Placental extract ameliorates liver fibrosis in a methionine- and choline-deficient diet-induced mouse model of non-alcoholic steatohepatitis

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ABSTRACT

Non-alcoholic steatohepatitis (NASH) is a severe form of fatty liver disease that is defined by the presence of inflammation and fibrosis, which ultimately leads to cirrhosis and hepatocellular carcinoma. We previously showed that human placental extract (hPE) was intramuscularly injected to ameliorates liver injury in a methionine- and choline-deficient (MCD) diet-induced NASH model. In the present study, we investigated the effects of hPE using dB/db mice which exhibit obesity and insulin resistance and are thought to reproduce the pathological background of NASH. The MCD-diet induced liver atrophy accompanied by fibrosis around the liver sinusoids. hPE dose-dependently reduced the perivascular fibrosis. Moreover, αSMA-positive activated hepatic stellate cells increased in number in mice on the MCD diet, with this effect reversed by hPE treatment. hPE significantly decreased expression of Acta2, Col1a1, and Tgfb1 genes in hepatic stellate cells, and inhibited Smad phosphorylation. Moreover, hPE treatment increased the expression of the antioxidative genes Hmox1, Nqo1, Cat, and Sod1, and significantly enhanced nuclear factor erythroid 2-related factor 2 activity. Furthermore, hPE decreased the expression of Nox4 and attenuated the levels of intracellular reactive oxygen species. These results, along with our previous study, suggest that hPE effectively ameliorates liver fibrosis in NASH. This beneficial effect may, in part, be due to suppression of hepatic stellate cell activation.

Non-alcoholic fatty liver disease (NAFLD) is a common chronic liver disease and a major indicator of metabolic syndrome (22). Non-alcoholic steatohepatitis (NASH) is a more severe form of NAFLD that is broadly defined by the presence of steatosis with inflammation and progressive fibrosis. The progression of chronic inflammation and fibrosis in NASH can lead to the development of cirrhosis or hepatocellular carcinoma (5, 25).

Liver fibrosis is a phenomenon in which there is an accumulation of extracellular matrix proteins, including collagen, and is detected in most types of chronic liver disease. It is thought to be a wound-healing response to liver injury (3). Within the liver, fibrosis is a process aimed at maintaining homeostasis despite organ injury; however, an excess accumulation of extracellular matrix proteins causes irreversible collapse of the hepatic parenchyma. Recently, NASH was recognized as a major cause of liver fibrosis (4). NASH is thought to be a com-

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Abbreviations
NASH, non-alcoholic steatohepatitis; hPE, human placental extract; MCD, methione- and choline-deficient; HSC, hepatic stellate cell
nant of metabolic syndrome, which is characterized by obesity with insulin resistance. Because the incidence of metabolic syndrome is rapidly increasing, the prevalence of NASH is becoming an important medical issue worldwide. At present, there are no effective and safe therapeutic options available to treat liver fibrosis. Activated hepatic stellate cells (HSCs), formerly known as lipocytes, Ito cells or perisinusoidal cells, have been identified as major collagen producers within the injured liver (7). When affected by chronic liver disease, HSCs acquire dramatic fibrogenic properties (8) and could be the major cause of the progression of liver fibrosis.

Placental extract can be prepared by hydrolysis of the placenta with both hydrochloric acid and enzymatic digestion. Placental extract contains amino acids, nucleic acid bases, minerals, diketopiperazine, dipeptides, and unknown materials (11, 15, 19, 34). Human placental extract (hPE) has been prescribed clinically to treat chronic hepatitis, liver cirrhosis, and other hepatic diseases. In an experimental animal model of hepatitis, hPE reportedly ameliorated hepatic injury through liver regeneration and inhibition of inflammatory reactions and hepatocyte apoptosis (15, 32). Moreover, Shimokobe et al. reported that hPE is effective in NASH patients who were unresponsive to lifestyle intervention (26). In that study, patients treated for eight weeks with a hPE formulation exhibited a significant reduction in serum transaminases, and suppression of inflammation and hepatocyte damage was noted in liver biopsies. As it is safe and well tolerated, we hypothesized that hPE administration could be an effective approach to the treatment of NASH.

In an earlier study, we generated a NASH model by feeding a methionine- and choline-deficient (MCD) diet with high-salt loading (8% NaCl in the drinking water) to mice deficient in the vasoprotective molecule RAMP2 for 5 weeks (36). In this model, fibrosis was observed in regions adjacent to the sinusoids in control mice but the fibrosis was less pronounced in hPE-treated mice. Liver inflammation and oxidative stress were also suppressed in the hPE-treated mice. We concluded that these beneficial effects of hPE are in part attributable to its protective effects on liver sinusoidal endothelial cells.

In the present study, we generated a NASH model using dB/db mice which exhibit obesity and insulin resistance due to a genetic leptin deficiency, and are thought to best reproduce the pathological background of NASH. After feeding the MCD diet to dB/db mice, we evaluated the efficacy of hPE by intramuscular injection, focusing specifically on its effect on liver fibrosis. Furthermore, to clarify the underlying mechanistic effects of hPE, we investigated the expression levels of hepatic fibrosis-associated genes, molecules related to modulating oxidative stress, and the cytokine signaling pathway.

MATERIALS AND METHODS

Human placental extract (hPE). Human placental extract was produced by Japan Bio Products Co., Ltd. (Tokyo, Japan) through the hydrolysis of human placenta with hydrochloric acid and pepsin.

Animals. Seven-week-old dB/db male mice purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) were used for the study. All mice were maintained according to a strict procedure under specific pathogen-free conditions in an environmentally controlled (12-h light/dark cycle; room temperature, 22 ± 2°C) breeding room at the Division of Laboratory Animal Research, Department of Life Science, Research Center for Human and Environmental Sciences, Shinshu University. Before the operative procedures, the mice were anesthetized through intraperitoneal injection of a combination anesthetic that included 0.3 mg/kg of medetomidine (Nippon Zenyaku Kogyo Co. Ltd., Koriyama, Japan), 4.0 mg/kg of midazolam (Astellas Pharma Inc. Tokyo, Japan), and 5.0 mg/kg of butorphanol (Meiji Seika Pharma Co. Ltd., Tokyo, Japan). All animal handling procedures were in accordance with a protocol approved by the Ethics Committee of Shinshu University School of Medicine.

NASH model. The study protocol is shown in Fig. 1A. The MCD diet (calorie percentages for carbohydrates, protein, and lipid were 58.2%, 18.3% and 23.5%) was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Mice were fed a normal diet until they were 8 weeks old, after which they were fed either the normal control diet or the MCD diet for 8 weeks (challenge period). Mice were intramuscularly administered 0.4 mL of hPE (50.4–806.4 mg/kg) or control saline twice a week during the challenge period (12, 21, 24).

Histology and immunohistochemistry. Livers excised from mice were fixed for 24–48 h in 10% formalin and embedded in paraffin, after which the tissues were cut into 5-μm-thick sections. The sections were used for sirius red staining or immunohistochemistry. For immunohistochemical analysis, the liver sections were first incubated with mouse anti-human
α-smooth muscle actin (αSMA) antibody (Agilent Technologies, CA) and then stained using simple stain MAX-PO (M) and simple stain DAB solution (NICHIREI BIOSCIENCE INC, Tokyo, Japan). Images of each section were obtained using a microscope (BZ-9000; KEYENCE, Osaka, Japan). Images of 5 randomly selected intralobular 400x fields were taken, and the staining-positive areas were calculated using Image J software.

**Cell culture and drug treatments in vitro.** RI-T (JCRB1088; JCRB Cell Bank Osaka, Japan) cells, a rat hepatic stellate cell line, were cultured in RPMI1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in humidified air containing 5% CO₂. RI-T cells were treated with hPE for 24 h and then stimulated with transforming growth factor β1 (TGF-β1) for 24 h.

Rat hepatic stellate cells (HSCs) were isolated from a 12-week-old male Sprague-Dawley (SD) rat (SLC, Shizuoka, Japan). After digestion with collagenase and pronase, HSCs were separated by centrifugation through a Stractan gradient (15). Isolated rat HSCs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in humidified air containing 5% CO₂. Cells were treated with hPE for 24 h (14, 35).

**Quantitative real-time PCR (qPCR).** Total RNA was extracted from rat liver tissues using RNeasy Mini Kit (Qiagen) and then submitted to cDNA synthesis using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa Bio Inc., Japan). Quantitative real-time PCR was performed using FastStart Essential DNA Green Master in a LightCycler 96 System (Roche). Primer sequences are described in Table 1. The data were analyzed using the 2−ΔΔCT method and normalized with rat Gapdh mRNA as endogenous control.

**Western blot analysis.** RI-T cells were lysed in a cell lysis buffer containing protease and phosphatase inhibitor cocktails. Twenty micrograms of protein were loaded into 10% SDS-PAGE gels, electrophoresed under reducing conditions, and transferred onto PVDF membranes. Blots were probed with rabbit anti-human SMAD3 (Abcam, MA, USA), rabbit anti-human phospho-SMAD3 (Ser423, Ser425) (Thermo Fisher Scientific, USA), and mouse anti-human β-actin (Santa Cruz Biotechnology, CA, USA) antibodies and sheep anti-mouse horseradish peroxidase (HRP)–conjugated secondary antibodies (GE Healthcare, UK). The bounded antibodies were detected with WesternSure PREMIUM Chemiluminescent Substrate (LI-COR, Lincoln, NE) and captured using a LAS-3000 LuminoImage analyzer (Fujifilm, Tokyo, Japan).

**Luciferase assay.** Three tandem copies of the ARE consensus sequence of Nqo1 were synthesized, annealed, and subcloned into the pGL4.26 luciferase reporter vector (Promega Corporation, Madison, Wisconsin). RI-T cells were transfected with Lipofectamine 3000 reagent (Invitrogen, Grand Island, NY, USA). A Nuclear factor erythroid 2-related factor 2 activity (Nrf2)-Luc reporter construct was added to RI-T cells with pGL4.74 (Promega Corporation), serving as an internal control in order to normalize the transfection efficiency. Twenty-four hours after transfection, cells were treated with TGF-β (3 ng/mL) and a serial dose of hPE (1.4, 2.8, 5.6 mg/mL) for 24 h. Luciferase activity was detected by Dual-Glo Luciferase Assay System (Promega Corporation).

**Intracellular reactive oxygen species (ROS) level.** The ROS levels in RI-T cells were evaluated using 2',7'-dichlorofluorescin diacetate (DCFH-DA) staining. Briefly, RI-T cells were seeded at a concentration of 5.0 × 10⁴ cells per well and allowed to adhere for 24 h. Subsequently, cells were washed and treated with a serial dose of hPE (1.4, 2.8, 5.6 mg/mL).

| Gene   | Sequence (5’ to 3’)               |
|--------|----------------------------------|
| GAPDH  | Fw: GGCACAGCTCAAGGCTGAGAATG      |
|        | Rv: ATGGTGTTGGAAGACGGCAGTA       |
| ACTA2  | Fw: TGTGCTTGACTCTGGAGATG         |
|        | Rv: GATACCTCGGCCTACACGG          |
| COL1A1 | Fw: GAGTGAGGCACCCAGCATGA         |
|        | Rv: AGCCCGGATGTCACAAGAAG         |
| TGFBI  | Fw: AGAAGTACACCCCGGTGCTA         |
|        | Rv: TGTGTGATCTTGGTTGTTGCTA       |
| TGFBR1 | Fw: CCTAAATCCCCCAGACAGGC         |
|        | Rv: TGGCTTTTCAGTAGTGGGATG        |
| NOX4   | Fw: GGATCACAGAAGGTCCCTAGC        |
|        | Rv: AGAAGTTTACCGGGTTCACC         |
| HMOX1  | Fw: GTCCACAGATTTGTCCAGGG         |
|        | Rv: GGAGCCATACACAGCTAGG          |
| NQO1   | Fw: TCGGAAGCATTCCAGAGGTTGG       |
|        | Rv: GGGCCAAATACACCTGACG          |
| CAT    | Fw: GCGGATTCTCTGAGAGAGTGG        |
|        | Rv: GACTGTGGAAGATTCGAGG          |
| SOD1   | Fw: AGGGCGCTATTCATCTGCAA         |
|        | Rv: CCCATGCTGCGCTTCAATGG         |

Table 1: Primers used for quantitative real-time PCR
Liver weight changes in dB/db mice fed the MCD diet
At the end of the challenge period (week 8), the livers were resected from all mice and weighed to calculate the liver weight/body weight (LW/BW) ratio. Mice on the MCD diet showed prominent liver atrophy, decreasing in weight to about 20% of that seen in mice fed the normal diet (mean liver weight: normal diet, 3.611 ± 0.115 g; MCD diet, 0.780 ± 0.026 g) (Fig. 1C). hPE administered to mice fed the MCD diet dose-dependently elevated the mean LW/BW ratio relative to hPE (0 mg/kg) group with the increase reaching statistical significance at the highest dose (806.4 mg/kg): 21.00 ± 0.62 mg/g vs. 24.24 ± 0.75 mg/g (Fig. 1D).

Liver fibrosis in dB/db mice fed the MCD diet
We next assessed liver fibrosis through Sirius red staining of liver sections (Fig. 2A). Although dB/db mice fed the normal diet exhibited fatty liver changes with ballooning of the hepatocytes, there was no apparent fibrosis. On the other hand, mice fed the MCD diet not only exhibited the ballooning degeneration of hepatocytes but also showed fibrotic changes (compare red staining in the upper panels of Fig. 2A). The fibrotic changes were detected mainly around the vascular walls of the central veins, portal veins, and sinusoids, but were also seen between parenchymal hepatocytes in some areas. Although the lowest dose of hPE (50.4 mg/kg) did not affect fibrosis, higher doses of hPE (201.6 and 806.4 mg/kg) dose-dependently reduced the fibrotic area within the livers of dB/db mice fed the MCD diet (compare red staining in the lower panels of Fig. 2A). We then relatively quantified Sirius red stained-positive areas in the liver sections (Fig. 2B). The fibrotic area in liver sections from mice fed the MCD diet with no hPE treatment was assigned a value of 1, and the fibrotic areas in other sections were normalized to that group. Mice fed the normal diet showed no apparent fibrosis. The fibrotic area in mice fed the normal diet was calculated to be approximately 10% of that in mice fed the MCD diet. The lowest dose of hPE (50.4 mg/kg) did not reduce the fibrotic area. The two higher hPE doses (201.6 and 806.4 mg/kg) showed a tendency to reduce the fibrotic area, but the effect did not reach statistical significance (201.6 mg/kg hPE, 0.67 ± 0.08; 806.4 mg/kg hPE, 0.60 ± 0.18).

αSMA-positive HSC distribution in dB/db mice on MCD diet
Activated HSCs, the major collagen-producing cells in the injured liver, are immunopositive for αSMA.
Mechanism of placental extract

Therefore, we immunostained liver sections from dB/db mice for αSMA (Fig. 3A). Mice fed the normal diet showed no immunopositivity for αSMA, whereas mice fed the MCD diet were strongly positive for αSMA (compare brown staining in the upper panels of Fig. 3A). αSMA-positive cells were mainly detected along the sinusoids. hPE treatment (50.4–806.4 mg/kg) reduced the αSMA immunopositive area in the livers of mice fed the MCD diet (see lower panels of Fig. 3A). We quantified the αSMA immunopositive areas in the liver sections (Fig. 3B). As with fibrotic area (Fig. 2B), the αSMA immunopositive area in sections from mice fed the MCD diet with no hPE treatment was assigned a value of 1, and the immunopositive areas in all other slides were normalized to that group. hPE (50.4–806.4 mg/kg) administration dose-dependently reduced the αSMA immunopositive areas relative to hPE (0 mg/kg)
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in HSCs. hPE treatment inhibited Smad phosphorylation at concentrations between 2.8 and 5.6 mg/mL relative to TGF-β group without hPE (Fig. 4B, C).

Anti-oxidative effect of hPE in HSCs in vitro
Total RNA was extracted from rat primary HSCs which were incubated with TGF-β in the presence of hPE. qPCR analysis showed that hPE treatment increased the expression of the anti-oxidative genes Hmox1, Nqo1, Cat, and Sod1 relative to the control (Fig. 5A). These antioxidants and enzymes are partially sufficient to stimulate Nrf2 pathway. hPE treatment significantly enhanced NRF2 activity relative to TGF-β group without hPE in a dose-dependent manner (Fig. 5B). Furthermore, hPE decreased the expression of Nox4 (Fig. 5C) and at-

Effect of hPE on the expression of tissue-related genes and the TGF-β/Smad signaling pathway in HSCs in vitro
hPE treatment significantly decreased expression of the Acta2 gene, which is related to the activation of HSCs, and of fibrosis-related genes, such as Col1a1 and Tgfb1, while the expression of Tgfr1 was unchanged (Fig. 4A). TGF-β is known as a crucial mediator in liver fibrosis and is an upstream activator of Smad signaling. Changes in Smad phosphorylation levels were determined by western blot analysis in order to evaluate the effect of hPE on this pathway in HSCs. hPE treatment inhibited Smad phosphorylation at concentrations between 2.8 and 5.6 mg/mL relative to TGF-β group without hPE (Fig. 4B, C).
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fractions, the inside fraction had broad peaks at retention times between 20 and 30 min, corresponding to molecular weights of between 1.4 to 6.5 Da. Meanwhile, the outside fraction showed multiple peaks at retention times between 32 to 46 min, corresponding to molecular weights of less than 1.4 Da, with these same minor peaks present in the inside fraction (Fig. 6B). These results suggest that the molecules, responsible for suppressing hepatic stellate RI-T cell activation has a molecular weight within the range of 1.4 to 6.5 Da.

DISCUSSION

Animal models are useful for studying the patho-

tenuated the levels of intracellular ROS relative to TGF-β group without hPE in TGF-β-induced RI-T cells in a dose-dependent manner (Fig. 5D).

Effect of hPE fractions on suppressing hepatic stellate RI-T cell activation

hPE was separated into two fractions using a dialysis membrane (inside and outside fractions). The suppression of hepatic stellate RI-T cell activation was evaluated using qPCR of Acta2 mRNA. The expression of Acta2 markedly decreased in both fractions relative to TGF-β (Fig. 6A). The inside fraction showed a significantly higher suppression of Acta2 expression than the outside fraction (Fig. 6A). Regarding the molecular weight distributions of these fractions, the inside fraction had broad peaks at retention times between 20 and 30 min, corresponding to molecular weights of between 1.4 to 6.5 Da. Meanwhile, the outside fraction showed multiple peaks at retention times between 32 to 46 min, corresponding to molecular weights of less than 1.4 Da, with these same minor peaks present in the inside fraction (Fig. 6B). These results suggest that the molecules, responsible for suppressing hepatic stellate RI-T cell activation has a molecular weight within the range of 1.4 to 6.5 Da.

Animal models are useful for studying the patho-
In an earlier study, to accelerate the damage of sinusoidal endothelial cells, we modified the MCD model by combining it with high-salt loading and used mice deficient in Receptor activity modifying protein 2 (RAMP2), a molecule involved in mediating endothelial viability (36). Using that model, we showed that hPE treatment ameliorated NASH pathology by preventing the damage of sinusoidal endothelial cells. In the present study, we further investigated the effect of hPE using dB/dB mice which exhibit obesity and insulin resistance due to leptin-deficiency, and are thought to better reproduce the pathological background observed in human NASH. In dB/dB mice, Sirius red staining revealed that the MCD diet evoked fibrosis around the liver sinusoids, and that effect was reproducibly ameliorated by hPE treatment. Clinically, 0.56 mg/kg hPE is prescribed for chronic hepatitis. The effectiveness of drugs in mice is generally 10 times lower than in humans, which makes the hPE dosage used in this study comparable to the clinical dosage. Usage of hPE as medicine is limited to be injected subcutaneously or intramuscularly. Considering reducing a patient burden, oral administration of it may be better than them. To do that, we have been tackling re-

**Fig. 4** hPE attenuates activation of hepatic stellate cells and its TGF-β/Smad signaling pathway. (A) Expression of fibrosis-related genes (*Acta2*, *Col1a1*, *Tgfβ1*, and *Tgfbr1*) in hPE-treated rat primary hepatic stellate cells. The cells were treated with 5.6 mg/mL of hPE for 24 h. All data were normalized with *Gapdh* RNA. (B) SMAD3 phosphorylation level. RI-T cells were treated with TGF-β (3 ng/mL) and hPE (2.8 and 5.6 mg/mL) in serum-free RPMI 1640 for 16 h. Cells were lysed and 20 μg of soluble protein was separated by electrophoresis on an SDS-PAGE gel. Protein phosphorylation was detected by Western blot analysis. (C) Representative images of Western blot and densitometry analysis. Results represent means ± SEM (*n* = 9 for each, *P* < 0.05, ***P* < 0.001, significantly different from Control or TGF-β).
Mechanism of placental extract

...may be mediated by HSCs in addition to sinusoidal endothelial cells, as the suppressive effect of hPE on HSC activation ameliorates liver fibrosis in NASH.

The MCD diet model well replicates the histological features of fibrosis observed in human NASH, however, its metabolic context differs greatly. For example, although dB/db mice are obese, the MCD diet caused body weight loss, contrary to the human disease (16), and a reduction in the liver weight/body weight ratio. Thus, further studies using other models will be required for a complete assessment.

We found that numbers of αSMA-positive activated HSCs were increased by the MCD diet, with this effect reversed by hPE treatment. Even the lowest dose of hPE (50.4 mg/kg) significantly reduced the αSMA-positive area in the liver, and the effect was dose-dependent. Activated HSCs have been identified as the major collagen-producing cells in the injured liver (7, 8), therefore, they may be the major reason for the progression of liver fibrosis. This suggests that the beneficial effects of hPE on NASH may be mediated by HSCs in addition to sinusoidal endothelial cells, as the suppressive effect of hPE on HSC activation ameliorates liver fibrosis in NASH.

Fig. 5 Enhancing anti-oxidative enzymes and suppressing intracellular ROS in hPE-treated hepatic stellate cells. (A) Expression of anti-oxidative enzyme genes (Hmox1, Nqo1, Cat, and Sod1) in hPE-treated rat primary hepatic stellate cells. Cells were treated with 5.6 mg/mL of hPE for 24 h. All data were normalized with Gapdh RNA. (B) NRF2 activity in hPE-treated RI-T cells. NRF2-Luc reporter construct was co-transfected with pGL4.74 (for normalization) into RI-T cells. After 24 h of transfection, cells were treated with TGF-β (3 ng/mL) and hPE (1.4, 2.8, 5.6 mg/mL) for 24 h. Luciferase activity was detected by Dual-Glo Luciferase Assay, and the data was shown as -fold activation relative to the level obtained with transfection of an empty pGL4.74 vector. (C) Nox4 gene expression in hPE-treated rat primary hepatic stellate cells. The Gapdh gene was used to normalize qPCR data. (D) Intracellular ROS levels in hPE-treated RI-T cells. Cells were treated with hPE (1.4, 2.8, 5.6 mg/mL) in serum-free RPMI 1640 for 24 h and then TGF-β (3 ng/mL) was added to the medium. ROS levels were measured through incubation with H2DCFDA. Representative data are shown as means ± SEM (n = 9 for each). *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from control or TGF-β. †P < 0.05, vs. None.
of the effect of hPE on liver fibrosis in NASH. Although several therapeutic strategies have been attempted in efforts to treat NASH, there is as yet no consensus as to how best to evaluate NASH patients or treat them (1). Against that background, hPE is an attractive therapeutic candidate with which to suppress progression from simple fatty liver to NASH.

An excess production and deposition of extracellular matrix proteins (6, 10) such as collagens, occurs when HSCs are activated by multiple different chemokines and cytokines released by Kupffer cells, HSCs, and hepatocytes (33) in hepatic fibrosis. The present study revealed that hPE reduced the expression of Acta2, indicating that hPE inhibited the activation of HSCs. This idea is supported by the decreased expression of Col1a1 in hPE-treated HSCs. In addition, as expression of Tgfb decreased upon hPE treatment, inhibition of HSC activation by hPE may be partly explained by the decline in Tgfb gene expression because TGF-β is one of most important fibrogenic mediators. Furthermore, hPE treatment inhibited Smad phosphorylation in TGF-β-activated HSCs. These results indicate that inhibition of HSC activation by hPE may occur through attenuation of the TGF-β/Smad signaling pathway in TGF-β-activated HSCs. Therefore, inhibition of HSC activation by hPE may be a key approach to suppress the progression of hepatic fibrosis.

In this study, hPE treatment attenuated the levels of intracellular ROS in HSCs which may be achieved through a number of ways. hPE induced the production of anti-oxidative enzymes, such as HMOX1, NQO1, CAT, and SOD1, and their expression is regulated by NRF2. As hPE activated NRF2 in TGF-β-activated HSCs, increased expression of those enzymes could be due to NRF2 activation by hPE. Additionally, hPE decreased the expression of Nox4. The production of NOX is induced by TGF-β and activation of NOX4 generates ROS during HSC activation (13, 18). Therefore, the decline of ROS levels in hPE-treated HSCs could potentially be attributed to the suppression of Nox4 gene expression by hPE. These results indicate that hPE exhibits effective multifunctional activities in activated HSCs. However, it is still unclear which of the components of hPE play a role in this. It has been reported that some amino acids (tryptophan, phenylalanine, and tyrosine) or uracil have antioxidant activity (28, 31) as they exhibited inhibitory activity on cytochrome P450-dependent lipid peroxidation. In fact, we also confirmed that our hPE had an inhibitory effect on lipid peroxidation and contains, at least, these small anti-oxidative molecules (data not shown). Although it remains unclear if they can activate NRF2 following expression of anti-oxidative enzymes and a decrease in the expression of Nox4, it cannot be excluded that they may play a partial role in the reduction of intracellular ROS in hPE-treated HSCs.

Fig. 6 Measuring molecular weight distribution of the active fraction of hPE. (A) Relative expression of Acta2 in RI-T cells. Cells were pretreated with hPE (5.6 mg/mL), inside fraction (5.6 mg/mL), and outside fraction (5.6 mg/mL) or received no pretreatment for 24 h, and then treated with and without TGF-β1 (3 ng/mL) for 24 h. All data (mean ± SEM, n = 3 for each) were normalized by Gapdh RNA. Representative data are shown. Significantly different from as follows, \( ^* P < 0.01 \) vs. None, \( ^{**} P < 0.01 \) vs. TGF-β, \( ^{**} P < 0.01 \) vs. TGF-β with hPE (5.6 mg/mL), \( ^{**} P < 0.01 \) vs. TGF-β with hPE (5.6 mg/mL). (B) The fractions from hPE (5 mg/mL in water) were analyzed by HPLC (Superdex 30 Increase 10/300 GL column) in 20 mM phosphate buffer (pH 7.2) containing 0.25 M NaCl at a flow rate of 0.5 mL/min. The eluted fractions were monitored at 214 nm. Molecular weights of the peaks were estimated by a calibration curve with glycy1-glutamic acid (186), vitamin B12 (1,355), insulin chain B (3,495), and aprotinin(6,511).
Considering the properties of these molecules, it is speculated that the effect of hPE may be due to the actions of multiple components. In this respect, hPE may be similar to other substances that have multiple functions, such as flavonoids (30). As flavonoids have various active components, it is predicted that they have multiple modes of action. Therefore, the advantage of using such materials is that they may be effective in diseases involving multiple pathways, such as NASH (23).

In order to gain insight into the molecules responsible for preventing HSC activation, hPE was partially purified and its molecular weight was estimated. HPLC analysis indicated that the molecules responsible are potentially located in the inside fraction, ranging from 1.4 to 6.5 Da. However, there have been reports of biologically active substances identified in placental extract with molecular weights of approximately 200 Da or less (2, 34, 35), so our fraction may have different components than those identified in previous reports. In another report, Togashi et al. identified collagen fragments of 25 to 43 kDa in hPE. Considering their molecular weights, these differences may be due to differences in the preparative methods of hPE, in particular the degree of enzyme digestion. In fact, there were no protein bands at 25 kDa or above in SDS-PAGE analysis of our hPE (data not shown). It has also been demonstrated that these large collagen fragments have antioxidant activity in hPE (27). Moreover, since it has been reported that other collagen peptides exhibit antioxidant activity, such as tetra- or henicosa-peptides (9), we suggest that our inside hPE fraction consisting of molecules with molecular weights of 1.4 to 6.5 Da may contain collagen peptides, as well as peptides carrying homologous sequences to the described collagen polypeptides. Our future research will focus on the identification of these small molecules in the inside fraction of our hPE.

In order to understand the mechanisms of the demonstrated anti-fibrotic effect of hPE at the cellular level, primary cultures of rat HSCs and RI-T cells (29) were treated with hPE. It has been reported that rat HSCs cultured on plastic dish spontaneously undergo myofibroblastic transdifferentiation (“activation”) from day 2–3 (37). On the other hand, RI-T cells require inducer for “activation” such as TGF-β (20). *In vivo* study, we demonstrated that HSCs were activated in MCD NASH model mice. Moreover, we used RI-T cells under “activation” state *in vitro* study. Therefore, it is suggested that there is a correlation between them.

Our results showed that hPE could ameliorate perivascular fibrosis in MCD-induced NASH model dB/dB mice. The effect of hPE on HSCs may be mediated by suppressing the activation of HSCs, attenuation of the TGF-β/Smad signaling pathway, and decreasing ROS levels. Further clinical trials on the efficacy of hPE in humans are recommended as it may be a useful therapy to prevent progression from simple fatty liver to NASH.

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CONFLICT OF INTEREST STATEMENT
Taiichi Kaku and Hong Seok Lim are a stockholder of Japan Bio Products Co., Ltd.
Akihiro Yamauchi, Takahiro Tone, Andrea de Toledo, Kyoko Igarashi, Koji Sugimoto, Haruka Miyai, Dawei Deng, Junichi Nakamura, and Eiichi Hirano are employers of Japan Bio Products Co., Ltd.

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