (Iwaki, Osaka, Japan) and observed using confocal laser scanning microscopy (A1 Confocal Laser Microscope System, Nikon Instruments, Tokyo, Japan).[21] We encapsulated Bax siRNA into the nonselective MEND and the LSEC-transfer efficient MEND. Each purified MEND formulation was stored at 4 °C and used within 2 weeks.

siRNA transfection and Jo2 application

The Fas/Fas ligand system plays important roles in hepatitis and other liver diseases.[22,23] As a model of acute liver damage, we have administrated the anti-Fas antibody in mice.[12] Mice were anesthetized with isoflurane. One microgram per gram of bodyweight of siRNA was diluted to 150 μL with saline and injected via the tail vein.[24,25] Twenty-four hours after siRNA administration, 0.2 mg/kg bodyweight of anti-Fas antibody was injected i.p. to induce acute liver failure. The mice were divided into three groups: (i) the control group ($n = 7$), in which mice were injected with saline only; (ii) the nonselective group ($n = 7$), in which siRNA was administrated via the nonselective MEND; and (iii) the LSEC-transfer efficient group ($n = 7$), in which the LSEC-transfer efficient MEND was used to deliver siRNA. At 0, 2, 6 and 24 h after the anti-Fas antibody treatment, blood samples were collected and centrifuged for 10 min at 4 °C at 1200 *g*. The supernatants were collected and stored at −80 °C. The mice in each group were killed, and the livers were removed and divided into three specimens: one section was fixed in 10% buffered formalin for subsequent histological analysis, silver impregnation and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining; another was fixed in 2% glutaraldehyde for transmission electron microscopy analysis; and the third was snap-frozen in liquid nitrogen and stored at −80 °C until use for real-time RT–PCR and western blot analysis.

Biochemical assays

To estimate the extent of liver injury, the serum level of ALT was determined using an autoanalyzer (Fuji Dri-chem 7000 V; Fujifilm, Tokyo, Japan).

Histological analysis

The liver specimens were stained with hematoxylin–eosin (HE) and silver impregnation to observe liver damage, and changes to the sinusoidal structure were examined using standard histological techniques. To detect the sinusoidal changes, the relative ratio of the sinusoidal area stained with silver impregnation around the portal vein was calculated at ×400 magnification under a microscope (BZ-9000, BZ Analysis Application; Keyence, Osaka, Japan). Apoptosis in the liver tissue was detected by the appearance of apoptotic cells in the TUNEL assay (In Situ Apoptosis Detection Kit; Takara Bio, Otsu, Japan), and the ratio of TUNEL positive/total hepatocytes was calculated with a microscope at ×400 magnification.

Transmission electron microscopy

The liver tissues were removed 24 h after the anti-Fas antibody treatment and were cut into small pieces (~1 mm^3). The specimens were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer and then were dehydrated through a graded series of ethanol, passed through propylene oxide and embedded in EPON 812. Ultra-thin sections were mounted on copper grids and stained with uranyl acetate and lead citrate before observation using a Hitachi H-7000 transmission electron microscope (Hitachi, Tokyo, Japan).

Total RNA extraction and quantitative RT–PCR

Frozen liver tissue samples were homogenized, and total cellular RNA was isolated using a NucleoSpin RNA Kit (Takara Bio, Otsu, Japan). The RNA concentrations were determined by measuring the absorbance at 260/280 nm with a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Complimentary DNA was synthesized using AMV Reverse Transcriptase (Promega, Madison, WI, USA) and random primers (Takara Bio). Briefly, a mixture of 1 mM deoxyribonucleotide triphosphate (Fermentas Life Sciences, Burlingame, ON, Canada), 0.025 mg/mL random primer, 0.25 U/mL reverse transcriptase and 500 ng of total RNA was incubated at 30 °C for 10 min, 37 °C for 60 min and 95 °C for 5 min before storage at −80 °C. RT–PCR primers were designed using Primer Express Software for Real-Time PCR version 3.0 (Applied Biosystems, Foster City, CA, USA) with GenBank sequences. Primers were purchased from Hokkaido System Science (Hokkaido, Japan). The Bax (NM_007527) primer sequences were 5′-CAGGATG CGTCCACCAAGA-3′ and 5′-CTGCCACCCGGAAGAAGAC-3′. The Bak (NM_007523.2) primer sequences were 5′-GGAATGCCTACGAACTCTTCA-3′ and 5′-CCAGCTGA TGCCACTCTTAAA-3′. The glyceraldehyde 3-phosphate dehydrogenase (NM_008084; endogenous control) primer sequences were 5′-CCAGCCTCGTCCCGTAGA-3′ and 5′-CGCCCAATACGGCCAAA-3′. RT–PCR was performed

using SYBR Green Real-time PCR Master Mix-Plus (Toyobo, Osaka, Japan) and an Applied Biosystems 7300 Real-time PCR system (Applied Biosystems) following the procedure recommended by the manufacturers.[26,27]

Protein extraction and western blot analysis

Frozen liver tissue samples were homogenized in a buffer consisting of 150 mM/L NaCl, 50 mM Tris–HCl, 1% NP-40 and proteinase inhibitors. The samples were centrifuged and the supernatants were collected for analysis. The samples were separated on 12% SDS polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). Anti-Bax (2772) and anti-cleaved caspase-3 (9661) antibodies (Cell Signaling, Beverly, MA, USA) were used as primary antibodies. A secondary goat antirabbit antibody conjugated to horseradish peroxidase was purchased from Zymed Laboratories (San Francisco, CA, USA). Protein quantities were determined by densitometry ($n = 3$ independent experiments).

Statistical analysis

Pairwise comparisons of subgroups were performed using Student's *t*-test. Comparisons between multiple treatments were performed using one-way ANOVA, followed by the Bonferroni–Dunn multiple comparisons post-hoc test. In all cases, $P < 0.05$ was considered significant.

RESULTS

Inhibition of Bax expression suppresses LSEC apoptosis

THE EXPRESSION OF Bax mRNA in the Bax siRNA(+) group was significantly lower than that in the Bax siRNA(−) group at 0, 12 and 24 h after administration of the anti-Fas antibody (Fig. 1a) and at 0, 2, 4 h after STS treatment (Fig. 1b). Bax and cleaved caspase-3 protein expression was significantly suppressed in the Bax siRNA(+) group compared with the Bax siRNA(−) group at 12 and 24 h after administration of the anti-Fas antibody (Fig. 1c) and at 2 and 4 h after STS treatment (Fig. 1d).

Novel MEND aggregates in liver sinusoid

The rhodamine-DOPE-labeled MEND signal overlapped with the isolectin B4-stained hepatic sinusoid, indicating that the MEND accumulated in the liver sinusoid. The pattern of accumulation of the LSEC-transfer efficient MEND indicated that this MEND had greater efficacy than the nonselective MEND toward LSECs (Fig. 2).

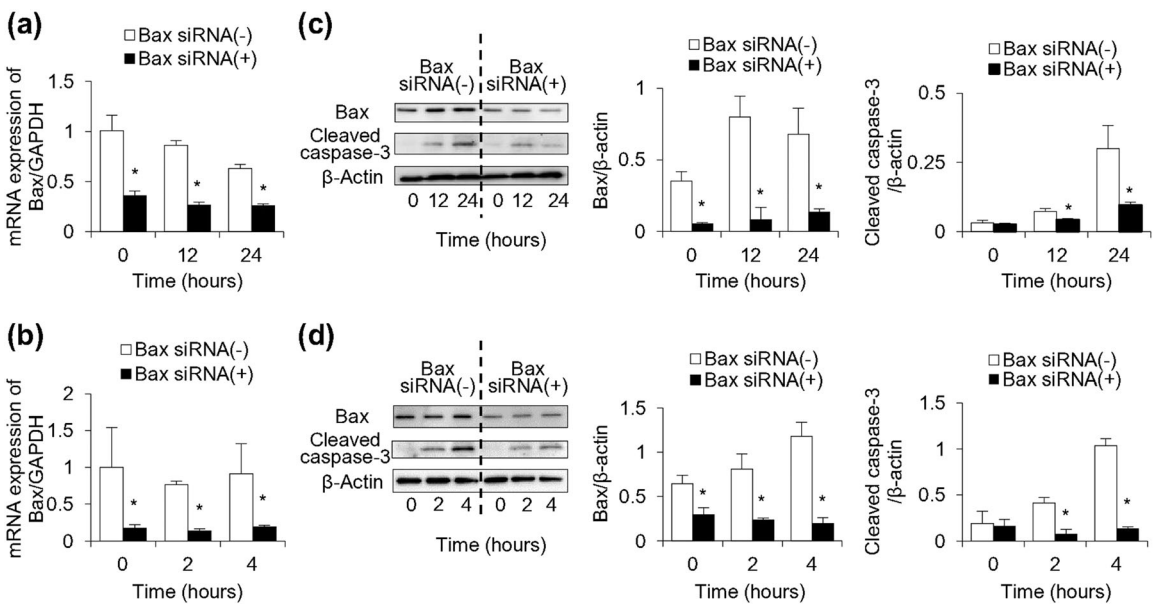

Figure 1 *In vitro* quantitative reverse transcription polymerase chain reaction (RT–PCR) and western blot analyses. (a,b) The expression of Bax mRNA was measured by real-time RT–PCR after administration of (a) the anti-Fas antibody and (b) staurosporine (STS) ($n = 3$ each group). (c,d) Representative immunoblots and densitometry analysis of Bax and cleaved caspase-3 protein expression after administration of (c) the anti-Fas antibody and (d) STS; β-actin served as the loading control ($n = 3$ independent experiments). Mean ± standard deviation. *$P < 0.05$ versus the Bax siRNA(−) group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

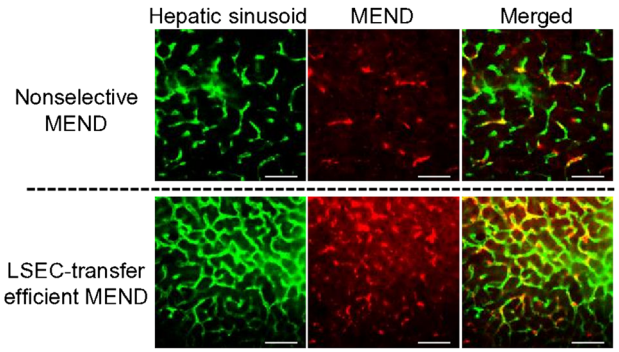

Figure 2 Accumulation of each multifunctional envelope-type nano device (MEND). Accumulation of the nonselective MEND and the liver sinusoidal endothelial cell (LSEC)-transfer efficient MEND *in vivo*. The green and red colors represent isolectin B4 staining of the hepatic sinusoid and the rhodamine-DOPE-labeled MEND, respectively. Scale bars, 50 μm.

Inhibition of Bax in LSECs reduces liver damage

All mice survived at 24 h after the administration of an anti-Fas antibody. Serum ALT was significantly lower in the LSEC-transfer efficient group than in the control and nonselective groups at 6 and 24 h after administration of the anti-Fas antibody (Fig. 3a). At 48 h after administration of the anti-Fas antibody, serum ALT recovered to almost normal, 51 ± 29 IU/L (mean ± standard deviation; $n = 4$, data not shown).

Light microscopy photographs of the HE-stained liver tissues 24 h after administration of the anti-Fas antibody are shown in Figure 3(b). There was less accumulation of inflammatory cells and less disruption of the sinusoidal structure in the LSEC-transfer efficient group compared with the other groups. Necrotic changes were unclear in all of the groups.

Protecting LSECs results in a decrease in hepatocyte apoptosis

Bax mRNA expression in the nonselective and LSEC-transfer efficient groups was significantly lower than that in the control group 24 h after administration of the anti-Fas antibody (Fig. 4a). Bax protein expression in the nonselective and LSEC-transfer efficient groups was suppressed compared with that in the control group 24 h after administration of the anti-Fas antibody (Fig. 4b). Cleaved caspase-3 protein expression was significantly lower in the LSEC-transfer efficient group compared with the other groups (Fig. 4b). Bak mRNA expression in the nonselective group and LSEC-transfer efficient groups was lower than that of the control group, though these differences were not significant (Fig. 4a).

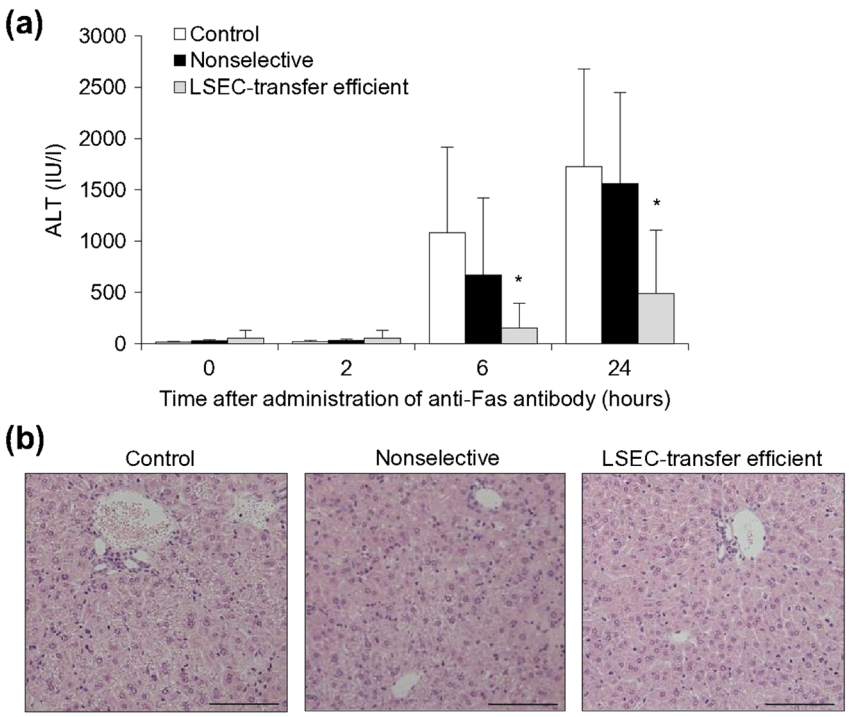

Figure 3 Liver enzymes *in vivo* and hematoxylin–eosin (HE) staining of liver tissues. (a) Changes in serum alanine aminotransferase (ALT) in the control, nonselective and liver sinusoidal endothelial cell (LSEC)-transfer efficient groups at 0, 2, 6 and 24 h after anti-Fas antibody treatment ($n = 7$ each group). Mean ± standard deviation. *$P < 0.05$ versus the other groups. (b) Light microscopy images of HE-stained liver tissues 24 h after anti-Fas antibody treatment (original magnification ×200). Scale bars, 100 μm.

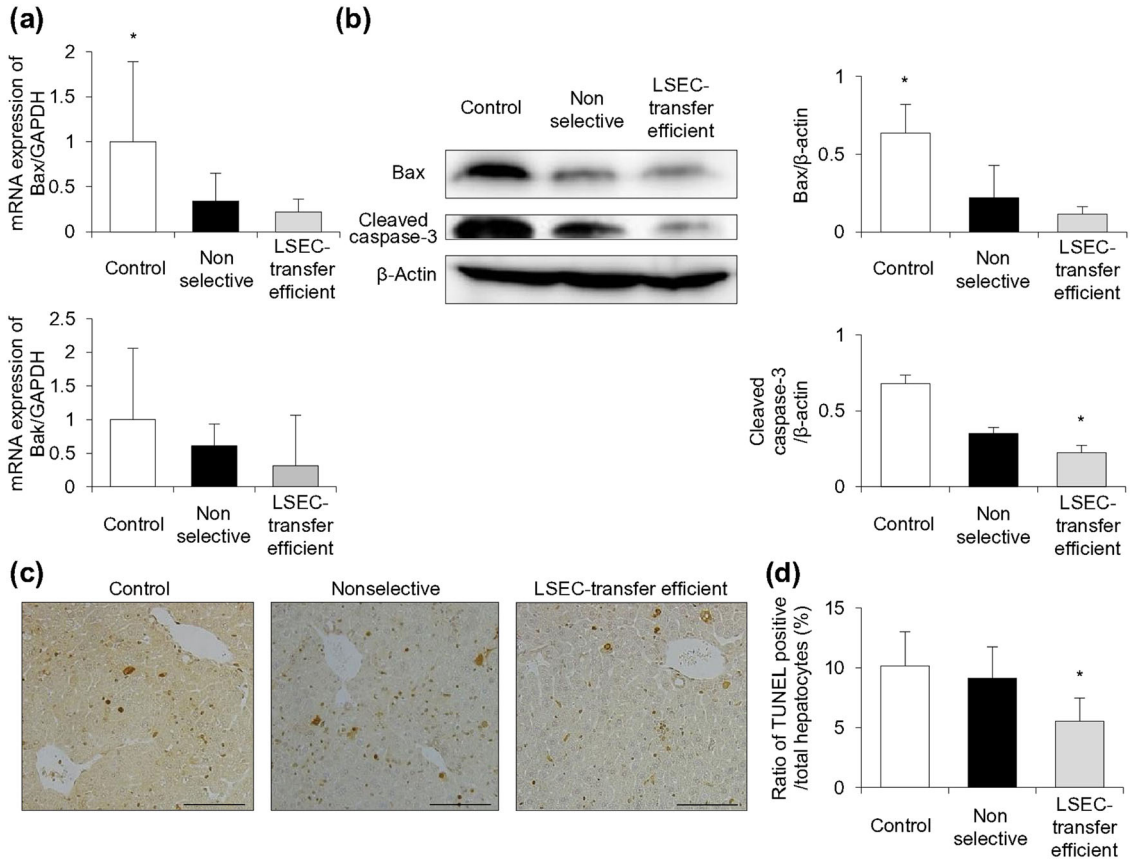

Figure 4 *In vivo* quantitative reverse transcription polymerase chain reaction (RT–PCR), western blot and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay of liver tissues. (a) Bax and Bak mRNA expression in liver tissues was measured by real-time RT–PCR 24 h after anti-Fas antibody administration ($n = 7$ each group). (b) Representative immunoblots and densitometry analysis of Bax and cleaved caspase-3 protein expression 24 h after anti-Fas antibody treatment; β-actin served as the loading control ($n = 3$ independent experiments) (right). (c) TUNEL assay of liver tissues 24 h after administration of the anti-Fas antibody (original magnification ×200). Scale bars, 100 μm. (d) Ratio of TUNEL positive/total hepatocytes ($n = 7$ each group). Mean ± standard deviation. $^*P < 0.05$ versus the other groups. LSEC, liver sinusoidal endothelial cell.

There were fewer TUNEL positive hepatocytes in the LSEC-transfer efficient group than in the control and nonselective groups (Fig. 4c). The ratio of TUNEL positive/total hepatocytes in the LSEC-transfer efficient group was significantly lower than that in the other groups (Fig. 4d).

Suppressing LSEC apoptosis maintains the sinusoidal structure

At 24 h after anti-Fas antibody treatment, the silver-stained sinusoidal structures were better maintained in the LSEC-transfer efficient group compared with the other groups (Fig. 5a). The relative ratio of silver-stained sinusoidal structure around the portal vein was significantly higher in the LSEC-transfer efficient group compared with the other groups (Fig. 5b).

The transmission electron microscopy images of the sinusoidal structure 24 h after anti-Fas antibody administration are shown in Figure 5(c). In the control and nonselective groups, the sinusoidal endothelial lining was destroyed and detached into the sinusoidal space, and Disse's spaces were enlarged. In contrast, the sinusoidal endothelial lining was preserved in the LSEC-transfer efficient group.

DISCUSSION

HEPATOCYTE APOPTOSIS IS the main cause of acute liver failure, and LSEC apoptosis occurs during acute liver failure.[1,2,4,28–32] It is necessary to elucidate the

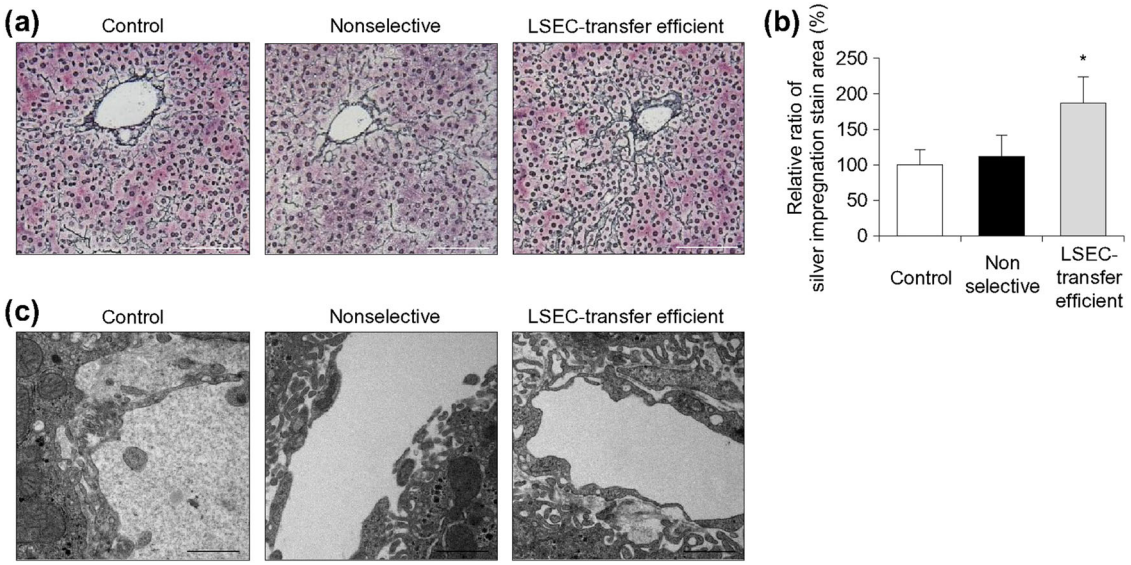

Figure 5 Silver impregnation assay of liver tissues and transmission electron microscopy of liver tissues. (a) Silver staining of liver tissues 24 h after administration of the anti-Fas antibody (original magnification ×200). Scale bars, 100 μm. (b) Relative ratio of the silver-stained sinusoidal area around the portal vein ($n = 7$ each group). Mean ± standard deviation. $^*P < 0.05$ versus the other groups. (c) Transmission electron microscopy of liver tissues 24 h after anti-Fas antibody administration (×6000). Scale bars, 1 μm. LSEC, liver sinusoidal endothelial cell.

pathogenic mechanism by which apoptosis occurs in hepatocytes and LSECs to prevent apoptosis-induced acute liver failure. However, the precise apoptotic mechanism has not been clarified due to the lack of a suitable method for suppressing apoptosis in particular cells of the liver, specifically in hepatocytes and LSECs. Previously, we demonstrated that LSECs are impaired before hepatocyte injury in acute liver failure.[12] Moreover, LSECs play protective roles during inflammation and toxicity.[33,34] Therefore, we hypothesized that LSEC apoptosis prior to hepatocyte apoptosis is strongly associated with acute liver damage. To evaluate this hypothesis, we investigated the efficient suppression of LSEC apoptosis using a novel DDS, namely, a MEND, in a mouse model of acute liver failure. Compared with the nonselective suppression of liver apoptosis, the efficient suppression of LSEC apoptosis decreased the amount of acute liver damage. The present study also revealed that maintaining the hepatic sinusoidal structure by suppressing LSEC apoptosis diminished acute liver injury.

Bax, a pro-apoptotic factor in the mitochondrial apoptosis pathway, is expressed in the liver and promotes liver apoptosis.[10,11] The siRNA-mediated inhibition of Bax suppresses hepatocyte apoptosis *in vitro*.[9,35] Bax is also expressed in LSECs, and the inhibition of Bax has been suggested to suppress LSEC apoptosis.[32,36] However, the function of Bax in LSECs is unknown. The present study clearly indicated that inhibiting Bax in LSECs also has an anti-apoptotic effect *in vitro*.

In an animal model of acute liver failure, the siRNA-mediated inhibition of Bax suppresses hepatocyte and liver damage.[8,9] However, it was unclear whether Bax in LSECs, as opposed to in hepatocytes, has a significant effect on acute liver failure. We previously developed a MEND that is covered with a lipid envelope membrane as a novel DDS for siRNA.[17,18] Various modifications to this MEND can be implemented to specifically deliver the device to target cells within organs.[18,37–40] We previously developed a MEND that accumulates in the liver and is incorporated mainly into hepatocytes.[19,20] However, this MEND is nonselective and targets both hepatocytes and LSECs.[13,14] A novel MEND was developed to inhibit Bax expression and suppress apoptosis in LSECs more efficiently than nonselective MEND.[13,41] We previously reported that the LSEC-transfer efficacy of the novel MEND is higher than that of the nonselective MEND.[13,14]

In this study, a fluorescent staining assay indicated greater accumulation of the novel MEND compared with the nonselective MEND in the sinusoidal structure. Moreover, compared with the nonselective MEND, the novel MEND can encapsulate siRNA to significantly suppress the expression of a specific gene (Tie2) in LSECs.[14] Based on the

results of the current study, the LSEC-transfer efficient MEND is effective as a DDS for Bax siRNA to suppress LSEC apoptosis.

The results of this study indicate that the LSEC-transfer efficient MEND, which suppresses LSEC apoptosis, reduces hepatocyte apoptosis and acute liver damage. Acute liver damage decreases after protecting against or inhibiting LSEC apoptosis.[42,43] LSECs are located between the blood and hepatocytes and play an important role in the stability of hepatocytes.[33] Disruptions in the sinusoidal structure caused by LSEC injury elicit hemorrhage-induced biochemical events and disturb the sinusoidal continuity, leading to secondary hepatocyte damage during acute liver failure.[30,44] In addition, the deterioration of hepatic microcirculation induced by LSEC damage leads to hepatocyte injury.[45–48] The electron microscopy findings of the present study revealed that only the efficient suppression of LSEC apoptosis prevented LSEC disturbances and maintained the morphologies of the sinusoidal structures and Disse's space. We elucidated the importance of efficiently suppressing LSEC apoptosis for maintaining sinusoidal structure. Therefore, the prevention of LSEC apoptosis should become a target for treating acute liver failure.

In this study, despite the administration of a nonselective MEND to inhibit Bax in hepatocytes, it was impossible to suppress liver damage. Hikita *et al.* reported that the suppression of acute liver damage induced by a large amount of anti-Fas antibody was insufficient in mice with Bax-deficient hepatocytes.[36] This result is likely associated with the fact that inhibiting Bax in LSECs, not just hepatocytes, is important for the siRNA-mediated treatment of acute liver failure. Suppressing apoptosis in the entire liver via i.v. siRNA administration was reported to decrease acute liver damage, but this suppression effect was due to the administration of approximately threefold the amount of siRNA as was used in the present study.[6,7,9]

In this study, the dose of Bax siRNA delivered via a nonselective MEND was considered too low to cover the therapeutic range for protecting against LSEC damage. However, the LSEC-transfer efficient MEND enabled the effective reduction of LSEC damage using only a small amount of Bax siRNA. As mentioned above, the novel MEND may be an effective DDS for clinical applications in the treatment of acute liver failure. However, the effect duration, amount of accumulation, and side-effects on other organs of both the siRNA and MEND must be investigated prior to their clinical application.

Bax siRNA sealed in each MEND may be assumed to be action of non-parenchymal cells other than LSECs. Bax expression of non-parenchymal cells in the nonselective and LSEC-transfer efficient groups was predicted to be inhibited almost equally. This may be explained by the fact that non-parenchymal cells contain many Kupffer cells and hepatic stellate cells in addition to LSECs.[49] However, we did not describe the effect of Bax siRNA sealed in each MEND on non-parenchymal cells in the present study. It was not expected to separate LSECs from non-parenchymal cells with sustaining the efficacy of Bax siRNA.[50] Further investigations are needed to support our results of Bax inhibition in LSECs.

In conclusion, suppressing LSEC apoptosis via the efficient inhibition of Bax in LSECs maintains the sinusoidal structure, thereby reducing hepatocyte apoptosis and liver damage during acute liver failure. In clinical settings, there is no effective treatment for acute liver failure other than liver transplantation. Protecting LSECs and the sinusoidal structure with MEND could be clinically useful for treating acute liver failure.

ACKNOWLEDGMENT

WE THANK DR Saito of the Department of Laboratory Medicine, Jikei University School of Medicine, for providing the liver sinusoidal endothelial cell line M1.

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