GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Protein S Exacerbates Chronic Liver Injury and Fibrosis

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Protein S is a vitamin K—dependent glycoprotein produced mainly in the liver with anticoagulant, anti-inflammatory, immune-modulatory, and antiapoptotic properties. Protein S exacerbates acute liver injury by prolonging the survival of liver immune cells. However, the effect of protein S on chronic liver injury and fibrosis is unknown. Here, we investigated whether human protein S can affect chronic liver injury and fibrosis. Liver injury/fibrosis was induced by carbon tetrachloride injection in mice overexpressing human protein S and in wild-type mice. Human protein S transgenic mice receiving carbon tetrachloride showed significantly higher circulating levels of liver transaminases, increased liver expression of inflammatory cytokines, significantly more extended liver fibrosis, and areas with DNA breakage after chronic injury compared with wild-type mice. Wild-type mice infused with exogenous human protein S exhibited exacerbated liver injury and increased number of hepatic stellate cells compared with untreated mice. Human protein S inhibited apoptosis and increased Akt pathway activation in hepatic stellate cells. The anti-apoptotic activity of protein S may play a role in chronic liver injury and subsequent liver fibrosis. (*Am J Pathol* 2018, 188: 1195–1203; <https://doi.org/10.1016/j.ajpath.2018.01.007>)

Liver fibrosis is the final stage of chronic liver damage caused by viruses, toxins, excessive alcohol intake, and autoimmunity.¹ It is characterized by abnormal deposition of collagen-rich extracellular matrix proteins associated with destruction of the liver parenchymal architecture.² Chronic inflammation may lead to apoptosis of hepatocytes releasing by-products that may in turn stimulate liver resident immune cells [natural killer cells, natural killer T (NKT) cells, Kupffer cells] and recruited inflammatory leukocytes to secrete proinflammatory and profibrotic cytokines.¹ During liver injury profibrotic cytokines, including transforming growth factor β 1, activate hepatic stellate cells (HSCs), leading to excessive secretion and deposition of connective tissue components such as collagen I, tenascins, and periostin that ultimately may result in liver fibrosis and cirrhosis.¹

Protein S (PS) is a vitamin K—dependent glycoprotein synthesized in the liver that functions as a cofactor of activated protein C for inactivating coagulation factors Va and VIIIa to inhibit activation of the clotting cascade.³

Independent of its anticoagulant activity, PS can suppress inflammation and apoptosis through its Tyro3, Axl, and Mer tyrosine kinase receptors.⁴ After binding to Tyro3, Axl, and Mer receptors, PS activates several molecules involved in intracellular signaling pathways such as NF- κ B and Akt, leading to inhibition of inflammatory cytokine secretion and suppression of cell apoptosis.^{5–9} Inhibition of apoptosis of lung epithelial cells by PS attenuates pulmonary fibrosis and its inhibitory activity on apoptosis of islet β cells ameliorates diabetes mellitus in mice.^{10,11} However, administration of exogenous PS has been shown to exert detrimental effects in a mouse model of acute alcoholic hepatitis by its ability to activate and prolong the survival of NKT cells.¹²

We hypothesized that PS administration would worsen the pathologic state in chronic liver injury and fibrosis. To

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demonstrate this hypothesis we compared the severity of liver injury/fibrosis between transgenic mice overexpressing human PS (hPS) and wild-type (WT) mice and between mice treated with human PS and untreated mice.

Materials and Methods

hPS transgenic (hPS-TG) mice were generated as previously described and backcrossed with mice with C57BL/6J backgrounds for >10 generations.¹² Male hPS-TG mice (8 to 10 weeks old) were used in the experiments. Male littermates were used as control mice. To evaluate the efficacy of exogenous hPS approximately 8- to 12-week-old male WT mice weighing 18 to 22 g were purchased from Nihon SLC (Hamamatsu, Japan). Mice were maintained in a specific pathogen-free environment and subjected to a 12-hour light/dark cycle in the animal house of Mie University.

Ethical Statement

The Committee for Animal Investigation of Mie University approved the experimental protocols (approval no. 24-50), and all procedures were performed in accordance with approved institutional guidelines.

Model of Chronic Liver Injury and Fibrosis

Liver fibrosis was induced by intraperitoneal injection of 600 μ L/kg carbon tetrachloride (CCl_4 ; Sigma-Aldrich, St. Louis, MO) dissolved in corn oil at a concentration of 20% and administered twice a week for 8 weeks.¹³ Mice receiving intraperitoneal injection of the same volume of corn oil during the same period were used as controls.

There were four treatment groups in the experiments performed to induce liver fibrosis with the use of hPS-TG mice: WT mice treated with corn oil (WT/OIL) or CCl_4 (WT/ CCl_4), and hPS-TG mice treated with corn oil (hPS-TG/OIL) or CCl_4 (hPS-TG/ CCl_4).

To investigate the effects of hPS administration, WT mice were treated with exogenous hPS (0.23 mg/kg of mouse weight per day) through osmotic minipumps (model 2004; Alzet Corporation, Palo Alto, CA) for 8 weeks, and CCl_4 was administered intraperitoneally for 8 weeks, using the same dose and protocol for CCl_4 as described above. The osmotic minipump of each mouse for hPS administration was replaced for a new minipump on the fifth week of the experiment because the 2004 minipump model lasts only 4 weeks. Control mice received saline through osmotic minipumps and vehicle by intraperitoneal injection.

Blood and Liver Sampling

Euthanasia of mice was performed 3 days after the last CCl_4 injection by pentobarbital overdose, blood was collected in heparinized tubes, the liver was excised, and one portion of it was fixed in 10% formalin for histologic examination and

another part was stored at -80°C for subsequent extraction of mRNA.

Liver Collagen Staining and Quantification

Formalin-fixed liver tissue was embedded in paraffin, and tissue sections 3-mm thick were prepared and stained with hematoxylin and eosin. Tissue fibrotic areas were detected by staining with Masson's trichrome and quantified with WinRoof-2013 image processing software (Mitani Corp., Fukui, Japan). Briefly, microscopic lung fields were randomly photographed in each mouse from all groups with the use of an Olympus BX50 microscope and an Olympus DP70 digital camera (Tokyo, Japan). The liver area stained with collagen was then measured with WinRoof software, and the values were averaged for each individual mouse.

Immunohistochemistry

Immunohistochemical staining with anti-mouse α -smooth muscle actin (α -SMA) (Dako, Kyoto, Japan) was performed with the avidin-biotin-peroxidase complex technique. Positive cells were counted in at least 50 high-power fields, and the results were expressed as a percentage of total cells. Double staining of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) and α -SMA was performed at the Biopathology Institute Corporation (Oitaken, Kokutoshi, Japan), using rabbit anti- α -SMA (Abcam, Cambridge, MA; ab5694), Alexa Fluor 594 goat anti-rabbit IgG, and SlowFade Gold Antifade reagent with DAPI (ThermoFisher Scientific, Waltham, MA).

Evaluation of Apoptosis

DNA fragmentation in liver tissue was evaluated by the TUNEL method with the use of a commercially available kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions. The total area of cells with DNA breakage was measured with the WinRoof image processing software as described above.

Model of Acute Liver Injury

To induce acute liver injury, WT and hPS-TG mice were separated into WT/OIL, WT/ CCl_4 , hPS-TG/OIL, and hPS-TG/ CCl_4 groups as described above, but only one dose of CCl_4 (600 μ L/kg) or similar volume of corn oil was intraperitoneally administered before sacrifice after 24 hours. The blood was collected for measuring liver transaminases, and liver specimens were excised for hematoxylin and eosin staining and for isolation of liver nonparenchymal cells. The methods for isolation and counting of nonparenchymal cells by flow cytometry and the list of commercial antibodies have been previously reported.¹²

Cell Culture

LX-2 human HSC line was kindly provided by Dr. Takato Ueno from the Research Center for Innovative Cancer Therapy of Kurume University, and primary human HSCs were purchased from ScienceCell Research Laboratories (Carlsbad, CA). LX-2 cells were cultured in Dulbecco's modified Eagle's medium and primary human HSCs in stellate cell medium that contained 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin.

Cell Experimental Procedures

Human HSC line, LX-2, or the primary human HSCs were stimulated with 20 µg/mL hPS and cultured for further 16 hours in the presence or absence of 10% fetal bovine serum. Total protein was extracted from cells with radio-immunoprecipitation assay buffer, electrophoresed on SDS-PAGE, and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA). Flow cytometry was used to assess apoptosis of primary human HSCs after cell staining with annexin V and propidium iodide as previously described.¹²

Western Blot Analysis

Phosphorylated Akt, total Akt, and cytochrome P450 2E1 (CYP2E1) and cleaved caspase-3 were detected by Western blot analysis with the use of anti-p-Akt (S473), antibody (rabbit monoclonal antibody; clone D9E; Cell Signaling Technology, Danvers, MA), anti-Akt (rabbit monoclonal antibody; clone C6/E7; Cell Signaling Technology), rabbit polyclonal anti-CYP2E1 (Bioworld Technology, St. Louis Park, MN), rabbit anti-caspase-3 (Cell Signaling Technology), and anti-rabbit IgG-horseradish peroxidase (goat polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies.

Biochemical Analysis

The extent of liver injury was assessed by measuring the activity of plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT), using a Wako Transaminase CII-Test kit according to the manufacturer's protocol (Wako Pure Medical, Tokyo, Japan). Human PS was measured by enzyme immunoassays as described.¹²

RNA Isolation and RT-PCR

Total RNA was extracted from liver tissues with the use of TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Reverse transcription was performed according to standard methods. The expression levels of the mRNAs were determined by real-time PCR with the use of SYBR Premix Ex Taq (Takara, Liaoning, China). The primers used in the experiments were mouse

glyceraldehyde 3-phosphate dehydrogenase, forward: 5'-CCCTTATTGACCTCAACTACATGGT-3', reverse: 5'-GAGGGGCCATCCACAGTCTTCTG-3'; mouse transforming growth factor β1, forward: 5'-ACTCCACGTG-GAAATCAACGG-3', reverse: 5'-TAGTAGACGATGGG-CAGTGG-3'; mouse chemokine (C-C motif) ligand 2, forward: 5'-ATGCAGGTCCCTGTCATGCTTC-3', reverse: 5'-ACTAGTTCACTGTCACTGGTC-3'; mouse α-SMA, forward: 5'-CAGGATGCAGAAGGAGATCAC-3', reverse: 5'-TGTTGCTAGGCCAGGGCTAC-3'; mouse collagen I (Col1a1), forward: 5'-TAAGGGTCCCCAAT-GGTGAGA-3', reverse: 5'-GGGTCCCTCGACTCCTA-CAT-3'; mouse CYP2E1, forward: 5'-ACAGAGACCACC-AGCACAAC-3', reverse, 5'-AATGTCTCTGGATCTG-GAACT-3'; mouse tumor necrosis factor α, forward: 5'-ACGTGGAAGTGGCAGAAGAG-3', reverse: 5'-CTCCT-CCACTTGGTGGTTTG-3'; mouse IL-6, forward: 5'-CTGGTCTTCTGGAGTACCATAG-3', reverse: 5'-AAGT-CAGATACCTGACAACAGG-3'; mouse IL-17, forward: 5'-GGCCCTCAGACTACCTCAAC-3', reverse: 5'-TCTC-GACCCTGAAAGTGAAGG-3'; human glyceraldehyde 3-phosphate dehydrogenase, forward: 5'-GGAGCGA-GATCCCTCCAAAAT-3', reverse: 5'-GGCTGTTGTCA-TACTTCTCATGG-3'; human α-SMA, forward: 5'-GTGT-TGCCCCCTGAAGAGCAT-3', reverse: 5'-GCTGGGA-CATTGAAAGTCTCA-3'; human Col1a1, forward: 5'-AACCTGGATGCCATCAAAC-3', reverse: 5'-TCCA-TGTAGGCCACGCTGTTC-3'; and human fibronectin 1, forward: 5'-CGGTGGCTGTCAAGTCAAAG-3', reverse, 5'-AAACCTCGGCTTCCTCCATAA-3'.

Statistical Analysis

Results are presented as means ± SEM. Statistical difference between groups was calculated by analysis of variance with *post hoc* analysis, using the Fisher's predicted least significant difference test. The StatView version 4.5 package for Macintosh (Abacus Concepts, Berkeley, CA) was used. Statistical significance was defined as $P < 0.05$.

Results

Protein and mRNA Expression of CYP2E1

The protein expression of CYP2E1 was significantly different between WT/OIL and WT/CCl₄ mice and between hPS-TG/OIL and hPS/CCl₄ mice but not significantly different between WT/CCl₄ and hPS-TG/CCl₄ mice. CYP2E1 mRNA expression was unchanged between all groups (Supplemental Figure S1).

Increased Plasma PS Level in WT Mice with Liver Injury

As expected, the plasma hPS level significantly increased in hPS-TG mice compared with WT mice (Supplemental Figure S2). The plasma PS levels significantly increased

Figure 1 Human protein S (hPS) overexpression predisposes to chronic liver injury and attenuates apoptosis of hepatic stellate cells. Wild-type (WT) and hPS transgenic (TG) mice received carbon tetrachloride (CCl₄) or corn oil injection twice a week for 8 weeks. **A:** PS, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) enzyme activities were measured as described in *Materials and Methods*. mRNA expression of tumor necrosis factor (TNF)-α and chemokine (C-C motif) ligand 2 (CCL2) was evaluated by RT-PCR. Liver enzymes and mRNA relative levels of TNF-α and CCL2 were significantly increased in hPS-TG/CCl₄ mice compared with their WT counterparts. **B:** Hematoxylin and eosin staining of mouse livers. Increased fatty degeneration and cell infiltration (arrow) was observed in hPS-TG/CCl₄ mice compared with WT/CCl₄ mice. **C:** Increased area of cells with DNA fragmentation assessed by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) method in hPS-TG/CCl₄ mice and representative images of areas with DNA breakage (arrow). **D:** Western blot analysis showed reduced caspase-3 activation in hPS-TG/CCl₄ mice. **E:** Flow cytometric analysis showed hPS-mediated inhibition of apoptosis of primary human hepatic stellate cells. **F:** Western blot analysis showed enhanced Akt activation by hPS in starved [FBS(-)] LX-2 hepatic stellate cell lines. Data are expressed as means ± SEM. *n* = 4 mice in the WT/OIL group (**A–C**); *n* = 5 mice in the hPS/OIL group (**A–C**); *n* = 7 mice in both the WT/CCl₄ and hPS/CCl₄ groups (**A–C**); *n* = 3 in each group (**D–F**). **P* < 0.05 versus WT/OIL; †*P* < 0.05 versus WT/CCl₄; ‡*P* < 0.05 versus hPS(-)/FBS(-). Scale bars: 50 μm (**B**). Original magnification, ×200 (**B**). FBS, fetal bovine serum.

in the WT/CCl₄ group compared with their WT/OIL counterparts on the third, fifth, and seventh week after starting the experiment (Supplemental Figure S2).

Mice Overexpressing hPS Have Increased Chronic Liver Injury and Increased Area of Cells with DNA Fragmentation

Compared with mice of the WT/CCl₄ group, mice of the hPS/CCl₄ group significantly showed more conspicuous cell infiltration and fatty degeneration in the liver, increased plasma

concentrations of ALT and AST, enhanced liver tissue mRNA expression of tumor necrosis factor α and chemokine (C-C motif) ligand 2, and extensive areas of liver cells with DNA fragmentation (Figure 1, A–C). However, hPS/CCl₄ mice showed a significant reduction of caspase-3 activation in the liver tissue compared with WT/CCl₄ mice (Figure 1D).

hPS Decreases Apoptosis of HSCs *in Vitro*

An *in vitro* experiment showed that hPS significantly inhibited starvation-mediated apoptosis of human primary HSCs

Figure 2 Mice overexpressing human protein S (hPS) are prone to liver fibrosis. Wild-type (WT) and hPS transgenic (TG) mice received carbon tetrachloride (CCl₄) or corn oil injection twice a week for 8 weeks. **A:** Masson's trichrome stain of the liver and quantification by WinRoof (Mitani Corp., Fukui, Japan). Increased staining of collagen (**black arrows**) in the liver from hPS-TG mice compared with WT mice. **B:** Immunohistochemical staining with antibody against α -smooth muscle actin (α -SMA) and quantification of cells per high-power field (HPF). Increased staining of α -SMA(+) cells (**white arrows**) in the liver from hPS-TG mice compared with WT mice. **C:** mRNA expression of α -SMA and collagen I (Col1a1) was measured by RT-PCR. Increased expression of α -SMA and Col1a1 in the liver from PS-TG mice compared with WT mice. Data are expressed as means $\pm$ SEM. $n = 3$ mice in the WT/OIL group; $n = 3$ mice in the hPS/OIL group (**A–C**); $n = 5$ mice in both the WT/CCl₄ and hPS/CCl₄ groups (**A–C**). * $P < 0.05$ versus WT/OIL (**A–C**); † $P < 0.05$ versus WT/CCl₄. Scale bars: 50 μ m (**A**). Original magnification, $\times 200$ (**B**).

compared with untreated cells (Figure 1E). Analysis of the Akt signal pathway revealed that hPS significantly increased phosphorylation of Akt in LX-2 HSC lines (Figure 1F).

hPS-TG Mice Are Prone to Liver Fibrosis

After chronic liver injury with CCl₄, hPS-TG mice showed significantly enhanced collagen deposition and extensive number of α -SMA(+) cells in the liver compared with their WT counterparts (Figure 2, A and B). The relative mRNA expressions of α -SMA and Col1a1 were also significantly

enhanced in liver tissue from hPS-TG/CCl₄ mice compared with WT/CCl₄ mice (Figure 2C).

hPS Exerts No Effect on Fibrogenic Activity of Primary Human HSCs

In the *in vitro* study, hPS showed no effect on mRNA expression of Col1a1, α -SMA, or fibronectin-1 in the presence or absence of transforming growth factor β 1 in primary human HSCs (Supplemental Figure S3).

Figure 3 Administration of exogenous human protein S (hPS) is associated with exacerbation of chronic liver injury and increased number of hepatic stellate cells. Wild-type (WT) mice received carbon tetrachloride (CCl₄) or saline (SAL) injection twice a week and hPS or SAL through subcutaneous minipumps for 8 weeks. **A:** Hematoxylin and eosin staining of mouse livers. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzyme activities were measured with commercial kits. Increased fatty degeneration, cell infiltration (**arrow**), and enhanced plasma liver enzyme liver in mice receiving hPS. **B:** Staining of α-smooth muscle actin (α-SMA), nuclei, and DNA fragments by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). Increased number of α-SMA(+) cells in mice treated with hPS. Data are expressed as means ± SEM. *n* = 6 mice in the WT/OIL group (**A**); *n* = 5 mice in the WT/CCl₄ + SAL group (**A**); *n* = 6 mice in the WT/CCl₄ + Exo-hPS group (**A**); *n* = 3 in all groups (**B**). **P* < 0.05 versus WT/OIL; [†]*P* < 0.05 versus WT/CCl₄. Scale bars: 50 μm (**A**). Exo, exogenous; HPF, high-power field.

Administration of Exogenous hPS Is Associated with Exacerbation of Chronic Liver Injury and Increased HSCs *in Vivo*

WT mice received repeated injections of CCl₄ and were treated with or without hPS for 8 weeks. More cell infiltration and fatty degeneration were seen in the liver, and the plasma concentrations of AST and ALT significantly increased in WT mice treated with exogenous hPS compared with untreated WT mice (Figure 3A). In addition, reduction of the number of TUNEL and α-SMA double-positive cells were seen, but enhancement of the number of α-SMA(+) cells was seen in mice treated with exogenous hPS compared with saline-treated mice (Figure 3B).

Increased Percentage of Immune Cells and HSCs in hPS-TG Mice with Acute Liver Injury

The percentage of activated NKT cells and HSCs was significantly increased, whereas the percentage of T cells and apoptotic NKT cells was significantly decreased in the liver tissue from hPS-TG/CCl₄ mice compared with WT/CCl₄ mice (Supplemental Table S1).

Overexpression of hPS Worsens Acute Liver Injury

WT and hPS-TG mice received one injection of CCl₄ and were sacrificed after 24 hours. The plasma concentrations of AST and ALT and the tissue mRNA expressions of tumor

Figure 4 Overexpression of human protein S (hPS) worsens acute liver injury. Wild-type (WT) and hPS transgenic (TG) mice received one intraperitoneal injection of carbon tetrachloride (CCl₄) or corn oil and were sacrificed after 24 hours. The plasma levels of liver enzymes and the mRNA expression of tumor necrosis factor (TNF)- α , IL-17, chemokine (C-C motif) ligand 2 (CCL2), and IL-6 were significantly enhanced in mice overexpressing PS. Data are expressed as means $\pm$ SEM. $n = 3$ mice in the WT/OIL and hPS/OIL groups; $n = 4$ mice in the WT/CCl₄ and hPS/CCl₄ groups. * $P < 0.05$ versus WT/OIL; † $P < 0.05$ versus WT/CCl₄. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

necrosis factor α , IL-17, CCL-2, and IL-6 were significantly increased in hPS-TG mice compared with WT mice (Figure 4).

Discussion

PS can protect against excessive blood coagulation activation by increasing the anticoagulant activity of activated protein C, directly inhibiting the prothrombinase complex, and accelerating fibrinolysis.^{3,4} PS is also protective against enhanced inflammatory responses by promoting phagocyte-mediated clearance of apoptotic cells, abolishing activation of the complement system, and inhibiting Tyro3, Axl, and Mer receptor-mediated expression of cytokines and cell apoptosis.^{14–17} However, overprotection by PS may also be counterproductive under certain conditions, including

cancer by inhibiting apoptosis of malignant cells and acute alcoholic hepatitis by prolonging survival of activated NKT cells.^{12,18} Here, we assessed the effect of hPS overexpression on chronic injury and fibrosis induced in the liver by CCl₄ and found that hPS-TG mice receiving CCl₄ injection had significantly increased liver injury, inflammatory changes, enhanced liver mRNA expression of inflammatory cytokines, and significantly enhanced area of liver cells with DNA fragmentation compared with their WT counterparts. CCl₄-associated injury may lead to liver fibrosis by increasing the number and differentiation of HSCs to myofibroblasts that secrete excessive extracellular matrix proteins.^{19,20} Here, we demonstrated that mice overexpressing hPS have less caspase-3 activation during liver injury and, with the use of an *in vitro* cell culture system that hPS can activate the antiapoptotic Akt signal pathway and decrease apoptosis of HSCs, pointing to a role for hPS in liver fibrosis. Consistent with this assumption, evaluation of tissue fibrosis markers revealed significantly enhanced collagen deposition, increased number of α -SMA-positive myofibroblasts, and significantly elevated mRNA expression of collagen I and α -SMA in the liver from PS-TG/CCl₄ compared with WT/CCl₄ mice. However, an *in vitro* study showed that PS is unable to directly stimulate the secretion of fibrotic factors from primary human HSCs. Overall, these observations suggest that high expression of hPS may promote chronic liver injury and fibrosis by prolonging the survival of activated HSCs.

Reports have shown reduced circulating levels of PS in patients with advanced liver fibrosis, but studies also report no significant difference compared with healthy controls.^{21–23} This discrepancy may be due to differences in disease stage of the patients. In this regard, a recent study performed in patients with different model for end-stage liver disease scores showed that the activity of PS relative to the international normalized ratio increases in parallel to the model for end-stage liver disease scores.²⁴ To date no published work has shown the sequential changes of plasma PS during the transition from hepatitis to liver fibrosis.²⁵ Here, we evaluated sequential changes in the plasma PS levels after CCl₄ injection and demonstrated that it increases in this model of chronic liver injury. Extrahepatic source of PS may also explain the increased circulating level of PS during liver injury. Lymphocytes, megakaryocytes, osteoblasts, cardiac muscles, vascular endothelial cells, lung, and kidney epithelial cells can synthesize and secrete PS.^{15,26–30}

To confirm the harmful effect of hPS, mice receiving several injections of CCl₄ were treated with exogenous hPS or saline alone for the same period of time, and the results were compared. Liver damage and inflammation were significantly worse in mice treated with exogenous hPS than in mice treated with saline, recapitulating the effect observed in mice with PS overexpression. In addition, we compared the number of HSCs in the liver between mice treated with exogenous PS and mice treated with saline and

found that exogenous administration of hPS enhances the number of HSCs in mice with liver injury. These results suggest that hPS can promote liver fibrogenesis by favoring the survival of HSCs during liver injury.

In a previous study, we demonstrated that overexpression of PS exacerbates the inflammatory response in acute alcoholic hepatitis.¹² To clarify whether overexpression of hPS worsens the acute inflammatory response caused by CCl₄, one intraperitoneal dose of CCl₄ was administered to hPS-TG or WT mice and the mice were sacrificed after 24 hours to make comparative analysis. hPS-TG mice receiving CCl₄ showed significantly higher plasma levels of liver enzymes and higher mRNA expression of inflammatory cytokines and chemokines than their WT counterparts, suggesting the injurious role of hPS in CCl₄-induced acute liver injury. Interestingly, there was a significantly increased number of T cells and HSCs and significantly increased activated NKT cells with significantly decreased apoptotic NKT cells in hPS-TG/CCl₄ mice compared with WT/CCl₄ mice, suggesting the participation of NKT cells and profibrotic HSCs in the chronic damage and fibrosis of the liver.

Conclusion

The results of this study suggest that hPS worsens chronic inflammation and tissue fibrosis in the liver.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.01.007>.

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