Inhibition of liver sinusoidal endothelial cells in rats with hepatic fibrosis by injection of VEGF 165 via the inferior vena cava

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Abstract

Background Although VEGF can maintain the normal phenotype of liver sinusoidal endothelial cells (LSECs), it has also been reported that VEGF exacerbates cirrhosis. The role of VEGF in the progression and recovery of cirrhosis has still remained controversial.

Methods We established a cirrhotic rat model by thioacetamide that was used as drinking water; besides, 0, 1, 2, and 4 µg VEGF165 were then continuously injected into the rats. The serum level of hyaluronic acid was measured by ELISA at 0, 1, and 4 weeks, separately. Serum levels of ALT, AST, direct bilirubin, indirect bilirubin, and ALB were detected by an automatic biochemical analyzer. In addition, the levels of VEGF165, CD44, MMP9, MMP2, HIF-1α, and endothelin were detected by Western blotting. The expression level of CD44 in LSECs was detected by immunohistochemistry. Changes of fenestrations of LSECs and basement membranes of blood vessels were observed by transmission electron microscopy.

Results With the increase of dosage and duration of VEGF treatment, the levels of liver function markers in the serum, the levels of CD44, HIF-1α, hydroxyproline and endothelin were significantly improved. With determination of the serum level of hydroxyproline in the blood, it was disclosed that the mentioned level was markedly decreased. In the Sirius Red staining, the stained red area was gradually reduced. Images captured by transmission electron microscopy also confirmed that the ultrastructure of LSECs tended to be normal.

Conclusion VEGF165 can accelerate the resolution of liver fibrosis by promoting fenestration structure formation in LSECs, as well as promoting material exchange between sinusoids and hepatocytes. Our findings may provide a new insight for the study of the role of VEGF in liver fibrosis.

Introduction

The age standardized incidence of cirrhosis increased significantly from 6.6/100000 in 1997 to 89.6/100000 in 2016, and in the same period, prevalence notably elevated from 42% in 1997 to 84% in 2016(1). Cirrhosis, the most common form of alcoholic liver disease, is the ultimate common pathway for many chronic liver diseases. It is within the top 20 causes of disability-adjusted life years and years of life lost,, accounting for 1.6% and 2.1% of the global burden(2).

Liver fibrosis is a wound healing response to a variety of chronic liver diseases, including chronic viral hepatitis, alcoholic liver disease and autoimmune hepatitis. Liver fibrosis is a serious public health problem that may develop into cirrhosis, eventually liver cancer, or even death. The development and validation of anti fibrotic therapy has become the central stage of the study of translational liver disease (3).

Liver sinusoidal endothelial cells (LSECs) are highly differentiated endothelial cells in the wall of hepatic sinuses. Fenestrae, vesicles and channels jointly control most of the endothelial transport between blood and tissues (4). The perisinusoidal space, also known as the space of Disse, is separated from the

sinuses by a layer of endothelial cells (5). The LSECs are typically characterized by the presence of pores (fenestrae). In some pathological conditions, LSEC undergoes "capillarization", which is characterized by the loss of fenestrations and the acquisition of vascular phenotype. Capillarization not only precedes fibrosis, but also changes the morphological characteristics of LSECs which is an integral part of the fibrosis process.

Vascular endothelial growth factor (VEGF) gene is located on human chromosome 6. Alternative splicing of human VEGF mRNA from a single gene gives rise to at least six different isoforms of 121, 145, 165, 183, 189 and 206 amino acid residues (6). Among these isoforms, VEGF165 is freely soluble and can induce angiogenesis, and it is also a predominant isoform secreted by benign and malignant cells (7, 8).

In addition, VEGF and its receptor (VEGFR) have been shown to play major roles not only in physiological angiogenesis, but also in pathological angiogenesis (e.g., cancer). It has been shown that although VEGF is a key factor in maintaining LSEC differentiation (9, 10), VEGF and VEGF-R2 are increased in fibrosis, suggesting that capillarization is due to disruption of signaling downstream of VEGF (11–13). The role of VEGF in the progression and recovery of cirrhosis has still remained elusive. In the present study, the effects of continuous infusion of VEGF165 in the inferior vena cava on sinusoidal capillary vascularization were assessed, and the role of VEGF in liver fibrosis was further explored to provide a new target for the treatment of liver fibrosis.

Materials And Methods

Model establishment and surgical procedures

Establishing a rat model of cirrhosis: Wistar male rats (body weight, 250–300 g) were randomly assigned to normal group and model group. In model group, 0.03% thioacetamide was used as drinking water in the first 5 weeks, and 0.04% thioacetamide was utilized in the last 5 weeks. The total duration of experiment was 10 weeks. The drinking water was replaced every 1–2 days. Western blotting was carried out to detect VEGF165 content in liver cells of rat models to ensure the drug concentration gradient. The efficiency of successfully established rat models was validated by Masson's trichrome staining and hematoxylin and eosin (H&E) staining.

Rats, after successful modeling, were randomly assigned to VEGF treatment group and control group (receiving normal saline). For disposal of the VEGF treatment, VEGF165 protein was dissolved in 2 ml normal saline at concentrations of 0, 1, 2, and 4 µg, respectively. ALZET osmotic pumps are miniature, implantable, research drug delivery systems. The pumps are capsule-shaped and range from 1.5 to 5.1 cm in length and 0.6 to 1.4 cm in diameter. Herein, the ALZET osmotic pump was fixed to the abdominal wall of the rat, and the injection wire was inserted into the inferior vena cava and fixed. VEGF165 was then injected into the inferior vena cava at a rate of 3 µl/h using an ALZET pump, and the liver and blood tissues were harvested at 0, 1, and 4 weeks, respectively. The rats were anesthetized with intramuscular injection of ketamine (75 mg/kg for cirrhotic rats and 150 mg/kg for normal rats), and the gross morphology of the liver and the degree of dilatation of the portal vein and common bile duct were

observed. The skin was sutured after taking the liver specimen, and feeding was continued until the next specimen was harvested. For disposal of the normal saline as control group, the mixture of heparin (50 µg) and normal saline (2 ml) was used as control.

Masson's trichrome stain

The formalin-fixed paraffin-embedded liver tissues were treated with graded concentrations of ethanol and then gradient into water for 2 min, rinsed with warm water at 40°C, stained with R1 staining nuclear solution for 60 s, R2 staining slurry for 30–60 s, R3 yellow-colored separation solution for 6–8 min, and R4 blue complex staining solution for 5 min. The tissues were rinsed with absolute ethanol, blown dry, then mounted with a non-toxic and environmentally friendly mounting medium, and photographed under a microscope.

H&E staining

The paraffin-embedded liver tissues were cut into sections, and H&E staining was carried out for microscopic observation.

Cell Function

An automatic biochemical analyzer was used to measure the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), direct bilirubin, indirect bilirubin, and albumin (ALB). The serum level of hyaluronic acid (HA) was measured by enzyme-linked immunosorbent assay (ELISA).

Hydroxyproline Levels

In this phase, 100 mg of liver tissues in each group were weighed after successful modeling, and then tissues were placed into a test tube. Next, 1 ml of hydrolysate was added, and boiling water bath canning was used for 20 min (adjusted pH value, 6.0-6.8). After that, 20 mg of activated carbon was added to adsorb unhydrolyzed tissues. After centrifugation at 3500 rpm, 1 ml supernatant was extracted and mixed well. The supernatant was incubated at 60°C for 15 min, and then centrifuged at 3500 rpm for 10 min after cooling. After zeroing the instrument with the help of double-distilled water, the optical path was 1 cm at 550 nm. The absorbance of each tube was measured, and the level of hydroxyproline in each group was detected.

Sirius Red Staining

Tissue sections stained with Sirius Red were scanned by Automated Cellular Imaging System III (ACIS III; ChromaVision Medical System Inc., San Juan Capistrano, CA, USA). The outline of each digitized tissue section was manually traced using a computer mouse. Areas of the portal vessels and central veins were manually outlined. The total area of each tissue section and the total area of the portal vessels and central veins were determined by an image analysis system. Fibrotic area was calculated as the total area stained with Sirius Red minus stained perivascular spaces(14).

Immunohistochemical Analysis For Detecting The CD44 Expression

The liver tissues were dewaxed in xylene, hydrated with graded concentrations of ethanol, inactivated with fresh 3% H₂O₂, and heated in a water bath with 0.01 mol/L sodium citrate buffer solution (pH 6.0) to about 95°C. The tissue sections were heated for 10–15 min, blocked with goat serum for 20 min, and then incubated with primary and secondary antibodies. According to instructions of DAB kit for chromogen development (immunohistochemistry), color development was carefully monitored under a microscope. The cells were counterstained with hematoxylin for 2 min, differentiated with HCL-alcohol three times for 2 s each, dehydrated with graded ethanol, and dipped in xylene, and neutral gum (cat. no. BD5044; Bioworld Technology, Inc., St. Louis Park, MN, USA) was dropped on the slice so that the cover slip was replaced before microscopic observation.

Detecting expression levels of VEGF165, CD44, matrix metalloproteinase 9 (MMP9), MMP2, HIF-1 α , and endothelin by Western blotting

Proteins were extracted from liver cells, including samples taken at the same dose and at different time points, in addition to samples taken at different doses and at the same time for detecting expression levels of CD44 by Western blotting. Preparation of 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was performed and the denatured linear protein molecules were loaded onto 5% polyacrylamide gel. Then, electrophoresis was carried out until the target protein was effectively separated. Next, electrophoresis was stopped, and the membrane was electrotransferred. The presence of immunoreactive protein was detected using an electrochemiluminescence (ECL) method to expose X-ray films. Processed films were scanned or photographed, and the integrated optical densities of the bands were analyzed using a LabWorks 4.0 gel image analysis system. The ratios of the integrated optical densities of the proteins to β -actin were calculated. The expression levels of CD44, MMP9, MMP2, HIF-1 α , and endothelin were detected in the same way.

Observation of the fenestrations of sinusoidal endothelial cells and the ultrastructure of liver

The tissue specimen was cut into less than 1 mm³ blocks, fixed in 2.5% glutaraldehyde overnight at 4°C, post-fixed in 2% osmic acid for 1 h, dehydrated in ethanol, and embedded in Epon 812, and ultrathin sectioning was carried out. A 0.5% solution of uranyl acetate was also prepared and used with the lead citrate for staining. The ultrastructure of liver and fenestrae were observed by transmission electron microscopy (TEM).

Isolation And Culture Of Sinusoidal Endothelial Cells

LSECs were isolated from normal rats according to the method of Braet. Normal rats were divided into six groups: in Group A, the cells were incubated with LSEC culture medium without VEGF165; in Group B, the cells were incubated with TAA 6H medium; in Group C, the cells were incubated with TAA 6H followed by 0 ng/ml VEGF165 18H medium; in Group D, the cells were incubated with TAA 6H followed by 40 ng/ml VEGF165 18H medium .

Immunofluorescence Assay

CD31

The slides with crawled cells were washed with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.5% TritonX-100 (prepared with PBS) at room temperature for 20 min. Normal goat serum was added dropwise, and then adequate amount of diluted primary antibody (CD31, species: rabbit, dilution ratio of 1:100) was added dropwise and placed into a wet box and incubated overnight at 4°C. After that, diluted secondary antibody (fluorescent (Cy3)-labeled goat anti-rabbit IgG, dilution ratio of 1:100) was added dropwise and incubated for 1 h in a wet box.

Dil-Ac-LDL

The cell climbing slide of the fourth day was taken and incubated in endothelial cell medium (ECM) containing Dil-Ac-LDL 10 (µg/mL) for 4 h. The ECM was then aspirated and the climbing sheets were fixed with 4% paraformaldehyde for 15 min.

The above-mentioned two steps were continued as follows: To stain the nuclei, cells were incubated with DAPI (1 µg/mL in PBS) for 15 min in the dark, and mounted with mounting solution containing anti-fluorescence quencher, and then images were visualized under a fluorescence microscope.

Phalloidin Staining

The slides with crawled cells were fixed with 4% paraformaldehyde in culture plates, and the diluted phalloidin staining solution (5 µg/mL) was added dropwise and incubated at 37°C for 1 h. To stain the nuclei, cells were incubated with DAPI (1 µg/mL in PBS) for 5 min in the dark, mounted with mounting solution containing anti-fluorescence quencher, and then observed under a fluorescence microscope.

VEGF165 was added to the culture medium at 100 ng/ml for 15 min, and the structures of fenestrated endothelial cells were photographed by 1 h scanning electron microscopy. The digestion solution was prepared with 100 mmol/L PIPES buffer (pH6.9) containing 1 mmol/L EGTA, 4% polyethylene glycol 6000 (PEG, Sigma-Aldrich, St. Louis, MO, USA), and 0.1% Triton X-100, and incubated at room temperature for 1 h. After that, the cell membrane was digested, and the remaining cytoskeleton was fixed and observed via scanning electron microscopy (SEM).

Male BALB/c rats (n = 30; age, 6-week-old; body weight, 220–250 g) were obtained from the Animal Experiment Center of Shandong University (Jinan, China) and fed in a standard laboratory at an air-conditioned room. All experimental protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (7), and approved by the Ethics Committee of Shandong Provincial Hospital affiliated to Shandong University (Jinan, China). All procedures were carried in accordance with the ARRIVE guidelines (<https://arriveguidelines.org>).

Data analysis

In the present study, Image J image analysis software was used to count the following variables: number of fenestrations (within unit surface area), cytoskeleton visualized by SEM, and cytoskeleton visualized by fluorescence microscopy. Besides, tissue sections stained with Sirius red were scanned by ACIS III. All data were statistically analyzed using SPSS 24.0 software (IBM, Armonk, NY, USA), and t-test was used for analysis of measurement data.

Results

1. Surgical procedures

The ALZET osmotic pump was fixed to the abdominal wall of the rat and the injection wire was fixed to the inferior vena cava.

2. Masson's trichrome staining (Figure 1)

The normal hepatic lobular architecture was fully destroyed. Around the central vein, increased amounts of fibrous tissues were observed with activated fibroblasts. The formation of septa, fibrous cords, regenerative nodules of hepatocytes, and pseudo-lobules was observed, along with typical pathological changes of cirrhosis.

3. HE staining (Figure 1)

After H&E staining, degeneration and necrosis of hepatocytes, nodular regeneration of hepatocytes, inflammatory cell infiltration in portal area and central vein, diffuse proliferation of hepatic connective tissues, destruction of hepatic lobular structure, and formation of fibrous septa surrounding pseudo-lobules were microscopically observed.

4. Biochemical analysis (Figure 2)

Serum levels of ALT, AST, direct bilirubin, indirect bilirubin, and ALB were measured by an automatic biochemistry analyzer. Serum levels of hyaluronic acid (HA) were measured by radioimmunoassay.

Compared with 0 µg group, it was revealed that the serum levels of hydroxyproline, direct bilirubin, and indirect bilirubin, hyaluronic acid, AST, and ALT were significantly increased in the normal group, while the serum level of ALB was decreased. The serological and histological analyses indicated that the mouse model of liver fibrosis was successfully constructed.

With the elevation of the dosage and duration of VEGF165 treatment, serum levels of AST, ALT, indirect bilirubin, and hyaluronic acid were remarkably decreased, whereas serum levels of ALB and direct bilirubin were notably increased ($P < 0.05$). These results suggest that VEGF attenuates fibrosis and improves liver function in cirrhotic rats.

5. Hydroxyproline content (Figure 3)

With increase dosage and duration of VEGF165 treatment, hydroxyproline content was noticeably reduced ($P < 0.05$), demonstrating that the degree of liver fibrosis gradually improved.

6. Sirius Red staining(Figure 3)

With elevation of dosage and duration of VEGF165 treatment, the red portion of Sirius Red staining, which indicated liver fibrosis, significantly decreased from 25.4% (0 ug, 1 week) to 3.3% (4 ug, 4 weeks), reflecting that the degree of liver fibrosis gradually improved ($P < 0.05$).

7. Detecting the expression level of CD44 by immunohistochemical analysis(Figure 3)

After immunohistochemistry, it was unveiled that the expression level of CD44 was remarkably increased with elevation of dosage and duration of treatment, suggesting that it contributes to the clearance of hyaluronic acid from the blood by the liver.

8. Expression level of VEGF165 in liver (Figure 4)

Compared with the normal group, the expression level of VEGF165 in the liver of the mice in the model group was significantly decreased. With the increase of the dosage and duration of VEGF165 treatment, the expression level of VEGF165 in the liver of the mice was significantly increased ($P < 0.05$), indicating that the method of pumping the inferior vena cava was effective and could increase the concentration of VEGF165 in the liver.

9. Detecting the expression level of CD44 by Western blotting (Figure 4)

The results of Western blotting showed that the expression level of CD44 was notably elevated with increasing dosage and duration of VEGF165 treatment ($P < 0.05$), demonstrating that the endocytic exocytosis function of liver cells was improved.

10. The expression level of endothelin (Figure 4)

The expression level of endothelin was markedly decreased with elevation of dosage and duration of VEGF165 treatment ($P < 0.05$).

11. The expression level of HIF-1 α (Figure 5)

With the escalation of dosage and duration of VEGF165 treatment, the expression level of HIF-1 α was remarkably decreased ($P < 0.05$), indicating that the local hypoxia status of the liver improved.

12. Detecting expression levels of MMP9 and MMP2 by Western blotting (Figure 5)

With the increase of dosage and duration of VEGF165 treatment, no remarkable changes were observed in the expression levels of MMP9 and MMP2, which may be due to the inconsistent peak secretion time of MMP9 and MMP2 in different groups.

13. Perisinusoidal structure in normal liver and hepatic fibrosis (Figure 6)

When the model of liver fibrosis was successfully established, the cellular changes observed by electron microscopic included mitochondrial swelling, infiltration of collagen fibers into Di's space, and formation of basement membrane of sinusoidal endothelial cells. With the application of VEGF, the fenestrations reformed, the mitochondrial swelling was reduced, the structure of membrane was clear, the microvillus structure was normal, and the Die's space was reproduced.

It was revealed that with the extension of duration of VEGF therapy, the number of open fenestrations also increased, indicating that the structure of liver cells gradually recovered. In addition, the electron microscopy of endocytosis and exocytosis showed that VEGF can improve exchange of substances.

14. Cellular localization via immunofluorescence microscopy: CD31 (200X) and Dil-Ac-LDL (200X)

As illustrated in Figure 7, red fluorescence is positive for CD31, with a positive rate of > 90%, and the cell purity is > 90%; Figure 7 displays that red fluorescence is positive for Dil-Ac-LDL, with a positive rate of > 90%, and the cell purity is > 90%, both suggesting accurate localization of sinusoidal endothelial cells.

15. Myofilament changes of LSECs after different treatments (Figure 7)

Blue light represents the nucleus, red light shows VEGF-R1, green light indicates myofilaments, and the degree of myofilament adaptations was judged by the naked eye. group A > B means that fenestrations of LSECs could close together due to TAA; group D > C > B represents that VEGF could reopen the closed fenestrations of LSECs induced by TAA, indicating that the skeleton of LSECs gradually returned to normal status.

16. Changes in fenestrations after different treatments (Figure 8)

The aim of this study was to indicate that VEGF could prolong the maintenance of the fenestrations in LSECs and reopen the fenestrations after loss of LSECs. With microscopically observation of the cytoskeleton, formation of fenestrations and changes in endothelial permeability could be explained.

Conclusion

At present, the only effective treatment for advanced cirrhosis is liver transplantation, however, this is limited by several factors, including donor shortage, a risk of rejection, and high costs (3). Stem cell therapy is another attractive approach to liver fibrosis that has been recently studied. Preliminary results were found to be promising, however, design of experiments was not appropriate to demonstrate its safety and efficacy in liver cirrhosis (15). Therefore, new therapies for reversing fibrosis are urgently required.

Hepatic fibrosis is a main feature of multiple chronic liver disease due to chronic damage to the liver (16). After formation of a chronic injury, hepatic stellate cells (HSCs) are activated and transdifferentiated into

myofibroblast-like cells (MFCs) that secrete large amounts of extracellular matrix (ECM). In the process of liver fibrosis, HSC is taken as the final target cell into consideration in all kinds of fibrogenic factors. HSCs are normally quiescent, and when the liver is injured by inflammatory or mechanical stimuli or other insults, HSCs are activated and their phenotype changes from quiescent to activated. On one hand, activated HSCs are involved in the formation of liver fibrosis and the reconstruction of intrahepatic structures through hyperplasia and secretion of ECM; on the other hand, involvement is realized through cell contraction to increase the pressure in the liver sinusoids. Accumulation of ECM distorts the hepatic vasculature and causes shunting of portal and arterial blood, impairing the exchange of substances between hepatocytes and sinusoidal blood (17), leading to capillarization of sinusoids. Thus, promoting angiogenesis can improve hepatic microcirculation, thereby reducing fibrosis (18, 19).

Treatment of cirrhosis is currently standardized and specifically developed to reduce activation of HSCs, inhibit fibrosis, increase degradation of matrix components, and reduce activated myofibroblasts (20).

It is noteworthy that LSECs, stellate cells (SCs), and Kupffer cells (KCs) not only support hepatocytes, but also contribute to the inflammatory response (21, 22).

In addition, LSECs is a highly specialized endothelial cell, representing the interface between one side of blood cells and the other side of liver cells and HSCs. In fact, the combination of fenestrations, absence of septa, and absence of basement membranes make them the most permeable endothelial cells in mammals. They also have the highest endocytosis capacity of human cells. Under physiological conditions, LSECs can regulate the tension of hepatic blood vessels and maintain low portal vein pressure, although there are significant changes in hepatic blood flow during digestion. Furthermore, LSEC maintains the static state of HSC, thus inhibiting the development of intrahepatic vasoconstriction and fibrosis. Under pathological conditions, hepatocytes play an important role in the development of chronic liver disease. In fact, they become capillary, lose their protective function, promote angiogenesis and vasoconstriction. LSECs also play an important role in the occurrence, development, aging of hepatocellular carcinoma and liver injury related to inflammation and infection (23) .

Capillarization is defined as the loss of LSEC fenestration in vivo with the development of organized basement membrane. In vitro studies have shown that differentiated LSECs (i.e., fenestrated LSECs) prevent the activation of HSCs and the rapid reversal of activated HSCs to quiescence, while LSECs lose this role in their dedifferentiation or capillarization (24). Therefore, the disorder of LSEC phenotype is a critical step in the process of fibrosis (25).

VEGF is one of the most widely studied angiogenic growth factors, which is essential for the migration, proliferation of endothelial cells and the formation of new blood vessels (26). VEGF can promote the fenestration and increase the permeability of endothelial cells. Bioinformatics and experimental data show that CD147 promotes the progress of liver fibrosis via VEGF-A/VEGF receptor-2 (VRGFR-2) signaling-mediated crosstalk between hepatocytes and LSECs.

It has been found that the administration of exogenous VEGF at the site of tissue injury is an effective therapeutic approach to promote tissue repair and regeneration. In patients with cirrhosis, a significant decrease in VEGF levels has been reported, thus it can be concluded that the use of VEGF may play a pivotal role in making delay or reversing the progression of liver fibrosis (9, 28).

You et al. examined the effects of recombinant human endostatin Endostar on hepatic sinusoidal capillarization in a mouse model of liver fibrosis, and it was found that endostar therapy was related to the reduced levels of VEGFR1 and VEGFR2 in liver tissues ($P < 0.01$), as well as with decreased hepatic sinusoidal endothelial cell capillarization in a mouse model of liver fibrosis, and this effect may involve the VEGF pathway (5).

VEGF gene injection or simultaneous preoperative injection of recombinant adenoviral vectors containing the VEGF gene has been shown to be effective in stimulating liver regeneration in cirrhotic rats (29). The above-mentioned studies suggested the possibility of VEGF in the treatment of liver fibrosis.

Due to the short half-life and rapid diffusion of VEGF into the extracellular fluid, it is difficult to maintain an effective local concentration (30), and multiple injections are therefore essential to maintain VEGF concentrations during the experiment, which may increase the risk and cost of this potential therapy. In addition, excessive VEGF at the injection site and the spread of VEGF may cause some drug side effects. Therefore, an efficient and safe drug delivery system is required to improve its local therapeutic efficiency and reduce possible adverse effects (27).

Our previous study showed that VEGF could inhibit the fibrotic process in cirrhosis. However, in vivo transfection was inefficient and unstable. On this basis, we developed a new method for drug delivery using a mini-pump to ensure the stability and long-term effect of the drug, improve the success rate, and increase the reliability. With detection of VEGF165 in the liver tissue, the level of VEGF165 in the liver tissue is gradually increased with the increase of the dosage and duration of VEGF165 (see Figure 4).

After modeling, we found complete destruction of normal lobular architecture, regenerative nodules of hepatocytes, and signs of cirrhosis of pseudo-lobule formation by Masson's trichrome and H&E staining methods. Additionally, with making comparison between the normal group and 0 μg group, it was unveiled that the serum levels of hydroxyproline, direct bilirubin, indirect bilirubin, hyaluronic acid, ALT, and AST were increased, while serum level of ALB was decreased. As displayed in Figures 1-3, histological and serological analyses indicated that the rat model of hepatic fibrosis was successfully constructed.

As dosage and duration of VEGF165 treatment increased, the liver function of cirrhotic rats gradually improved, mainly reflecting a significant decrease in serum levels of ALT, AST, indirect bilirubin, and hyaluronic acid, while a notable increase in the serum level of ALB; besides, as shown in Figure 2, direct bilirubin also showed a decreasing trend, suggesting that VEGF improved liver function and reduced fibrosis in liver tissues in cirrhotic rats.

With detecting the serum level of hydroxyproline in the blood, it was disclosed that the mentioned level was markedly decreased, and the degree of liver fibrosis was gradually improved (Figure 3).

At the same time, fibrosis was assessed by Sirius Red staining (31). In the Sirius Red staining, as depicted in Figure 3, the stained red area was gradually reduced, indicating that the degree of fibrosis in the liver was decreased.

Additionally, CD44, which was weakly expressed in normal liver, was found to be present in large amounts in cirrhotic liver. The distribution pattern of CD44 was similar to that of hyaluronic acid, and CD44 was mainly localized in infiltrated lymphocytes and KCs, which contributed to the clearance of hyaluronic acid from the blood by the liver and also reflected the recovery of liver function (32).

In addition, a significant increase in HIF-1 α , reflecting the state of liver blood flow, indicates that the blood supply and nutrient supply of fibrotic liver tissues are rich, and it also contributes to the recovery of liver function (see Figure 5). Endothelin, as a factor reflecting the elasticity of blood vessels, significantly decreased, indicating that the degree of hepatic vasoconstriction was reduced and restoration of hepatic blood flow was noted as well (see Figure 4). Images captured by TEM also confirmed that the ultrastructure of LSECs tended to be normal, and the decline of liver fibrosis was confirmed microscopically as well.

The results of in vitro experiments also confirmed that thioacetamide could disrupt myofilament function and make close the fenestration, while VEGF165 could promote myofilament recovery and reopen the fenestration.

The findings of the present research confirmed that VEGF can improve local microcirculation and protect liver function after liver fibrosis. Meanwhile, the increase of circulating blood volume can allow macrophages to reach various parts of liver earlier. MMP2 and MMP9 secreted by macrophages can penetrate into the fibers formed by decomposition in the already fibrotic liver tissue. In the current experiment, the total cell protein levels of MMP2 and MMP9 were unchanged, while VEGF increased the density of microvessels in the fibrotic tissue. VEGF does not contribute to fibrinolysis in fibrotic liver tissues, and when factors related to liver damage are present, addition of VEGF causes a greater damage to the liver due to restoration of hepatic blood supply, which may justify why different experiments have concluded that VEGF has controversial influences on liver fibrosis (27).

In summary, our findings may provide a new insight for VEGF in the study of the decline or even disappearance of liver fibrosis, and present a potential target for the treatment of liver fibrosis.

Declarations

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Author Contribution statement

Zhigang Sun, Tianyi Dong, Zhun Zhang, Xingsong Tian and Hao Xu conceived and planned the experiments. Tiantian Wang, Zhigang Sun, and Chenyu Zhang carried out the experiments. Zhigang Sun, Zhun Zhang took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Figures

Masson's trichrome staining

HE staining

Figure 1

Masson's trichrome staining and HE staining of liver tissues

Figure 2

Serum levels of ALT, AST, direct bilirubin, indirect bilirubin, ALB and hyaluronic acid (HA)

Figure 3

Hydroxyproline content and the area of fibrosis(above).Tissue section stained with Sirius red and Immunohistochemical detection of CD44 expression(below)

Figure 4

Detection of expression level of VEGF165(above) ,CD44 (middle) and endothelin (below) by Western blot

Figure 5

Detection of expression level of HIF-1α (above) ,MMP9 (middle) and MMP2 (below) by Western blot

↑ Red blood cell
↓ Perisinusoidal space
→ Canaliculi microvilli
← Fenestrations

↑ Edema of hepatocyte mitochondrial membrane
↓ Infiltration of collagen fibers into Di's space
→ Formation of basement membrane of sinusoidal endothelial cells

↑ Red blood cell
↓ Fenestrations reopened
→ Microvilli start to become normal

↑ Red blood cell
↓ Intercellular junction
→ Microvilli structure became normal
← The number of reopened fenestrations further increased

Figure 6

Electron microscopic observation of normal perisinusoidal structure and hepatic fibrosis

Figure 7

Myofilament changes of LSECs after different treatments

Figure 8

Changes in fenestrations after different treatments