Human recombinant endostatin Endostar attenuates hepatic sinusoidal endothelial cell capillarization in CCl_4-induced fibrosis in mice

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Abstract. The aim of the present study was to detect the effect of the recombinant human endostatin Endostar on hepatic sinusoidal capillarization in CCl_4-induced murine models of liver fibrosis. The liver fibrosis model was induced in BALB/c mice using intraperitoneal injection of CCl_4 for 6 weeks. Animals were divided into the following six treatment groups: Group 1, normal animals; group 2, CCl_4-induced liver fibrosis; group 3, CCl_4+Endostar 20 mg/kg/day for 6 weeks; group 4, CCl_4+Endostar 10 mg/kg/day for 6 weeks; group 5, CCl_4+Endostar 20 mg/kg/day for 4 weeks; and group 6, CCl_4+Endostar 10 mg/kg/day for 4 weeks. The average number of fenestrae per hepatic sinusoid was determined using transmission electron microscopy. Vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) 1 and 2 expression was detected by western blot analysis. There were significant differences in the number of fenestrae per sinusoid between the normal control and untreated model fibrotic mice ($P<0.01$), and between the untreated model and Endostar-treated mice ($P<0.05$). Endostar treatment was associated with reduced levels of VEGFR1 and VEGFR2 in liver tissues ($P<0.01$), as well as with decreased hepatic sinusoidal endothelial cell capillarization in CCl_4-induced mouse models of liver fibrosis, and this effect may involve the VEGF pathway. However, further studies are required to confirm its involvement in other causes of liver fibrosis.

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Abbreviations: SEC, sinusoidal endothelial cells; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; TEM, transmission electron microscopy

Key words: carbon tetrachloride, hepatic fibrosis, Endostar, endostatin, vascular endothelial growth factor, hepatic sinusoidal cell capillarization

Introduction

Liver sinusoidal endothelial cells (SECs) are highly specialized fenestrated cells, without a basement membrane, which constitute the walls of the liver sinusoid. They are unique among other vascular endothelia (1). They serve as the first contact of the liver with the hepatic blood circulation, and the fenestrated structure is important for filtering selected molecules and substances that enter the liver, as well as controlling the exchange between the sinusoidal lumen and the perisinusoidal space (space of Disse). Due to the fenestrae and their lack of basement membrane, circulating lymphocytes come into direct contact with hepatocytes (2).

Dysfunction of SECs is probably one of the initial events in liver injury. Defenestration and capillarization of the sinusoidal endothelium may be major contributors to hepatic failure in cirrhosis (1). Studies have shown close interactions between SECs and hepatic stellate cells (HSCs), as SECs both prevent HSC activation and promote reversion of activated HSCs to a non-activated phenotype (3). Therefore, preserving SEC fenestration is essential for avoiding liver fibrosis and cirrhosis.

Endostar is a recombinant human endostatin introduced by Chinese scientists (4,5). Endostar was approved by the China State Food and Drug Administration in 2005 for the treatment of non-small cell lung cancer (4,6,7), and is considered one of the most valuable anti-angiogenic agents. It suppresses vascular endothelial growth factor (VEGF)-stimulated proliferation, migration, and tube formation of human umbilical vein endothelial cells *in vitro* (4). A study demonstrated that combined treatment with Endostar and dexamethasone had synergistic effects against experimental hepatoma growth (8). This combination may therefore provide a novel strategy for improving the management of hepatoma or other angiogenesis-dependent malignancies.

In a previous study, it was demonstrated that Endostar decreased liver fibrosis and necrosis in a mouse model of liver fibrosis induced by CCl_4 and inhibited collagen synthesis in the HSC-T6 rat stellate cell line *in vitro* (9). The present study aimed to further explore the antifibrogenic effects of Endostar by investigating the impact of Endostar on SEC phenotype in CCl_4-induced fibrotic mice, and to better understand the mechanisms underlying this action.

Materials and methods

Mouse model of CCl_4-induced liver fibrosis. The Animal Care and Use Committee of Harbin Medical University (Harbin, China) approved all protocols and procedures. Endostar was purchased from Simcere Pharmaceutical Research (Nanjing, China). The animals were housed in an air-conditioned room at 23-25˚C with a 12 h dark/light cycle for one week prior to initiation of the experiment. All animals received appropriate care during the study with unlimited access to chow and water.

Male BALB/c mice weighing 18-20 g were obtained from Beijing Vital River Laboratory Animal Technology (Beijing, China). Liver fibrosis was induced in the remaining mice by intraperitoneal injection (i.p.) of CCl_4 (Beijing Brilliance Biochemical Company, Beijing, China; 40% CCl_4 in corn oil, 0.2 ml/100 g body weight, twice weekly) for 6 weeks as previously reported (10). The mice were divided into six treatment groups: Group 1, normal mice (n=7); group 2, CCl_4-induced liver fibrosis (n=10); group 3, CCl_4+Endostar (n=7; 20 mg/kg/day for 6 weeks, Endostar was administered simultaneously with CCl_4 injection for 6 weeks); group 4, CCl_4+Endostar (n=7; 10 mg/kg/day for 6 weeks, Endostar was given simultaneously with CCl_4 injection for 6 weeks); group 5, CCl_4+Endostar (n=7; 20 mg/kg/day for 4 weeks, CCl_4 only was administered to mice for 2 weeks, then Endostar was given to mice simultaneously with CCl_4 injection for another 4 weeks); group 6, CCl_4+Endostar (n=7; 10 mg/kg/day for 4 weeks, CCl_4 only was administered to mice for 2 weeks, then Endostar was given to mice simultaneously with CCl_4 injection for another 4 weeks).

After the mice were euthanized (via 2% pentobarbital i.p. at 0.3 ml/100 g body weight), liver samples were obtained from all control mice and mice with CCl_4-induced liver fibrosis, with or without Endostar treatment. A section of the liver was immediately snap-frozen in liquid nitrogen and stored at -80˚C until further use. Another section was embedded in paraffin and sliced into 4 to 5-μm sections.

Transmission electron microscopy (TEM). Samples were processed for TEM as described previously (11). Fresh specimens were fixed in 3% glutaraldehyde, washed with phosphate-buffered saline three times, and fixed in 1% osmic acid for 60 min. The samples were dehydrated through an alcohol series, embedded in EPON 812 epoxy resin (Hede Biotechnology Co., Ltd., Beijing, China), and then cut into 50-nm sections with an ultrathin microtome (EMUC7; Leica Microsystems GmbH, Wetzlar, Germany). After staining with uranyl acetate and lead citrate for 30 min, the sections were observed under a transmission electron microscope (HITACHI H-7650, Tokyo, Japan). Ten hepatic sinusoids with a diameter of 2-3 μm were randomly selected from each group and the average number of fenestrae per hepatic sinusoid was determined.

Table I. Number of hepatic sinusoidal endothelial cell fenestrae in the different groups.

Group (n)	Number of fenestrae
Normal (neither CCl_4 nor Endostar) (7)	7.43±0.98
Model (CCl_4 alone) (8)	2.38±0.91
Lose-dose Endostar (6 weeks) (5)	4.60±0.90[a]
High-dose Endostar (6 weeks) (5)	4.80±0.84[a]
Low-dose Endostar (4 weeks) (6)	3.8±0.84[a]
High-dose Endostar (4 weeks) (7)	4.40±0.90[a]

[a]P<0.01, compared with model group.

Detection of protein levels of VEGF, KDR and FLT1 by western blot analysis. Protein was extracted from liver samples (Protein Extractor IV; DBI, Shanghai, China), homogenized, and assayed using the bicinchoninic acid method (Pierce BCA Protein Assay kit; Thermo Fisher Scientific, Rockford, IL, USA). Protein samples (40 μg) were resolved via SDS PAGE (80 V for 40 min on a 5% acrylamide stacking gel (Beijing Biochemical Company) and 120 V for 70 min on a 10 or 15% running gel), and then transferred to a nitrocellulose membrane (390 MA for 70 min or 80 V for 120 min; Hybond-C Extra Membrane 45; Amersham Biosciences, Uppsala, Sweden).

The membranes were soaked in Tris-buffered saline (10 mM Tris-HCl and 250 M NaCl), that contained 5% non-fat powdered milk and 0.1% Tween-20, for 2 h to block non-specific sites, and incubated with primary antibody overnight at 4˚C in blocking solution. The antibodies were as follows: Rabbit polyclonal anti-mouse VEGF (cat. no. sc-507), FLT1 (cat. no. sc-9029) and KDR (cat. no. sc-504) diluted 1:200 with Tris-buffered saline with Tween-20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-mouse ACTB (1:10,000; cat. no. SAB1403520; Sigma-Aldrich, St. Louis, MO, USA) and horseradish peroxidase (HRP)-linked goat anti-rabbit (cat. no. ZDR-5306) or anti-mouse (cat. no. ZDR-5307) IgG (1:10,000; Zhongshan Goldbridge Biochemical Company, Beijing, China). The resultant blots were washed and incubated with secondary antibody (HRP-linked goat anti-rabbit IgG) for 2 h at room temperature.

Immunoreactivity was visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific). Films were scanned using the Bio-Rad imaging system [ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Sensitivity comparison of the ChemiDoc™ MP Imaging System versus X-ray film was performed using blots of serial dilution of transferrin]. Individual levels of the above protein expression were normalized to β-actin.

Statistical analysis. The results are expressed as the mean ± standard deviation. Statistical analyses were performed

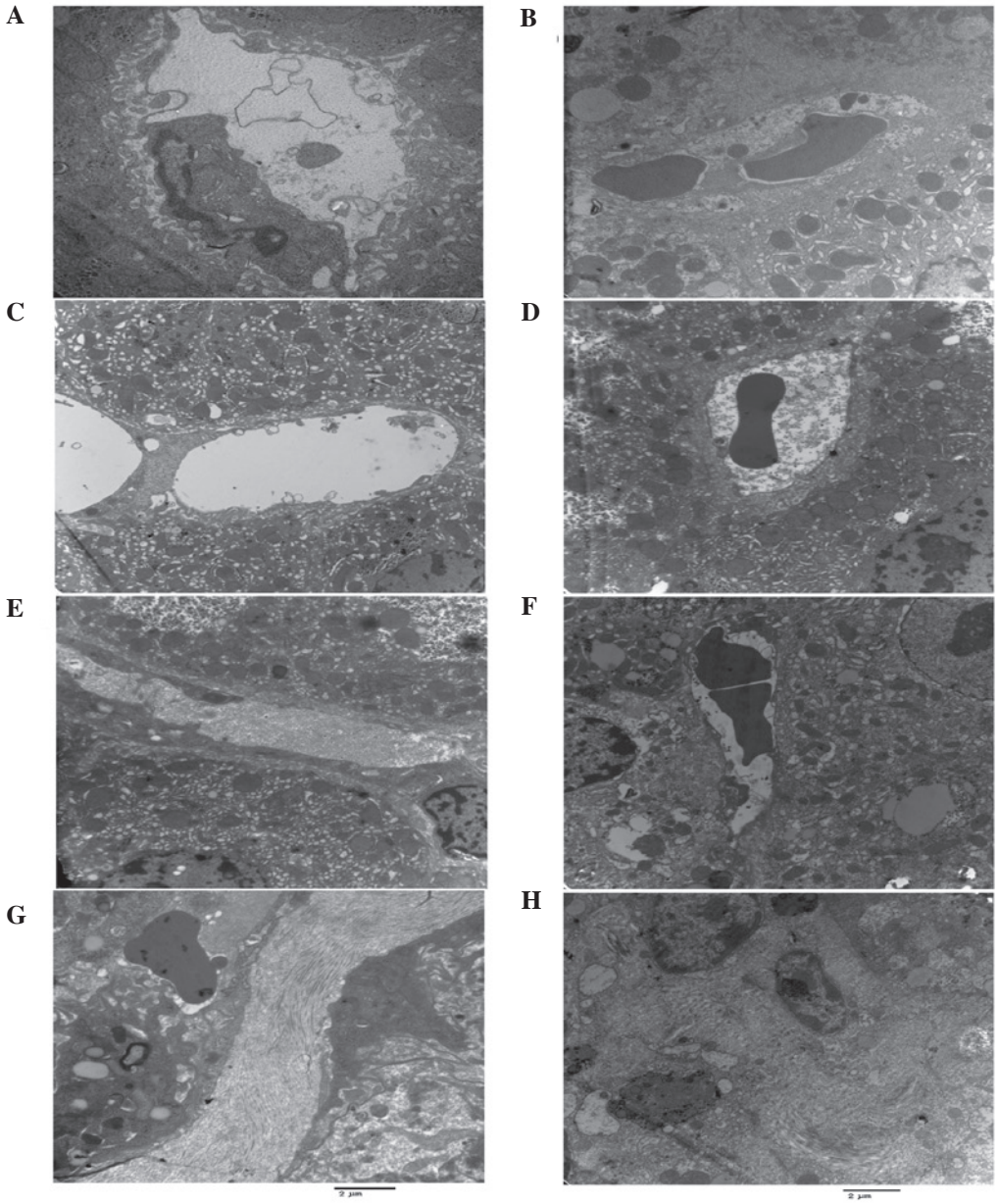

Figure 1. CCl_4-stimulated SEC capillarization, basement and membrane formation, and decreased hepatocyte microvilli. Endostar attenuated SEC capillarization and increased hepatocyte microvilli. TEM slides of the liver from mice in: (A) Group 1, normal mice (normal SECs and fenestrae); (B) group 2, CCl_4-induced fibrosis group [fenestrae, cell junctions (such as tight and ladder-like junctions on the cell surface) and microvilli of hepatocytes reduced and a basement membrane became apparent]; (C) group 3, Endostar 6-week group (high dose): Fenestrae, microvilli and cell junctions between SECs were similar to those of the normal control mice. TEM slides of the liver from mice in: (D) Group 4, Endostar 6-week group (low dose): Fenestrae, microvilli and cell junctions between SECs were similar to those of the normal control mice. (E) TEM slide of the liver from mice in the group 5, Endostar 4-week group (high dose): Fenestrae were marginally decreased compared with those of the normal control mice, the microvilli and cell junctions between SECs became apparent. (F) TEM slide of the liver from mice in group 6, Endostar 4-week group (low dose): Fenestrae and microvilli of hepatocytes were marginally decreased compared with those of the normal mice. (G and H) TEM slides of the liver from mice in group 2: A large quantity of collagen was generated. Stain, uranyl acetate and lead citrate; scale bar, 2 μm. SEC, sinusoidal endothelial cell; TEM, transmission electron microscopy.

using analysis of variance and the unpaired Student's t-test as appropriate. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA).

Results

Ultrastructural changes in fibrotic mice following Endostar treatment. There were significantly fewer fenestrae per SEC in the fibrotic mice relative to those of the normal mice ($P<0.01$; Table I; Fig. 1A and B). In all the groups administered Endostar, the number of fenestrae was significantly higher compared with the untreated fibrotic mice ($P<0.01$; Fig. 1C-F). There were no significant differences among the low dose and high dose of Endostar groups.

There were few or no collagenous fibers around the hepatic central venules in the healthy control group. In the untreated fibrotic model, mature collagen extended from the central vein and the portal area to the hepatic lobules, and the basement

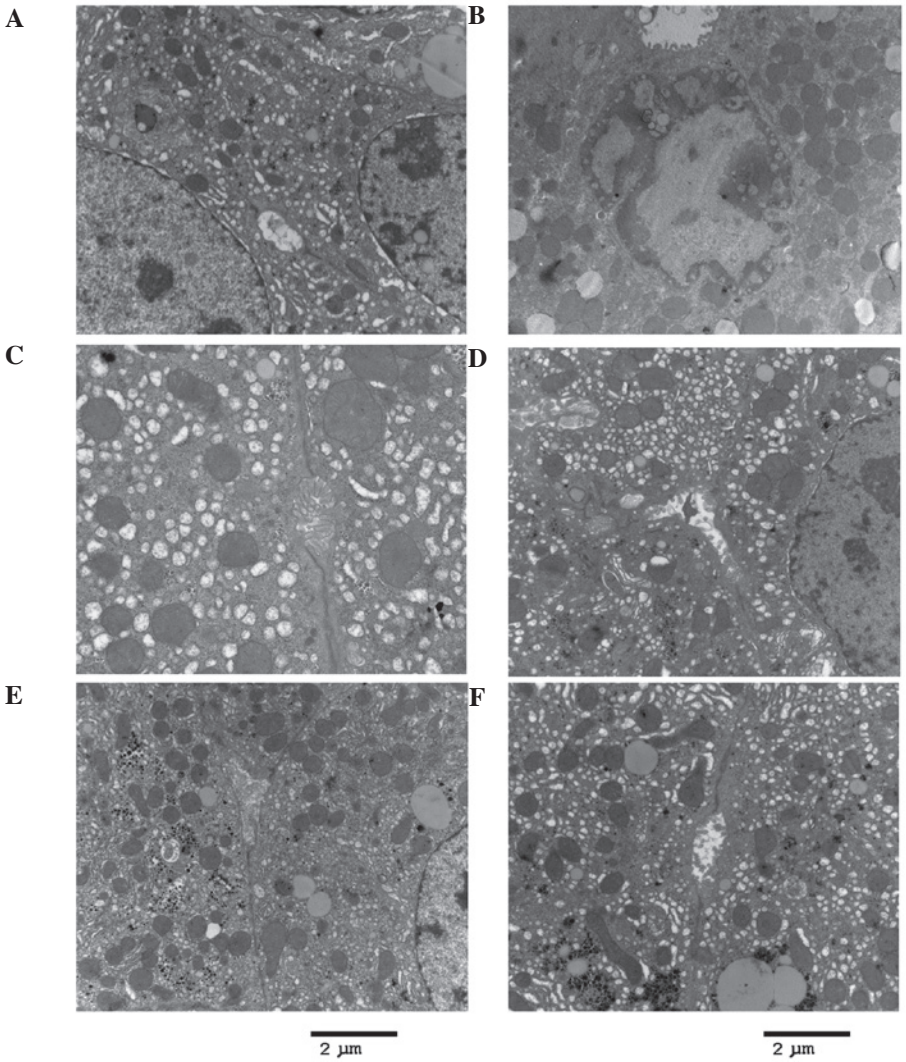

Figure 2. Endostar inhibited apoptosis, necrosis and inflammation in hepatocytes. Transmission electron microscopy slides of the liver from mice in: (A) Group 1, normal hepatocytes; (B) group 2, CCl_4-induced liver fibrosis (hepatocyte apoptosis is apparent); (C) group 3, Endostar 6-week group (high dose; exhibited hepatocyte fission and proliferation); (D) group 4, Endostar 6-week group (low dose; exhibited karyopyknosis in hepatocytes); (E) group 5, Endostar 4-week group (high dose; demonstrated regenerated hepatocytes); (F) group 6, Endostar 4-week group (low dose; demonstrated inflammatory cell infiltration). Stain, uranyl acetate and lead citrate; scale bar, 2 μm.

membrane was observed. In the Endostar groups, there were fewer collagenous fibers compared with the model group (Fig. 1G and H).

After 6 weeks of CCl_4 exposure, the junctions between hepatocytes in the untreated fibrotic models were destroyed, and the microvilli of hepatocytes in the perisinusoidal space and the intralobular bile ducts (cholangioles) disappeared (Fig. 2B). By contrast, the microvilli of hepatocytes were preserved in the Endostar groups (Fig. 2C-F). Whereas expansion and cholestasis were observed in the cholangioles of the fibrotic model mice (CCl_4 alone), they appeared normal in the Endostar groups (Fig. 2). It was also demonstrated that the extent of necrosis and inflammation in hepatocytes was less in the Endostar groups compared with that in the untreated model (Fig. 2).

Effect of Endostar on VEGF, KDR and FLT1 protein levels. VEGF protein levels were significantly higher in the fibrotic model mice than in the normal control mice ($P<0.01$). There were no significant differences between the untreated fibrotic mice and any of the groups treated with Endostar with regard to VEGF expression ($P>0.05$, all; Fig. 4). The levels of FLT1 and KDR in fibrotic model mice were significantly higher than those of the normal controls. However, levels of FLT1 and KDR were significantly lower in the four Endostar-treated groups relative to those of the model ($P<0.05$; Fig. 5).

Discussion

Hepatic fibrosis is a major complication in chronic liver diseases, increasing the risk of cirrhosis and ultimately resulting in hepatic dysfunction and hepatocellular carcinoma. Treating the cause of the liver disease may lead to fibrosis reversal. However, resorting to anti-fibrotic compounds represents an important complementary approach and a major therapeutic challenge.

In the present study, it was demonstrated that Endostar attenuates SEC capillarization and hepatic inflammation in a mouse model of liver fibrosis induced by CCl_4. In addition,

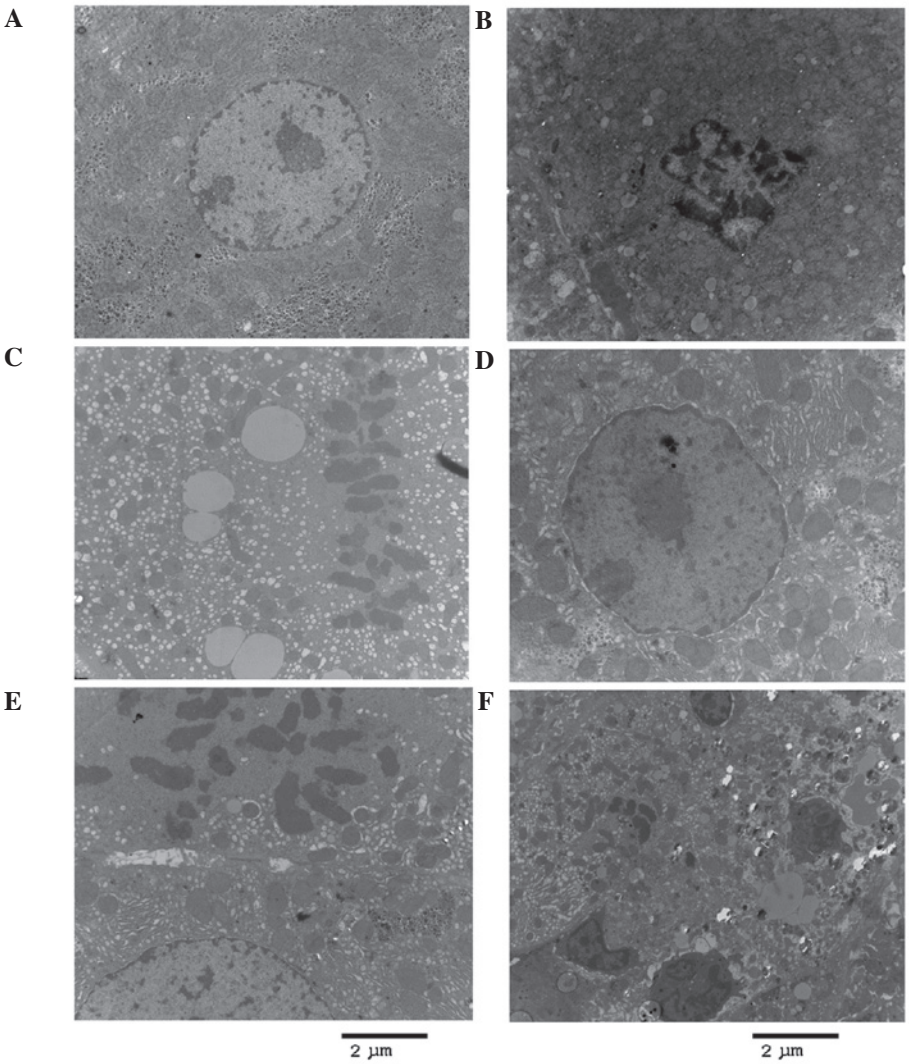

Figure 3. Endostar decreased the damage in cholangioles, microvilli and cell junctions between hepatocytes. Transmission electron microscopy slide of the liver from mice in: (A) Group 1, normal cholangioles, microvilli and cell junctions between hepatocytes; (B) group 2, CCl_4-induced fibrosis group (microvillus, cell junctions reduced, accompanied by cholangiole cholestasis); (C) group 3, Endostar 6-week group (high dose; microvilli and cell junctions appeared, and cholangioles were normal); (D) group 4, Endostar 6-week group (low dose; microvilli and cell junctions appeared, and cholangioles were normal); (E) group 5, Endostar 4-week group (high dose; microvilli, cell junctions and cholangioles were normal); (F) group 6, Endostar 4-week group (low dose; microvilli, cell junctions and cholangioles were almost normal). Stain, uranyl acetate and lead citrate; scale bar, 2 μm.

Endostar treatment was associated with reduced levels of VEGFR protein in liver tissues, suggesting that Endostar may exert its effects through the VEGF signaling pathway.

The results of the present study reinforce the concept that angiogenesis exhibits a major role in liver fibrogenesis. Evidence has been reported that angiogenesis modulates the formation of liver fibrosis, as well as the development of portal hypertension and hepatic carcinoma (12,13). Intrahepatic angiogenesis and sinusoidal remodeling occur in a number of chronic liver diseases. Anti-angiogenesis treatment may be a therapeutic approach in portal hypertension (14). In addition, it has been reported that anti-angiogenesis drugs, such as sorafenib and sunitinib, which are used in the treatment of carcinoma, inhibit not only hepatocellular carcinoma but also liver fibrosis (15-17).

Endostar is another anti-angiogenesis drug, which targets the VEGF-induced tyrosine phosphorylation of VEGFR-2 and ERK/MAPK signaling pathways in human umbilical vein endothelial cells (4). Endostar has also been shown to inhibit angiogenesis and hepatoma growth *in vitro* (8). In the present study, it was demonstrated that Endostar changed the SEC phenotype, leading to attenuated sinusoidal capillarization. In parallel, microvilli disappearance, inflammation, hepatocyte necrosis and bile duct alterations were attenuated in all Endostar groups. These results showed that anti-angiogenesis therapy may inhibit SEC sinusoidal capillarization and attenuate hepatocyte damage.

VEGF promotes angiogenesis by binding to and activating its receptors, FLT1 (VEGFR1), KDR (VEGFR2) and FLT4 (VEGFR3) (18). FLT1 and KDR are predominantly expressed in endothelial cells and cancer cells, while FLT4 is mainly expressed in lymphatic endothelial cells (19-21). VEGR and VEGFRs (KDR and FLT1) are important in the angiogenesis of the cirrhotic liver (22,23). Upregulation of VEGF expression may be a stimulating factor of angiogenesis in CCl_4 and chronic bile duct ligation induced-fibrosis models (24). In the present study, increased VEGF, FLT1 and KDR expression after 6 weeks of CCl_4 exposure was observed. Endostar was able to inhibit FLT1 and KDR expression, but not VEGF

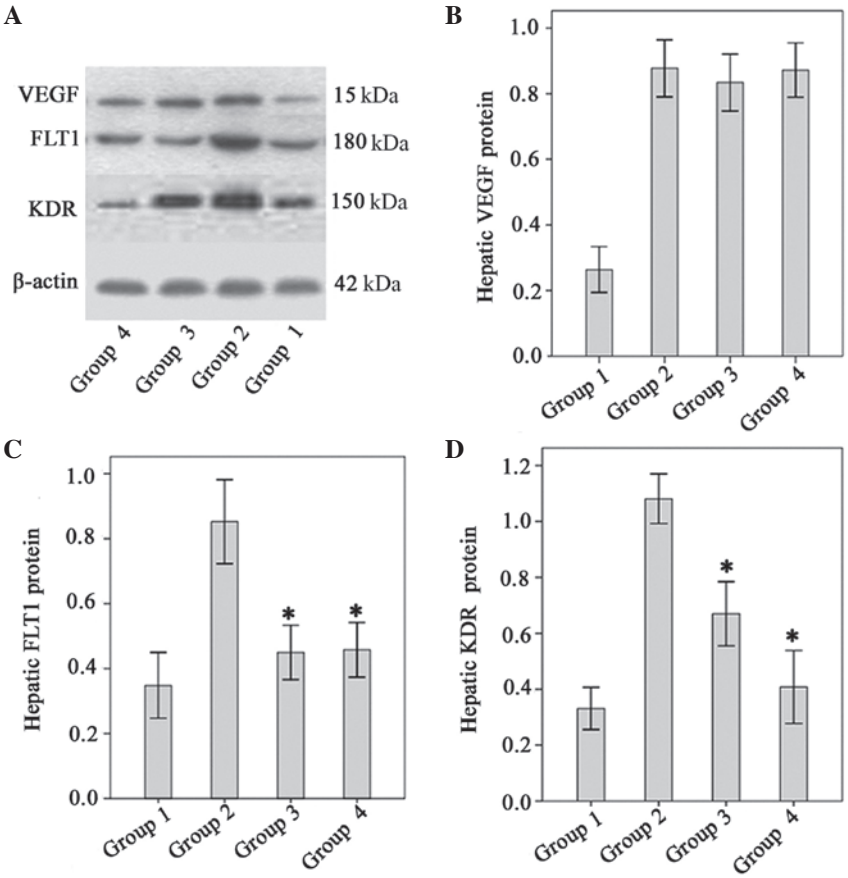

Figure 4. Endostar decreased FLT1 and KDR expression in the 6-week group. (A) Western blot analysis of VEGF, FLT1 and KDR in the liver. Quantification of the protein expression of (B) VEGF, (C) FLT1 and (D) KDR were normalized to an internal control (β-actin) and expressed as the mean ± standard deviation. Group 1, normal hepatocytes; group 2, CCl4-induced liver fibrosis; group 3, Endostar 6-week group (high dose); group 4, Endostar 6-week group (low dose). *P<0.01 vs. the CCl_4-induced liver fibrosis. VEGF, vascular endothelial growth factor.

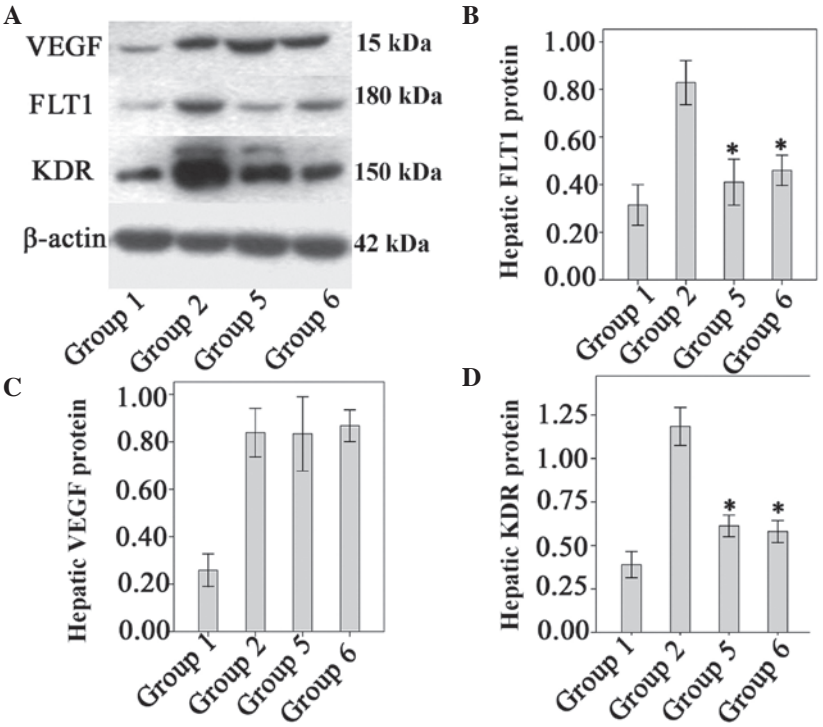

Figure 5. Endostar decreased FLT1 and KDR expression in 4 weeks groups. (A) Western blot analysis of VEGFR1, VEGF and VEGFR2 in the liver. Quantification of protein expression of (B) FLT1, (C) VEGF and (D) KDR. Data were normalized to the internal control (β-actin) and expressed as the mean ± standard deviation. *P<0.01, compared with the CCl_4-induced liver fibrosis group. Group 1, normal mice; group 2, CCl_4-induced liver fibrosis; group 5, CCl_4+Endostar (20 mg/kg/day for 4 weeks); group 6, CCl_4+Endostar (10 mg/kg/day for 4 weeks). VEGF, vascular endothelial growth factor.

expression. Whether Endostar may be a VEGFR blocker and inhibit VEGF binding to VEGFRs remains to be explored.

The molecular mechanism by which Endostar attenuates liver injury remains unknown. SECs may be the primary target cell in this process, and HSCs the secondary one. It has been well documented that VEGF, FLT1, and KDR expression increases in rat HSCs after CCl_4 intoxication (25). Moreover, it is known that paracrine signaling between SECs and HSCs modulates fibrogenesis, angiogenesis and portal hypertension in chronic liver disease (12,17,26,27). It was also demonstrated that Endostar inhibited collagen synthesis and downregulated tranforming growth factor-β1 expression in HSC-T6 cells *in vitro* (data not shown). The nature of the interactions between SECs and HSCs in chronic liver disease after Endostar treatment requires further investigation.

In conclusion, in the present study, Endostar treatment was associated with hepatic sinusoidal endothelial cell capillarization and reduced hepatocyte damage in CCl_4-induced fibrotic mice. These effects may involve the VEGF pathway. Endostar is therefore a promising agent for counteracting hepatic fibrosis. Further studies are required to confirm its involvement in other causes of liver fibrosis and in human chronic liver diseases.

Acknowledgements

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