Expression of TNF-α and VEGF in the esophagus of portal hypertensive rats

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Abstract

AIM: To investigate the expression of tumor necrosis factor-alpha (TNF-α) and vascular endothelial growth factor (VEGF) in the development of esophageal varices in portal hypertensive rats.

METHODS: Thirty male Sprague-Dawley (SD) rats in the model group in which a two-stage ligation of portal vein plus ligation of the left adrenal vein was performed, were divided into three subgroups (M7, M14 and M21) in which the rats were killed on the seventh day, the 14th and the 21st d after the complete portal ligation. Thirty male SD rats, which underwent the sham operation in the control group, were also separated into three subgroups (C7, C14 and C21) corresponding to the models. The expression of TNF-α and VEGF in the esophagus of all the six subgroups of rats were measured with immunohistochemical SP technique.

RESULTS: The portal pressure in the three model subgroups was significantly higher than in the corresponding control subgroups (23.82±1.83 vs 11.61±0.86 cmH2O, 20.90±3.27 vs 11.43±1.55 cmH2O and 20.68±2.27 vs 11.87±0.79 cmH2O respectively, P<0.01), as well as the number (9.3±1.6 vs 5.1±0.8, 11.1±0.8 vs 5.4±1.3 and 11.7±1.5 vs 5.2±1.1 respectively, P<0.01) and the total vascular area (78 972.6±3 527.8 vs 12 993.5±4 994.8 μm², 107 207.5±4 6461.4 vs 11 862.6±5 423.2 μm² and 110 241.4±49 262.2 vs 11 973.7±3 968.5 μm² respectively, P<0.01) of submucosal veins in esophagus. Compared to the corresponding controls, the expression of TNF-α and VEGF in M7 was significantly higher (2.23±0.30 vs 1.13±0.28 and 1.65±0.38 vs 0.56±0.30 for TNF-α and VEGF respectively, P<0.01), whereas there was no difference in M14 (1.14±0.38 vs 1.06±0.27 and 0.67±0.35 vs 0.50±0.24 for TNF-α and VEGF respectively, P>0.05) and M21 (1.20±0.25 vs 1.04±0.26 and 0.65±0.18 vs 0.53±0.25 for TNF-α and VEGF respectively, P>0.05). And the expression of TNF-α and VEGF in M7, (2.23±0.30 vs 1.14±0.38 and 1.65±0.38 vs 0.67±0.35 for TNF-α and VEGF respectively, P<0.01) and M14 (2.23±0.30 vs 1.20±0.25 and 1.65±0.38 vs 0.65±0.18 for TNF-α and VEGF respectively, P<0.01), but there was no difference between M7 and M14 (1.14±0.38 vs 1.20±0.25 and 0.67±0.35 vs 0.65±0.18 for TNF-α and VEGF respectively, P>0.05).

CONCLUSION: In the development of esophageal varices in portal hypertensive rats, increased TNF-α and VEGF may not be an early event, and probably play a role in weakening the esophageal wall and the rupture of esophageal varices.

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Key words: Portal hypertension; Esophageal varices; Tumor necrosis factor-alpha; Vascular endothelial growth factor

INTRODUCTION

It has been known that portal hypertension is responsible for the opening and dilatation of relative collateral vessels, which can lead to the development of varices at various locations. Esophageal varices are one of its most common and sometimes lethal complications. The studies on esophageal varices have been going on for more than one century, and have gained great achievements especially in the treatment, but the mechanism of their development remains to be clarified.

The functions of humoral substances in portal hypertension had been studied for a long time; however, most of the research was on their changes in the circulation. Recent studies suggested that the changes of humoral substances in portal hypertensive esophagus may be responsible for the development and rupture of esophageal varices[9-12].

Tumor necrosis factor-alpha (TNF-α), a 17 ku mononuclear-derived cytotoxic protein, plays an important role in the pathogenesis of multiple diseases[9-12]. The evidence indicates that the increased production of TNF-α has been implicated in the vasodilation associated with portal hypertension, which mainly mediated through an increased release of nitric oxide[9-12]. And further studies showed that TNF-α may activate not only the nitric oxide
synthase (NOS) gene but also the ET-1 gene\textsuperscript{[13-16]}. Furthermore, recent studies suggested that the overexpression of NOS and ET-1 in portal hypertensive esophagus may be responsible for the development and rupture of esophageal varices\textsuperscript{[5-8]}, but the expression and function of TNF-\(\alpha\) in portal hypertensive esophagus remain unclear.

Vascular endothelial growth factor (VEGF), termed on its ability to promote growth of vascular endothelial cells, is a glycoprotein that selectively induces endothelial proliferation, angiogenesis, and capillary hyperpermeability, and is known as a key regulator of blood vessel growth\textsuperscript{[19,20]}. Several studies showed that the VEGF gene is expressed in a wide variety of normal animals and human tissue\textsuperscript{[19,20]}, and it also plays an important role in some pathological conditions, which related to vessel changes\textsuperscript{[21-28]}. Though the serum VEGF levels in cirrhotic patients was significantly lower than that in the control\textsuperscript{[26-28]}, the expression of VEGF in portal hypertensive gastric mucosa, whether in patients or in animal models, had significantly increased\textsuperscript{[21-23]}. As to esophageal varices, another complication of portal hypertension, Genesca et al\textsuperscript{[25]} observed that the serum VEGF levels in cirrhotic patients without gastroesophageal varices was higher than that in patients with them, but how it is expressed in portal hypertensive esophagus remains unknown.

Since esophageal varices are characterized by the pathological changes of esophageal submucosal vein, and are also components during the progress of portal hypertension, we hypothesized that TNF and VEGF may be involved in the etiopathology of esophageal varices. So in the present study, we established a rat model of esophageal varices by a two-stage ligation of portal vein plus ligation of the left adrenal vein, then investigated the dynamic expression of TNF-\(\alpha\) and VEGF in the development of esophageal varices in portal hypertensive rats.

**MATERIALS AND METHODS**

**Preparation of esophageal variceal rat model**

Sixty male Sprague-Dawley rats weighing 225-275 g, obtained from the Laboratory Animals Center of Xiangya Medical College, were divided into the model group and the control randomly. Modified from Tanoue’s method\textsuperscript{[31]}, a two-stage ligation of portal vein plus ligation of the left adrenal vein was performed in the model group. In brief, with the rats under ether anesthesia, the portal vein was isolated after median laparotomy. A ligature was tied by a 3-0 silk around both an 18-gauge hypodermic needle lying alongside the portal vein and the portal vein, and then removal of the needle yielded a partial ligation of the portal vein. Subsequently, both ends of silk were drawn out through the abdominal wall after a round the portal vein, and placed hypodermically in the flank. In addition, the left kidney, adrenal gland, and adrenal veins were exposed, and the left adrenal vein was ligated, but no devascularization was conducted at the circumference of the left renal vein except removal of the retroperitoneal fat around the top of left kidney. Seven days after the operation, the ends of the silk in the flank were pulled simultaneously to make a complete portal ligation.

Thirty rats in model group were divided into three subgroups (M\(_1\), M\(_2\), and M\(_3\), \(n = 10\)), which were kiued on the seventh day, the 14\(^{th}\) day and the 21\(^{st}\) day after the complete portal ligation. The rats in the control group, which underwent a sham operation, were also separated into three subgroups (C\(_1\), C\(_2\), and C\(_3\), \(n = 10\)), corresponded to the model group. The rats fed with rat chow and water ad libitum before and after surgery. All procedures are under the animal protection council.

**Measurement of the portal pressure**

After the rats were anesthetized with ether and the superior mesenteric vein was exposed. A catheter perfused with heparin saline solution (200 units of heparin diluted in 1 mL of 0.9% saline solution) was inserted through the superior mesenteric vein into the portal vein. The portal pressure was measured from the height of the column of saline within the catheter, with the right atrium as the zero reference.

**Tissue preparation**

After portal pressure measurement, the rats were killed with a bolus of 0.5 mL potassium chloride via the mesenteric catheter. The lower esophagus (0.5 cm in length from esophagogastric junction) was excised, fixed in 10% buffered formalin, and embedded in paraffin. Serial transverse sections were cut at 4 \(\mu\)m, some stained with hematoxylin and eosin (HE) for pathological study and others for immunohistochemistry.

**Immunohistochemical procedure**

TNF-\(\alpha\) and VEGF were detected by the immunohistochemical SP technique according to the manufacturer’s instructions. In TNF-\(\alpha\) staining, the antigen enhancement was performed by immersing the slides in boiling 10 mmol/L citric acid buffer (pH 6.0) for 20 min. In VEGF staining, sections were treated with boiling EDTA solution for 20 min. After immersing in 0.3% \(\text{H}_2\text{O}_2\) in methanol for 10 min and incubating with normal goat serum for 10 min at room temperature, sections were incubated with the primary antibody of either anti-TNF\(\alpha\) (goat polyclonal antibody, Santa Cruz, diluted 1:50) or anti-VEGF antibody (mouse monoclonal antibody, Santa Cruz, diluted 1:50) at 4 °C overnight. Then, sections were incubated with biotinylated second antibody of either anti-mouse or anti-goat IgG for 10 min at room temperature, followed by 10-min incubation in an S-P complex solution. Finally, the peroxidase activity was visualized with 0.4% diaminobenzidine (DAB). Sections were rinsed in 0.01 mol/L phosphate buffer solution (PBS, pH 7.4) between every two steps. We used PBS instead of the primary antibody as negative control.

**Morphometric analysis**

With the computer software HPIAS-1000, the number of vessels and the total vascular area (diameter >20 \(\mu\)m) in the esophageal submucosa was calculated.

Immunohistochemical expression of TNF-\(\alpha\) and VEGF were detected with the corrected optical density (COD) values. Briefly, the optical density of immunoreactive
products in five randomly selected fields of each different esophageal section was measured by an image-based analysis system, and the measurement was standardized by subtracting the background intensity of each section.

**Statistical analysis**
All data were analyzed using the SPSS10.0 for Windows. The results were expressed as the mean±SD. Mann-Whitney U test was used to determine any significance between groups. 

**RESULTS**

**Portal pressure**
The mean portal pressure in all three model subgroups (M7, M14, and M21) was significantly higher than that in the corresponding control subgroups (C7, C14, and C21) (P<0.01). There was no difference in portal pressure between M14 and M21 (P>0.05), and it had significantly decreased in both than in M7 (P<0.05) (Table 1).

| Group | 7 d   | 14 d   | 21 d   |
|-------|-------|--------|--------|
| Model | 23.8±1.8 | 20.9±2.7 | 20.6±2.7 |
| Control | 11.6±0.8  | 11.4±0.8  | 11.8±0.7  |

**Esophageal morphometric analysis**

**Number of veins in submucosa** The number of submucosal veins in the three model subgroups (M7, M14, and M21) was significantly higher than that in the corresponding control subgroups (C7, C14, and C21) (P<0.01), but there was no difference between model subgroups (P>0.05) (Table 2).

| Group | 7 d   | 14 d   | 21 d   |
|-------|-------|--------|--------|
| Model | 9.3±1.5  | 11.1±1.8 | 11.7±1.5 |
| Control | 5.1±0.8  | 5.4±1.3  | 5.2±1.1  |

**Total vascular area in submucosa** The total vascular area in the three model subgroups (M7, M14, and M21) was significantly higher than in the corresponding control subgroups (C7, C14, and C21) (P<0.01). There was no difference in total vascular area between M14 and M21 (P>0.05), and it was significantly higher in both than that in M7 (P<0.05) (Figure 1, Table 3).

| Group | 7 d   | 14 d   | 21 d   |
|-------|-------|--------|--------|
| Model | 78 972±3 527 8 | 107 207±4 646 1.4 | 110 241±4 926 2.2 |
| Control | 12 993±4 994 8 | 11 862±6 423 2 | 11 973±7 968 5 |

**DISCUSSION**
In our study, a prehepatic portal hypertensive rat model was created, and the rats in model group developed more submucosal vessels than in the control. On the 14th d and the 21st d after the complete portal ligation, the portal pressure and the vascular area were stabilized basically; so we conclude that 7 d after the complete portal ligation should be considered the early stage, whereas 14 and 21 d, the persistent stage, in the development of esophageal varices.
TNF-α was first identified as an endotoxin-induced serum factor that can cause necrosis of tumors, by Carswell et al[31] in 1975, and this multifunctional cytokine can increase vascular permeability and cause both structural and metabolic changes in vascular endothelial cells[33]. Our results showed that overexpression of TNF-α was only detected in M₂ group, and the positive immunostaining areas were mainly in the surface of esophageal mucosa rather than submucosal vessels. Though other studies suggested that TNF-α can cause the overexpression of NOS and ET-1 in portal hypertensive gastric mucosa, our results showed that the overexpression of NOS and ET-1 was even earlier than TNF-α (data not shown). All findings indicated that TNF-α produced in local esophagus may play an important role at the persistent stage in the development of esophageal varices via other pathway instead of NOS and ET-1 pathway, and the pathogenesis of esophageal varices may be different from the portal hypertensive gastropathy (PHG). On the other hand, as an inflammatory factor, the overexpression of TNF-α in the surface of esophageal mucosa may increase mucosal susceptibility to injury, but its function in local portal hypertensive esophagus remains to be clarified.

VEGF was first described by Senger[34] in 1983 who partially purified a factor secreted by hepatocarcinoma cell lines that increased dye extravasation into the skin of guinea pigs. This peptide was also termed vascular permeability factor (VPF) since it can increase interstitial accumulation of intravenously injected dye and stimulate the production of ascites[34,35], or termed vasculotropin based on its ability to stimulate endothelial cell migration[40], and now this peptide is referred to as VEGF. VEGF can stimulate endothelial mitosis, migration and increase permeability of endothelial monolayers by binding to the three different receptors[38]. In our study, its expression in M₂ group was stronger than in other groups, which suggested that the synthesis of VEGF was not an early accident in the development of esophageal varices. Kroll et al[39] observed that the activation of VEGF receptor-2 leads to an up-regulation of NOS protein. Though we did not detect the expression of VEGF receptor-2 in our study, our data showed the overexpression of NOS in portal hypertensive esophagus, which seems not to be activated by VEGF produced at local esophagus indicating that the overexpression of NOS was earlier than VEGF (data not shown). The late-stage increased VEGF can probably cause edema of submucosal layer and lead varices losing their surrounding support, consequently resulting in the weakness of the esophageal wall and predisposes varices to the rupture by inducing vascular hyperpermeability.

Interestingly, we also noticed the controversy that whether a dilated submucosal vein plexus resulted from passive opening of pre-existent vascular channels or from the newly formed vessels. In our study, the model rats developed more submucosal vessels than the control. However, we only counted the vessels with diameter over 20 µm; there is always the possibility that the higher vessel number could be attributed to the dilatation of pre-existent veins not counted in the control group due to their small size. Nevertheless, the total vascular area in M₁ and M₂ was significantly higher than M; but there is no significant difference in the number of vessels, which indicated the majority of the dilatation of pre-existent veins in the early stage of esophageal varices. In addition, whether the overexpression of VEGF indicates the formation of new vessels at late stage of esophageal varices, our acute model cannot answer the question yet; however, it is more likely there is the passive opening of pre-existent vascular channels at early stage in the development of esophageal varices, and de novo synthesis of new vascular channels may appear at late stage.

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