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BASIC RESEARCH

Vascular endothelial growth factor attenuates hepatic sinusoidal capillarization in thioacetamide-induced cirrhotic rats

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Abstract

AIM: To investigate the effect of vascular endothelial growth factor (VEGF) transfection on hepatic sinusoidal capillarization.

METHODS: Enhanced green fluorescent protein (EGFP)/VEGF transfection was confirmed by immunofluorescence microscopy and immunohistoche-mistry both in primary hepatocytes and in normal liver. Cirrhotic rats were generated by thioacetamide (TAA) administration and then divided into a treatment group, which received injections of 400 μg of plasmid DNA encoding an EGFP-VEGF fusion protein, and a blank group, which received an equal amount of normal saline through the portal vein. The portal vein pressure was measured in the normal and cirrhotic state, in treated and blank groups. The average number of fenestrae per hepatic sinusoid was determined using transmission electron microscopy (TEM), while the relative abundance of VEGF transcripts was examined by Gene array.

RESULTS: Green fluorescent protein was observed in the cytoplasms of liver cells under immunofluorescence microscopy 24 h after transfection with EGFP/VEGF plasmid *in vitro*. Staining with polyclonal antibodies against VEGF illustrated that hepatocytes expressed immunodetectable VEGF both *in vitro* and *in vitro*. There were significant differences in the number of fenestrae and portal vein pressures between normal and cirrhotic rats (7.40 ± 1.71 *vs* 2.30 ± 1.16 and 9.32 ± 0.85 cmH_2O *vs* 17.92 ± 0.90 cmH_2O, $P < 0.01$), between cirrhotic and treated rats (2.30 ± 1.16 cmH_2O *vs* 4.60 ± 1.65 and 17.92 ± 0.90 cmH_2O *vs* 15.52 ± 0.93 cmH_2O, $P < 0.05$) and between the treatment group and the blank group (4.60 ± 1.65 cmH_2O *vs* 2.10 ± 1.10 cmH_2O and 15.52 ± 0.93 cmH_2O *vs* 17.26 ± 1.80 cmH_2O, $P < 0.05$). Gene-array analysis revealed that the relative abundance of transcripts of VEGF family members decreased in the cirrhotic state and increased after transfection.

CONCLUSION: Injection of a plasmid encoding VEGF through the portal vein is an effective method to induce the formation of fenestrae and decrease portal vein pressure in cirrhotic rats. Therefore, it may be a good choice for treating hepatic cirrhosis and portal hypertension.

Key words: Liver cirrhosis; Hepatic sinusoid capillarization; Fenestrae; Vascular endothelial growth factor; Transmission electrical microscopy; Ultrastructure; Gene array

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INTRODUCTION

Fenestrae are described as membrane-bound cytoplasmic holes with an average diameter of about 110 nm in transmission electrical microscopy (TEM) by Wisse in 1970[1,2]. Liver sinusoidal endothelial cells (LSECs) possess open fenestrae that perforate the hepatic endothelial lining, but lack a basal lamina. Fenestrae, vesicles and channels together control the bulk of trans-endothelial transport between blood and tissues[3]. Structural integrity of the

fenestrated sinusoidal liver endothelium is believed to be essential for the maintenance of the normal exchange of fluids, solutes, particles and metabolites between the sinusoidal blood and hepatocytes[4,5]. Although there are various causes and morphologies of hepatic cirrhosis, all forms of cirrhosis are characterized by a defenestrated sinusoidal endothelium and the presence of a subendothelial basement membrane[6-9]. It has been demonstrated that the disappearance of the normal filtration barrier in cirrhotic livers results in an impaired bidirectional exchange between the sinusoidal blood and parenchymal cells[10]. As a result, capillarization of the sinusoidal endothelium may be a major contributor to hepatic failure in patients with cirrhosis.

Vascular endothelial growth factor (VEGF), which plays a role in regulating vasculogenesis, induces angiogenesis and endothelial cell proliferation. In recent years, it has been proven that VEGF is relevant to the increased number of fenestrae and endothelial permeability in endothelial cells[10-13] and renal glomerulus[14]. Injection of VEGF-D plasmid into both normal and ischemic rat liver resulted in an increased number of new capillaries around hepatic sinuses[15]. Many studies have shown that, in the state of hepatic fibrosis and cirrhosis, both in patients and experimental models, VEGF is increased with harmful effects[16,17]. By contrast, other studies have shown decreased expression of VEGF in cirrhotic patients, and suggested that VEGF makes a helpful contribution[18,19]. After hepatectomy in cirrhotic rats, VEGF was found to effectively promote liver regeneration[20,21]. Moreover, human urokinase-type plasminogen activator gene administration via an adenoviral (Ad)-vector induced cirrhosis regression and ameliorated hepatic dysfunction with up-regulation of VEGF in a model of experimental liver cirrhosis[22,23].

Because defenestration and basement membrane formation result in a disordered exchange between the sinusoidal blood and hepatocytes, it is necessary to restore the function of liver sinusoidal endothelial cells in order to reverse cirrhosis. VEGF provides the perfect means to achieve this, because of it promotes fenestration and permeability. On account of these ideas, we studied the effects of VEGF transfection in cirrhotic rat livers.

MATERIALS AND METHODS

EGFP/VEGF transfection of hepatocytes

EGFP/VEGF was obtained from the Medical School of Shandong University (Jinan, China). Human VEGF-D cDNA was inserted into pEGFP-N1 plasmid between the constitutive cytomegalovirus promoter (pCMV) and enhanced green fluorescent protein (EGFP) to produce a plasmid encoding an EGFP-VEGF fusion protein (EGFP/VEGF plasmid), which was used as a tool to express VEGF consistently. EGFP was used to detect expression of the plasmid, and the expression of VEGF was detected by immunohistochemistry.

Primary hepatocytes were isolated from the livers of male Wistar rats (150-200 g) by collagenase perfusion as previously described[24]. Cells were plated on collagen-coated 6-well plates at a density of 3×10^5 cells/well in Williams' medium E supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 1% penicillin/streptomycin, and 100 nmol/L dexamethasone. The cells were incubated in 5% CO_2 at 37℃ to facilitate attachment, and the medium was exchanged after 4 h with serum-free Williams' medium E supplemented with 2 mmol/L L-glutamine, 1% penicillin/streptomycin, and 100 nmol/L dexamethasone. After overnight incubation in 5% CO_2 at 37℃, the medium was exchanged with serum-free Williams' medium E supplemented with 2 mmol/L L-glutamine and 1% penicillin/streptomycin. The EGFP/VEGF plasmid (50 μg) was added to the culture medium and the cells were incubated for 24 h. We investigated 5 dishes for each treatment in the present study.

Immunofluorescence microscopy and immunohistochemistry

Immunofluorescence microscopy was used to visualize the distribution of EGFP in hepatocytes. At the same time, immunocytochemical staining of VEGF was performed. Cultured hepatocytes were washed with phosphate buffered saline (PBS) 3 times, and fixed with acetone at room temperature for 10 min. Then, they were blocked with goat serum and permeabilized for 30 min with 0.1% Triton X-100 in PBS containing 1% bovine serum albumin (BSA). The cells were incubated overnight with rabbit anti-human VEGF polyclonal antibody (Santa Cruz) at a 1:50 dilution at 4℃. Subsequently, they were incubated with tetramethylrhodamine isomer R1-conjugated swine anti-rabbit IgG (Santa Cruz) at room temperature for 1 h. The distribution of VEGF was visualized by light microscopy.

Transfection of normal liver

Twenty healthy male Wistar rats, weighing 200-220 g, bought from the experimental animal center of Shandong Agricultural Research Center (Jinan, China) were divided equally into two groups. In the study group, each rat received an injection of 400 μg of the EGFP/VEGF plasmid through the portal vein. In the control group, each rat received an equal amount of normal saline in the same manner. All rats were sacrificed eight days after the operation. Liver samples were collected and fixed in 10% neutral-buffered formalin, embedded in paraffin and cut into 4-6 μm sections.

Immunohistochemistry

Immunohistochemistry was performed according to Hong-Lei Weng[25]. The integral score method was used to evaluate the positive cell distribution and intensity[26]. First, a proportion score was assigned, which represented the estimated proportion of positive-staining cells (0, none; 1, 0 to 1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; and 5, 2/3). Next, an intensity score was assigned, which represented the average intensity of positive cells (0, none; 1, weak, 2, intermediate; and 3, strong). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8. Then the score was compared between the study and control groups.

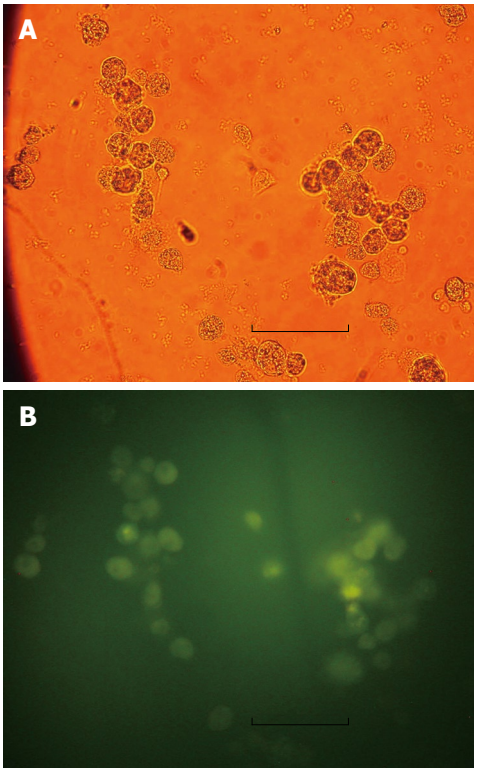

Figure 1 Hepatocytes incubated with EGFP/VEGF plasmid for 24 h. **A**: Light microscopy: no green fluorescent protein was found. Bar denotes 30 μm; **B**: Immunofluorescence microscopy: green fluorescent protein expression. Bar denotes 30 μm.

Transfection of cirrhotic liver

The portal vein pressures of 40 normal Wistar rats were measured and hepatic specimens were taken. Then, the rats received an oral administration of 0.03% thioacetamide (TAA) solution instead of feed water. After 5 wk, the concentration of TAA was increased to 0.04%. Ten wk later, 26 cirrhotic rats were identified and randomly divided into a treatment group and a blank group. The portal vein pressures of these rats were measured and cirrhotic liver specimens were obtained. Then, 400 μg of the VEGF/EGFP plasmid was infused through the portal veins of rats in the treatment group, while an equal amount of normal saline was given through the portal vein to rats in the blank group. After 2 wk, portal vein pressures were measured again; then, all rats were sacrificed and liver samples were collected. Comparisons of portal vein pressure were made between the normal and cirrhotic, treated and untreated rats. A randomized controlled trial was carried out to compare the treatment group and the blank group.

Transmission electron microscopy

Fresh specimens were first fixed in 3% glutaral, and then fixed in 1% glutaral after being washed with PBS 3 times for 60 min in total. The samples were dehydrated with an alcohol gradient, embedded in EPON812 epoxy resin, and then cut into 50-nm sections with an Ultrathin microtome. After being dyed with uranyl acetate and lead citrate for 30 min, the sections were observed under transmission electronic microscope (JEM-1200EX, Japan). Ten hepatic sinusoids with a diameter of 2-3 μm were randomly selected, and the average number of fenestrae per hepatic sinusoid in each state and group was determined. Valid fenestrae ran through LSECs.

Gene array

Angiogenesis microarrays were obtained from SuperArray Bio.Co. (Catalog No. ORN-024). Fresh specimens were put into 4℃ of RNA later and incubated for one night. The experiment was performed according to the Oligo GEArray assay protocol. Briefly, total RNA was extracted from tissue using TRIzol reagent (Invitrogen Life Technologies, USA). The quantity and purity of RNA were estimated by measuring A_{260} and A_{280}. A total of 3 μg of RNA was used to synthesize cDNA. The cRNA was then labeled by Biotin-16-dUTP (Roche Cat. No. 1-093-070) and amplified using a TrueLabeling-AMPTM linear RNA amplification kit. The membranes were hybridized with denatured cDNA probe and processed for chemiluminescent detection on X-ray film, and images were acquired using a flatbed desktop scanner. Subsequently, images of spots were converted into numerical data using the free ScanAlyze software and the raw data was saved as a Microsoft Excel file. All raw signal intensities were corrected for background signal by subtracting the minimum value to avoid the appearance of negative numbers, and were also normalized to the level of a housekeeping gene. These corrected, normalized signals were used to estimate the relative abundance of particular transcripts.

Statistical analysis

Results are expressed as mean ± SD, and statistical analysis was carried out using the Student's *t*-test and Spearman's rank correlation coefficient for paired data. A P value of less than 0.05 was considered to be significant.

RESULTS

VEGF expression in vitro and in vivo

Twenty-four hours after transfection of cultured hepatocytes with the EGFP/VEGF plasmid, green fluorescent protein was observed in the cytoplasms of liver cells under immunofluorescence microscopy, revealing expression of the plasmid (Figure 1). At the same time, staining of hepatocytes with polyclonal antibodies against VEGF illustrated that these cells expressed immunodetectable VEGF (Figure 2). Moreover, the transfection of liver cells following injection of the EGFP/VEGF plasmid into the portal veins of normal rats *in vivo* was also successful, because a considerable amount of VEGF was identified in the study group, but little was seen in the control group (Figure 3). VEGF was mainly observed in the cytoplasms of hepatocytes and some endothelial cells. The stain integral scores of the study group and control group were 6.0 ± 1.63 and 3.7 ± 2.31 respectively, allowing these groups to be differentiated from each other distinctively (Student's t test, $t = 2.74 > 2.62$, $P < 0.05$) (Figure 3).

Fenestrae and portal vein pressures

A comparison was carried out between normal, cirrhotic

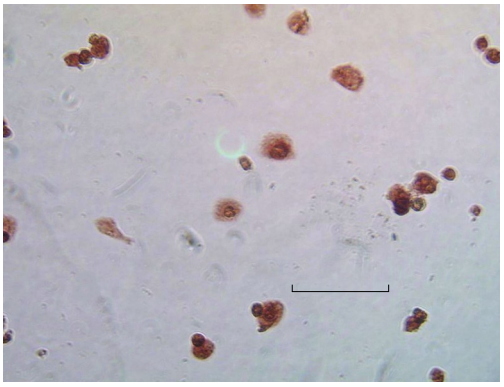

Figure 2 Immunohistochemistry: Positively stained hepatocytes for EGFP/VEGF. Bar denotes 30 μm.

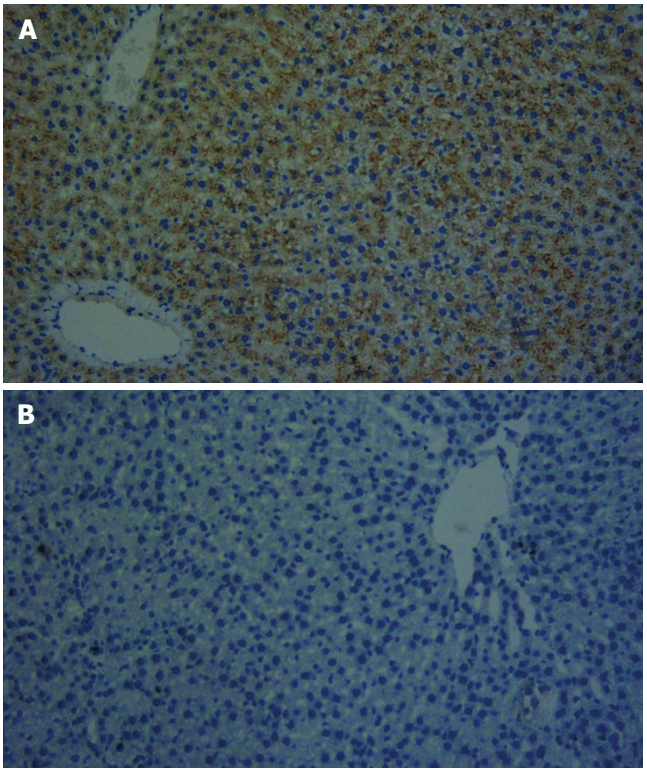

Figure 3 Immunohistochemistry × 200. **A**: Study group injected with EGFP/VEGF plasmid, VEGF-positive hepatocytes × 200; **B**: Control group injected with NS, VEGF-negative hepatocytes.

Table 1 The number of fenestrae and portal vein pressures in different groups (n = 11)

Group	The number of fenestrae	Portal vein pressures (mean ± SD) (cmH_2O)
Normal state	7.40 ± 1.71[d]	9.32 ± 0.85[d]
Cirrhotic state	2.30 ± 1.16	17.92 ± 0.90
Treated group	4.60 ± 1.65[a]	15.52 ± 0.93[a]
Blank group	2.10 ± 1.10	17.26 ± 1.80

Paired samples t test. Significant differences are shown both in fenestrae and pressure. [a]$P < 0.05$ *vs* Cirrhotic state; [d]$P < 0.01$ *vs* Blank group.

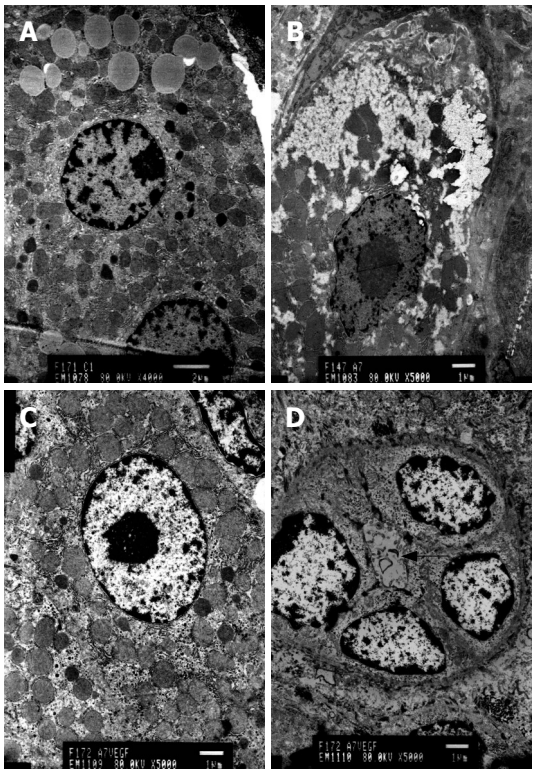

Figure 4 TEM. **A**: Normal hepatocytes, Bar denotes 2 μm; **B**: Cirrhotic state, hepatocyte apoptosis, Bar denotes 1 μm; **C**: Cirrhotic rats injected with the EGFP/VEGF plasmid, hepatocyte apoptosis decreased, Bar denotes 1 μm; **D**: Cirrhotic rats injected with EGFP/VEGF plasmid, Regenerated hepatocytes and cholangiole arrowhead, Bar denotes 1 μm.

and treated rats. The number of fenestrae per LSEC in cirrhotic and normal rats was 2.30 ± 1.16 and 7.40 ± 1.71, respectively (Table 1). The decrease in cirrhotic rats was significant (Student's t-test, $t = -7.965 < -6.548$, $P < 0.01$). The portal vein pressures were 17.92 ± 0.90 in cirrhotic rats and 9.32 ± 0.85 in normal rats (Table 1), revealing an obvious difference (Student's t-test, $t = 27.32 > 9.30$, $P < 0.01$).

After transfection with the EGFP/VEGF plasmid, the number of fenestrae in the treated group was 4.60 ± 1.65 (Table 1), representing a significant increase compared with cirrhotic rats (Student's t-test, $t = -4.14 < -3.56$, $P < 0.05$). The portal vein pressure in the treated group was 15.52 ± 0.93 (Table 1), representing a significant decrease compared with cirrhotic rats (Student's t-test, $t = 6.04 > 3.29$, $P < 0.05$).

A randomized controlled trial was performed to compare the treated group and the blank group. There was an obvious difference in the number of fenestrae (4.60 ± 1.65 *vs* 2.10 ± 1.10, Student's t-test, $t = 4.04 > 3.90$, $P < 0.05$) and in portal vein pressure (15.52 ± 0.93 *vs* 17.26 ± 1.80, Student's t-test, $t = -3.97 < -3.46$, $P < 0.05$), but there was no significant difference between the cirrhotic group and the blank group in these measures.

Ultrastructural changes in cirrhotic rats after EGFP/VEGF plasmid transfection

Transmission electron microscopy revealed hepatocellular apoptosis in rats with TAA-induced cirrhosis (Figure 4B); moreover, the fenestrae of endothelial cells disappeared

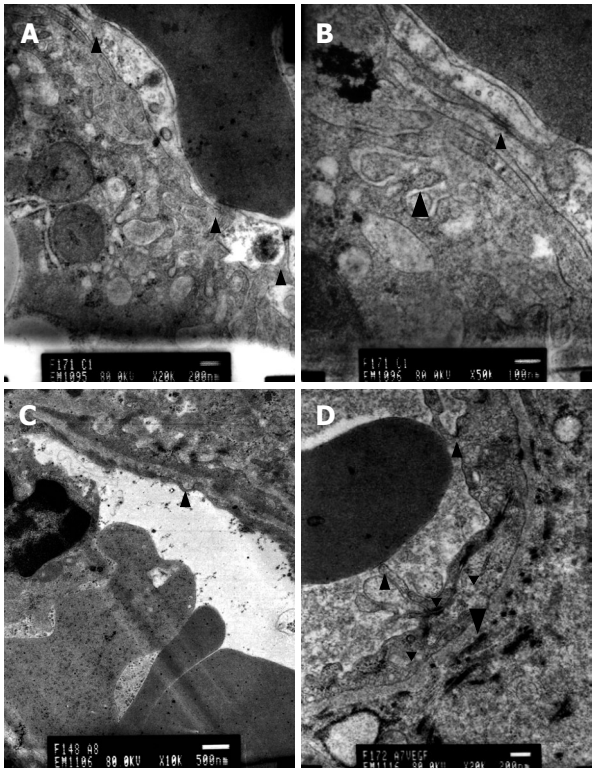

Figure 5 TEM. **A**: Normal state, LSECs and fenestrae (arrowhead). Bar denotes 200 nm; **B**: Normal state. Cell conjuction between LECs (small arrowhead) and microvilli of hepatocytes (large arrowhead). Bar denotes 100 nm; **C**: Cirrhotic state. Fenestrae, cell conjuction and microvilli of hepatocytes disappeared, and a basement membrane appeared (arrowhead). Bar denotes 500 nm; **D**: VEGF treated group. Fenestrae (small arrowhead), microvilli (large arrowhead) and cell conjunctions between LECs (triangle) appeared, Bar denotes 200 nm.

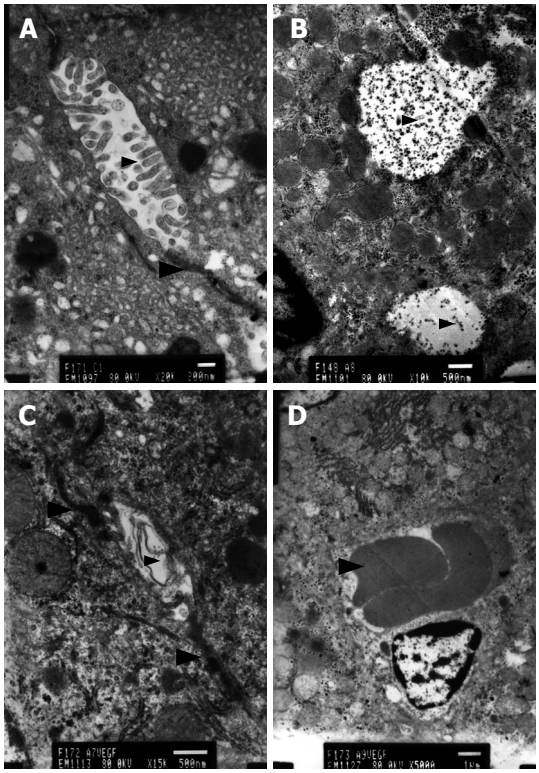

Figure 6 TEM. **A**: Normal state. Cholangiole, microvilli(small arrowhead) and cell conjuction between hepatocytes (large arrowhead). Bar denotes 200 nm; **B**: Cirrhotic state. Microvillus, cell conjuction disappeared and particles of bilirubin (arrowhead) overflowed. Bar denotes 500 nm; **C**: Treated group. Microvilli (small arrowhead) and cell conjunctions (large arrowhead) appeared and bilirubin overflow diminished. Bar denotes 500 nm; **D**: Treated group. Newborn capillary (arrowhead). Bar denotes 1 μm.

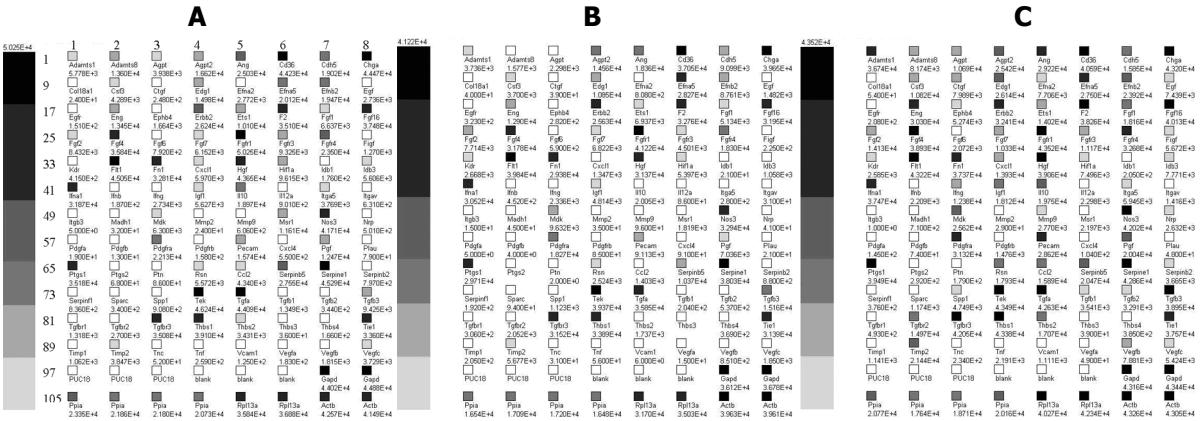

Figure 7 Representative expression profile of angiogenesis-related genes in the liver. The gene microarray included angiogenesis-related genes (1-96), negative control genes (97-99), blank controls (100-102), positive control genes (glyceraldehyde-3-phosphate dehydrogenase (103, 104), cyclophilin A (105-108), ribosomal protein L13a (109, 110) and b-actin (111,112). **A**: Normal state; **B**: Cirrhotic state; **C**: VEGF treated group.

and a basement membrane appeared (Figure 5C). Cell conjunction between hepatocytes was destroyed and particles of bilirubin overflowed into the cytoplasm of hepatocytes and LECs, even into the hepatic sinusoid (Figure 6B). The microvilli of hepatocytes in the space of Disse and the cholangiole were ablated in cirrhotic rats (Figure 5C and Figure 6B). However, this morphology changed after transfection with the EGFP/VEGF plasmid. The fenestrae, cell conjunctions, and microvilli of hepatocytes were restored, the basement membrane disappeared and cell apoptosis decreased. Newborn capillaries formed by a single liver endothelial cell emerged (Figure 6D).

Gene array

A total of 96 genes involved in angiogenesis were identified on the microarray (Figure 7 and Table 2). The levels of 32 genes were decreased by more than 50% in

Table 2 Angiogenesis-related genes on the angiogenesis microarray

Position	Description	Gene Name
1	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type Ⅰ motif, 1	Adamts1
2	Mus musculus a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 mot	Adamts8
3	Angiopoietin	Angiopoietin 1
4	Mus musculus angiopoietin 2 (Agpt2)	Angiopoietin2
5	Angiogenin	ANG
6	CD36 antigen	CD36
7	Cadherin 5	Cadherin 5
8	Chromogranin A	Chromogranin A
9	Procollagen, type ⅩⅧ, alpha 1	COL18A1
10	Colony stimulating factor 3 (granulocyte)	G-CSF
11	Connective tissue growth factor	Fisp12
12	Endothelial differentiation sphingolipid G-protein-coupled receptor 1	Edg1
13	Mus musculus ephrin A2 (Efna2)	Ephrin A2
14	Ephrin A5	Ephrin A receptor
15	Ephrin B2	Ephrin B2
16	Epidermal growth factor	EGF
17	Epidermal growth factor receptor	EGFR
18	Endoglin	Endoglin
19	Eph receptor B4	Ephrin B4
20	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	Neu/HER2
21	E26 avian leukemia oncogene 1, 5' domain	Ets-1
22	Mus musculus coagulation factor Ⅱ (F2)	Prothrombin kringle-1
23	Fibroblast growth factor 1	aFGF
24	Fibroblast growth factor 16	FGF16
25	Fibroblast growth factor 2	bFGF
26	Fibroblast growth factor 4	FGF4
27	Fibroblast growth factor 6	FGF6
28	Fibroblast growth factor 7	FGF7/KGF
29	Fibroblast growth factor receptor 1	FLG
30	Fibroblast growth factor receptor 3	FGFR3
31	Fibroblast growth factor receptor 4	FGFR4
32	C-fos induced growth factor	VEGF-D/FIGF
33	Kinase insert domain protein receptor	VEGFR2/FLK 1
34	FMS-like tyrosine kinase 1	VEGFR
35	Mouse fibronectin (FN) mRNA	Fn1
36	Chemokine (C-X-C motif) ligand 1	Gro1
37	Hepatocyte growth factor	HGF
38	Hypoxia inducible factor 1, alpha subunit	Hif1a
39	Inhibitor of DNA binding 1	ID1
40	Inhibitor of DNA binding 3	ID3
41	Interferon alpha family, gene 1	IFNA1
42	Interferon beta, fibroblast	IFN-b1
43	Interferon gamma	IFN r
44	Insulin-like growth factor 1	IGF-1
45	Interleukin 10	IL-10
46	Interleukin 12A	IL-12A
47	Integrin alpha 5 (fibronectin receptor alpha)	Integrin a5
48	Integrin alpha V	CD51
49	Integrin beta 3	CD61
50	MAD homolog 1 (Drosophila)	Smad1
51	Midkine	Midkine
52	Matrix metalloproteinase 2	Gelatinase A
53	Matrix metalloproteinase 9	Gelatinase B
54	Macrophage scavenger receptor 1	SR-A
55	Nitric oxide synthase 3, endothelial cell	NOS3
56	Neuropilin	Neuropilin
57	Platelet derived growth factor, alpha	PDGF a
58	Platelet derived growth factor, B polypeptide	PDGF b
59	Platelet derived growth factor receptor, alpha polypeptide	PDGFRa
60	Platelet derived growth factor receptor, beta polypeptide	PDGFRb
61	Platelet/endothelial cell adhesion molecule	PECAM1
62	Chemokine (C-X-C motif) ligand 4	PF4
63	Placental growth factor	Placental growth factor
64	Plasminogen activator, urokinase	PLAU
65	Prostaglandin-endoperoxide synthase 1	PTGS1
66	Prostaglandin-endoperoxide synthase 2	Cox-2

67	Pleiotrophin	PTN
68	Restin (Reed-Steinberg cell-expressed intermediate filament-associated protein)	Restin
69	Chemokine (C-C motif) ligand 2	Scya2
70	Serine (or cysteine) proteinase inhibitor, clade B, member 5	Maspin
71	Serine (or cysteine) proteinase inhibitor, clade E, member 1	PAI-1
72	Serine (or cysteine) proteinase inhibitor, clade B, member 2	PAI-2
73	Serine (or cysteine) proteinase inhibitor, clade F), member 1	Pedf
74	Secreted acidic cysteine rich glycoprotein	BM-40
75	Secreted phosphoprotein 1	Osteopontin
76	Endothelial-specific receptor tyrosine kinase	Tie-2
77	Transforming growth factor alpha	TGF-a
78	Transforming growth factor, beta 1	TGFb1
79	Transforming growth factor, beta 2	TGF b2
80	Transforming growth factor, beta 3	TGF b3
81	Transforming growth factor, beta receptor Ⅰ	ALK-5
82	Transforming growth factor, beta receptor Ⅱ	TGFbR2
83	Transforming growth factor, beta receptor Ⅲ	Betaglycan
84	Thrombospondin 1	THBS1
85	Thrombospondin 2	THBS2
86	Mus musculus thrombospondin 3 (Thbs-3)	THBS3
87	Mus musculus thrombospondin-4 mRNA	THBS4
88	Tyrosine kinase receptor 1	Tie1
89	Tissue inhibitor of metalloproteinase 1	Timp
90	Tissue inhibitor of metalloproteinase 2	TIMP2
91	Tenascin C	Tenascin C
92	Tumor necrosis factor	TNFa
93	Vascular cell adhesion molecule 1	VCAM-1
94	Vascular endothelial growth factor A	VEGF/VEGI
95	Vascular endothelial growth factor B	VEGF-B
96	Vascular endothelial growth factor C	VEGF-C

Table 3 Relative abundance of transcripts of VEGF and receptor family members

Gene	Normal state	Cirrhotic state	Treated group	Cirrhotic/normal	Treated/cirrhotic
VEGF-D/FIGF	5.790E-2	1.337E-2	2.936E-1	0.2309	21.950
VEGF-C	1.700E-1	1.099E-1	2.807E-1	0.6467	2.5530
VEGF-B	8.275E-2	5.057E-2	4.079E-1	0.6111	8.0650
VEGF/VEGI	8.344E-3	8.914E-4	2.536E-3	0.1068	2.8450
VEGFR	2.054E+0	2.368E+0	2.237E+0	1.1530	0.9448
VEGFR2/FLK1	1.892E-2	1.586E-1	1.338E-1	8.3800	0.8438

cirrhotic rats, and only 8 genes were enhanced by two-fold or greater compared with normal rats. After EGFP/VEGF transfection, 56 genes were increased by two-fold or greater in treated rats; and only one gene was decreased by more than 50% in comparison with cirrhotic rats. In cirrhotic rats, the expression levels of members of the VEGF family were decreased, and the expression levels of members of the VEGF receptor family were increased compared with the levels in normal liver. However, after administration of VEGF, expression of VEGF family members increased and that of receptor family members decreased. Furthermore, the level of VEGF-D was increased by 22-fold, while levels of VEGF, VEGF-B and VEGF-C were increased by more than 2-fold after EGFP/VEGF transfection (Table 3).

DISCUSSION

VEGF-D was first cloned by Yamada in 1997 from a human lung cDNA library[27]. VEGF-D binds VEGF receptor 2 (VEGF R2/Flk-1/KDR) and VEGF R3 (Flt-4)[28]. VEGF R2 and VEGF R3 are localized to vascular and lymphatic endothelial cells and are involved in signaling mediating angiogenesis and lymphangiogenesis[29]. There are numerous techniques and approaches that have been investigated for gene transfer to the liver. For gene therapy of hepatic diseases in animal experiments, exogenous genes were usually delivered to the liver through the portal vein and bile duct[30-33]. In our studies, EGFP/VEGF plasmid injection through portal vein was found to be an effective method.

The EGFP/VEGF plasmid was specifically designed for mammalian expression, and was constructed using a human VEGF-D cDNA and pEGFP-N1 plasmid. In the first stage of our studies, we tried to ascertain its expression efficiency, both *in vitro* and *in vivo*. *In vitro*, enhanced green fluorescent protein was observed in the cytoplasm of liver cells under immunofluorescence microscopy. At the same time the staining of hepatocytes with polyclonal antibodies against VEGF illustrated that the cells expressed immunodetectable VEGF. *In vivo*, transfection resulted in a comparatively high expression of VEGF protein. These findings demonstrate the efficient transfection of liver cells with the EGFP/VEGF plasmid *in vitro* and *in vivo*.

VEGF is an important regulator of angiogenesis and vascular permeability, whose expression in the adult is correlated with low permeability of the blood-brain barrier endothelium and high permeability of the fenestrated glomerular endothelium[14,34]. Fenestrae are critical for the maintenance of the high hydraulic conductivity of the glomerular capillary wall, and their loss results in a reduction in the glomerular filtration rate[35]. Defenestration is an early event in the pathogenesis of cirrhosis, and precedes the initiation of fibrosis. Sinusoidal capillarization is believed to be important in the initiation of perisinusoidal fibrosis by altering retinol metabolism[7]. In rats with TAA-induced cirrhosis, the fenestrae of endothelial cell disappeared and a basement membrane appeared. As a result, the microvilli of hepatocytes in the space of Disse were ablated, indicating that material exchange between hepatocytes and hepatic sinusoidal blood failed. Hepatocytes could not obtain the necessary nutrition and eliminate metabolites, which resulted in cell apoptosis. Cell conjunction between hepatocytes was damaged and particles of bilirubin overflowed into the cytoplasm of hepatocytes and endothelial cells, and even into the hepatic sinusoid, resulting in jaundice.

VEGF can promote fenestration and permeability of liver sinusoidal endothelial cells; therefore, it can improve the exchange between hepatic sinusoidal blood and hepatocytes, which argues for the development of VEGF gene therapy for cirrhosis. Roberts and Palade[36,37] showed that topical administration of VEGF-165 induced fenestrations in continuous microvascular endothelia of muscle and skin, and that tumor neovasculature induced by VEGF is fenestrated. Epithelial cells stably transfected with VEGF cDNA secreted a high level of VEGF and induced a seven- or eight-fold increase in the number of fenestrae and fused clustered vesicles in co-cultured endothelial cells, thus providing direct evidence of a role for VEGF in fenestrae induction. Endothelial cells pretreated with VEGF developed fenestrae and showed increased endothelial permeability[10]. However, to date, there have been few reports on using VEGF to heal cirrhosis *in vivo*. In our study, after injection of EGFP/VEGF plasmid into cirrhotic rats through the portal vein, the number of fenestrae increased obviously, accompanied with a decrease in portal vein pressure. Hepatocytes and microvillus regeneration demonstrated that the material exchange between hepatocytes and hepatic sinusoidal blood recovered. Furthermore, cell conjunction of hepatocytes was restored and overflow of particles of bilirubin lessened. Meanwhile, the basement membrane disappeared and cell apoptosis decreased. We suspect that the fenestrae and proliferation of LECs played important roles in this change.

At present, there are three points of view on the use of VEGF in cirrhotic disease. The first is that there is decreased VEGF in cirrhosis, and that administration of exogenous VEGF would have a helpful effect[18,19]. The second is that there is increased VEGF in cirrhosis, and that this VEGF has a harmful effect[16,17]. The last point of view is that the increased VEGF in cirrhosis is compensative, having a helpful effect[38]. Our angiogenic gene array supports the first point of view. We can see clearly that in rats with TAA-induced cirrhosis, the levels of VEGF family members decreased and the levels of VEGF receptors increased, as a reflective compensation. After VEGF/EGFP transfection, the abundance of VEGF family members increased, and that of VEGF receptors decreased, explaining the newborn capillaries we observed by transmission electron microscopy. In our previous study, the density of capillaries was also increased after VEGF-D plasmid hepatic situ injection[15].

Thus, the levels of transcripts of VEGF family members decreased in TAA-induced cirrhotic livers. Administration of a plasmid encoding an EGFP-VEGF fusion protein attenuated sinusoidal capillarization through increasing the number of fenestrae and the permeability of LECs, which improved the exchange between hepatocytes and sinusoidal blood. Consequently, hepatocytes had more nutrition and oxygen and thus preserved liver function to some extent. Therefore, VEGF gene transfer by injection through the portal vein might be an ideal method for the treatment of cirrhosis.

COMMENTS

Background

Hepatic sinusoidal capillarization has been thought to be a major contributor to hepatic failure in cirrhosis. It includes a defenestrated sinusoidal endothelium and the presence of a subendothelial basement membrane. However vascular endothelial growth factor (VEGF) has been proven to increase the number of fenestrae and permeability of liver sinusoidal endothelial cells (LECs).

Research frontiers

This study was designed to investigate the effect of VEGF transfection on hepatic sinusoidal capillarization by observing the ultrastructural change of cirrhotic endothelium as well as the relationship between the number of fenestrae and portal vein pressure.

Innovations and breakthroughs

Our study shows effective endothelial growth factor (EGFP)/VEGF transfection *via* injection of plasmid DNA into the portal vein decreases portal pressure in experimental cirrhosis induced by thioacetamide. This effect is mediated by an increase in the formation of fenestrae of liver sinusoids. Previous studies on the role of VEGF in portal hypertension were performed in models of non-cirrhotic pre-hepatic portal hypertension obtained through part portal vein ligation, and showed remarkable angiogenesis effect. The ultra-microstructure in the liver of those rats has not been specifically investigated. This study is more related to what happens in cirrhotic patients.

Applications

Portal hypertension is an almost unavoidable complication of cirrhosis, and it is responsible for the more lethal complications of this syndrome. Previous research has shown the role of hepatic sinusoidal capillarization in cirrhosis. To understand the mechanisms underlying hepatic sinusoidal capillarization and reverse them will be of benefit to developing a new therapeutic method.

Peer review

This paper describes the effect of VEGF transfection on hepatic sinusoidal capillarization in cirrhotic animals as compared to normal rats. The authors have shown histological evidence of partial reversal of cirrhosis.

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