Are humoral factors involved in the colonic mucosal lesion in portal hypertensive rats ?

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Abstract

Aims : **With a prehepatic portal hypertensive rat model, we explored the involvement of humoral factors to the occurrence of portal hypertensive colopathy (PHC), another clinical entity besides portal hypertensive gastropathy (PHG) in portal hypertension, by investigating the expression of inducible nitric oxide synthase (iNOS), endothelial constitutive NOS (ecNOS), endothelin-1 (ET-1), tumour necrosis factor alpha (TNF-α) and vascular endothelial growth factor (VEGF) in the colonic and gastric mucosa.**

Methods : **Portal hypertension was produced by a two-stage ligation of portal vein plus ligation of the left adrenal vein in male Sprague-Dawley rats. Two weeks after complete obstruction of the portal vein, the portal pressure was measured and the expression of iNOS, ecNOS, ET-1, TNF-α and VEGF in the colonic and gastric mucosa were detected by RT-PCR and immunohistochemistry methods.**

Results : **A 1.8fold ($P < 0.01$) elevation of the portal pressure was detected in the portal hypertensive rats as compared to control. Significantly up-regulation of the mRNA levels of iNOS ($P < 0.01$), ET-1 ($P < 0.05$) and TNF-α ($P < 0.01$), but not ecNOS and VEGF, were detected in the colonic mucosa of portal hypertensive rats compared with control. The mRNA of iNOS, ecNOS, ET-1, TNF-α and VEGF were all significantly increased at varied levels in the gastric mucosa as compared to control (P all < 0.05). No difference of the appearance and localization of immunostaining of iNOS, ecNOS, ET-1, TNF-α and VEGF in the colonic and gastric mucosa were seen between two groups.**

Conclusions : **These data suggest the involvement of the up-regulation of iNOS, ET-1 and TNF-α in the colonic mucosal lesion of portal hypertensive rats.** (Acta gastroenterol. belg., **2007**, 70, **271-276**).

Key words : portal hypertensive colopathy, endothelin-1, Nitric oxide synthase, tumour necrosis factor alpha, vascular endothelial growth factor.

Abbreviations : portal hypertensive colopathy (PHC) ; portal hypertensive gastropathy (PHG) ; inducible nitric oxide synthase (iNOS) ; endothelial constitutive NOS (ecNOS) ; endothelin-1 (ET-1) ; tumour necrosis factor alpha (TNF-α) ; vascular endothelial growth factor (VEGF) ; hepatic venous pressure gradient (HVPG) ; phosphate buffered saline (PBS).

Introduction

Studies about portal hypertension have been carried out for more than one century, nevertheless the term of portal hypertensive colopathy (PHC) was raised in recent decade (1-3). PHC and the colonic mucosal anomalies occurred subsequently in patients with portal hypertension, is a main cause of lower gastrointestinal bleeding in portal hypertensive patients (4,5). PHC is generally diagnosed based on the endoscopic features, which usually include vascular lesions, colitis-like abnormalities and rectal varices, or a combination of these features (5-9). Histomorphometric studies have shown PHC to be characterized by the proliferation, dilation and irregular thickening of the tortuous mucosal capillaries, with slight or without observable inflammation (1), which, to some extent, were similar to the histological features of portal hypertensive gastropathy (PHG) (10).

Colorectal varices commonly occurs with esophageal varices and PHG simultaneously, but the hepatic venous pressure gradient (HVPG) and severity of cirrhosis are not related to the presence of colorectal varices (11-13). This indicates that an elevated portal pressure is an important, but not unique, factor in the pathogenesis of PHC. From the studies of PHG, elevated portal pressure has been shown to be able to induce changes of local hemodynamics, thus cause congestion in the upper stomach and gastric tissue damage. These changes may then activate cytokines and growth factors, such as nitric oxide synthase (NOS), endothelin 1 (ET-1) and tumour necrosis factor alpha (TNF-α), etc. in the portal hypertensive gastric mucosa. The overproduction of these factors may together cause an increased susceptibility of gastric mucosa to damage and produce PHG in patients with portal hypertension (10,14-16).

Whether these humoral factors also involve in the pathogenesis of PHC, another disease entity originating from portal hypertension, as they do in the pathogenesis of PHG, it is, however, not known at present (16,17). Therefore, in this study, we established a prehepatic portal hypertensive rat model which mimics the histological changes of human PHC and PHG (18,19) by a two-stage ligation of portal vein plus ligation of the left adrenal vein, and then investigated the expression of inducible NOS (iNOS), endothelial constitutive NOS (ecNOS),

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ET-1, TNF-α and vascular endothelial growth factor (VEGF) in colonic and gastric mucosa.

Materials and methods

Preparation of portal hypertensive rat model

Twenty male Sprague-Dawley rats, weighing 225-275 g, obtained from the Laboratory Animals Centre of Xiangya Medical College of Central South University, Changsha, China, were divided into model group (n = 10) and control group (n = 10). These animals were housed separately and exposed to 12 h light-dark cycles with unlimited access to food and water. All animal experiments were done in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NRC1996) and were approved by the committee of Xiangya Medical College. The operation was performed as reported previously (20,21). Briefly, first ligation of portal vein plus ligation of the left adrenal vein was performed on the rats of model group, while the rats of control group only underwent a sham operation. Two weeks later, the portal vein was ligated completely on the rats of model group.

Measurement of the portal pressure

Two weeks after the complete portal ligation, the rats were anesthetized with ether and the superior mesenteric vein was exposed. A catheter perfused with heparin 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

Following the portal pressure measurement, the rats were killed with a bolus of 0.5 ml potassium chloride via the mesenteric catheter. Two pieces of the sigmoid (0.5-1.0 cm in length) and two pieces of the stomach (along the greater curvature) were excised, and rapidly frozen in liquid nitrogen or fixed in 10% buffered formalin.

Immunohistochemistry

Serial sections (5 μm) were examined with streptavidin-biotin peroxidase immunohistochemical staining according to the manufacturer's instructions (Maixin, Fujian, China). The antigen enhancement of the sections was performed with boiling for 20 min in EDTA solution (pH 8.5) for iNOS, ecNOS, ET-1 and VEGF staining or 10 mM citric acid buffer (pH 6.0) for TNF-α staining. Following the blocking of endogenous peroxidase with 0.3% H_2O_2 in methanol and incubating with normal goat serum for 10 min at room temperature, sections were separately incubated with the primary antibodies (Santa Cruz, Beijing, China), diluted 1:50 in 10% normal goat serum, at 4°C overnight. Afterward, sections were incubated with biotinylated IgG, diluted 1:100, for 10 min at room temperature. Finally, the peroxidase activity was visualized with 0.4% diaminobenzidine. Sections were rinsed in 0.01M phosphate buffered saline (PBS, pH 7.4) between every above step. We used PBS instead of the primary antibodies as negative control.

Semi-quantitative RT-PCR

Total RNA was extracted from the homogenate of the stomach or colon using silica gel-based spin column (RNeasy Kit, Qiagen) according to the manufacture's instructions. Ultraviolet spectrophotometer was used to determine its concentration and purity. Two μg of total RNA were used for first strand cDNA synthesis with Superscript reverse transcriptase II (Invitrogen). PCR was performed 32 cycles at 94°C for 30 s and 68°C for 1 min with the final volume 25 μL. The sequences of primers were used as follows. iNOS : sense 5' AGCATCACCCCTGTGTTCCACCA 3' and antisense 5' TGGGACAGTCTCCATTCCCA 3' (388bp) ; ecNOS : sense 5' TACGAAGAATGGAAGTG 3' and antisense 5' CCTTTGATCTCAATGTCG 3' (410bp) ; ET-1 : sense 5' GGAGCTCCAGAAACAGCTGTC 3' and antisense 5' CTGCTGATAGATACACTTCTTTCC 3' (432bp) ; TNF-α : sense 5' GCCACCACGCTCTTC-TGTCTACT 3' and antisense 5' GAGGTTGACTT-TCTCCTGGTATG 3' (384bp) ; VEGF : sense 5' TACCTCCACCATGCCAAGTG 3' and antisense 5' CTGTCTTTCTTTGGTCTGCATTCA 3' (356bp) ; GAPDH : sense 5' CGGTGTGAACGGATTTGGCCG-TAT 3' and antisense 5' GGCCTTCTCCATGGTGGT-GAAGAC 3' (307bp).

Ten μL of each PCR products was visualized on 1.5% agarose gel stained by 0.5% ethidium bromide. The intensities of the bands were measured with HPIAS-1000 (Olympus, Japan) and normalized to GAPDH.

Statistical Analyses

All computations were performed with the PRISM software (GraphPad, San Diego, CA, USA). Unpaired Students's t tests (two-tailed) were used to detect significant differences of the portal pressure and the mRNA levels of iNOS, ecNOS, ET-1, TNF-α and VEGF between the model group and sham-operated control. The potential relation between the increase of the mRNA levels of the different genes were analyzed by calculating Pearson correlation coefficients. Results were expressed as mean ± SEM. $P < 0.05$ was considered significant.

Results

General status and portal pressure

All the rats were alive till the end of the experiments. The weight of the rats of model group decreased

post-operationally, but no significant difference was detected between two groups over the four weeks (data not shown). Evident dilation of paraesophageal vein plexus, abdominal collaterals and mesentery vessels were observed in all the ten rats of model group two weeks later after the complete portal ligation was performed (Fig. 1A, B), whereas no such changes were observed in the sham-operated rats. The portal pressure of the rats of model group was significantly increased compared with control ($P < 0.01$, 20.90 ± 2.57 cmH_2O and 11.43 ± 1.03 cmH_2O, for model group and control, respectively).

Immunohistochemical study of portal hypertensive colonic and gastric mucosa

The positive immunoreactivity was visualized as brown-colored deposits. In gastric and colonic mucosa, positive immunoreactive deposits of iNOS, ecNOS or VEGF mainly located in the endothelia of submucosal vessels, muscularis mucosa and propria (Fig. 1C-J), and the immunoreactivity of ET-1 was observed in the submucosal vessels and submucosa. Positive immunoreactive deposits of TNF-α mainly located in the surface of mucosa, muscularis mucosa, propria of colon and stomach, as well as gastric submucosal vessels. Comparing with control, no difference of the appearance and localization of positive immunostaining of iNOS, ecNOS, ET-1, TNF-α and VEGF were observed in portal hypertensive colonic and gastric mucosa.

Gene Expression

As can be seen in Figure 2A, the levels of iNOS, ecNOS, ET-1, TNF-α and VEGF mRNA in the gastric mucosa was significantly increased in the portal hypertensive rats compared with control (range from 1.71fold to 2.35fold, P all < 0.05, Fig. 2B). The mRNA levels of ET-1 were significantly correlated with the mRNA levels of TNF ($P < 0.05$, $r = 0.76$) in the portal hypertensive gastric mucosa. The levels of iNOS, ET-1 and TNF-αmRNA in the portal hypertensive colonic mucosa were significantly elevated at 2.76fold ($P < 0.01$), 1.80fold ($P < 0.05$) and 2.41fold ($P < 0.01$), respectively, as compared to control (Fig. 2A, C). The mRNA levels of ET-1 were significantly correlated with the mRNA levels of iNOS and TNF-α ($P < 0.05$, $r = 0.67$ and $P < 0.01$, $r = 0.92$, respectively) in the colonic mucosa of the model group. A slight, non-significant correlation between the mRNA levels of TNF-α and iNOS ($P = 0.05$, $r = 0.63$) was detected in the portal hypertensive colonic mucosa. Although non-significant differences of the levels of ecNOS and VEGF mRNA were detected between two groups, 1.68fold and 1.24fold elevation for ecNOS and VEGF, respectively, appeared in the portal hypertensive rats as compared to control (Fig. 2A, C).

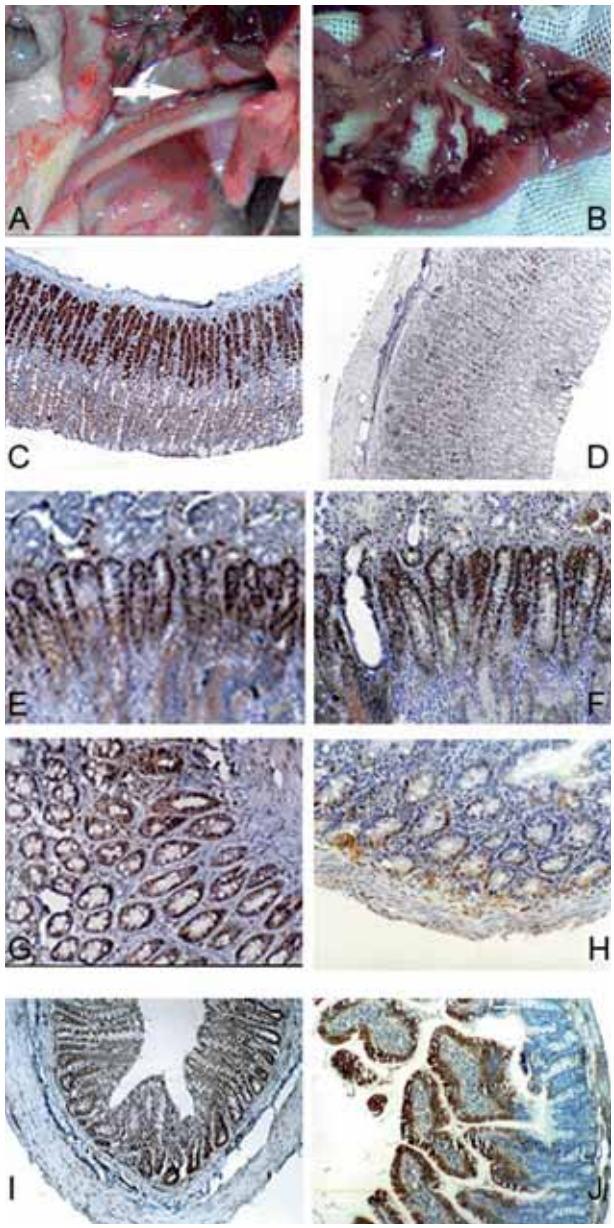

Fig. 1. — Dilated paraesophageal vein plexus (arrow) (A) or dilated mesentery vessels (B) in portal hypertensive rat. Immunostaining of ecNOS (C-F). Gastric mucosa in portal hypertensive rat (C, ×100) or control (D, ×100), colonic mucosa in portal hypertensive rat (E, ×200) or control (F, ×200). Immunostaining of VEGF (G-J). Gastric mucosa in portal hypertensive rat (G, ×200) or control (H, ×200), colonic mucosa in portal hypertensive rat (I, ×100) or control (J, ×200).

Discussion

In respect that the PHC occurs frequently in association with oesophageal varices and PHG in portal hypertensive patients (12), we developed a prehepatic portal hypertensive rat model by a two-stage ligation of portal vein plus ligation of the left adrenal vein. This method induces prevalent oesophageal varices in animals (20), which probably means to us that PHC occurs frequently

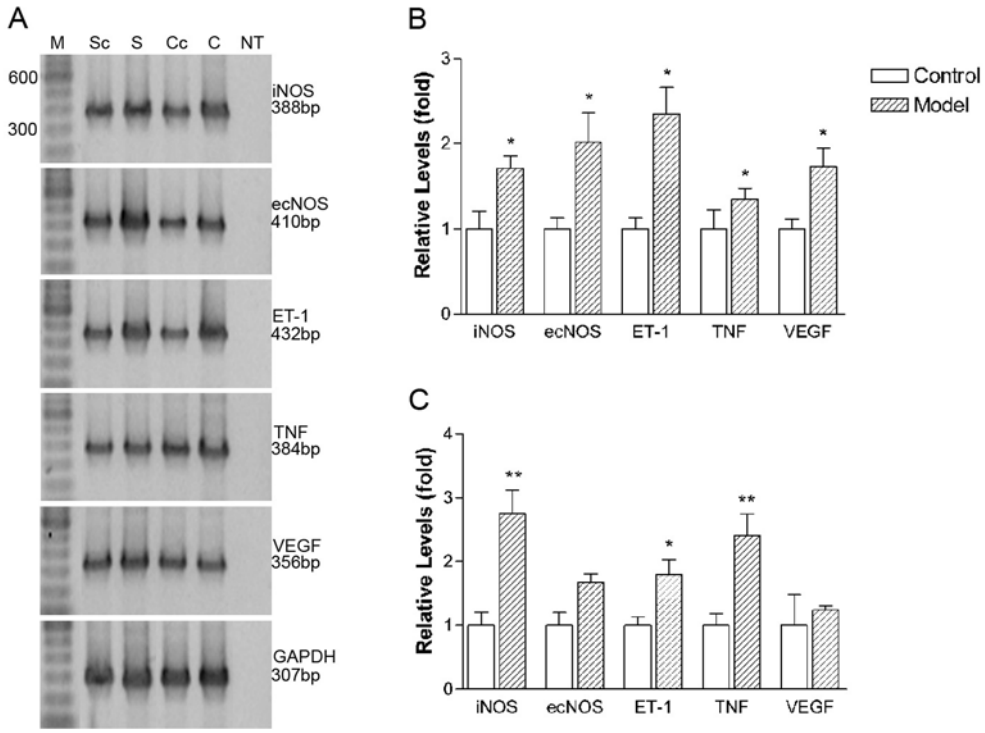

Fig. 2. — RT-PCR analyses of iNOS, ecNOS, ET-1, TNF-α and VEGF mRNA. Alterations of the mRNA levels of iNOS, ecNOS, ET-1, TNF-α and VEGF in the portal hypertensive stomach and colon (A). M, marker ; Sc, stomach of control ; S, stomach of portal hypertensive rat ; Cc, colon of control ; C, colon of portal hypertensive rat ; NT, negative control. Histogram summarizing and comparing of the mRNA levels of iNOS, ecNOS, ET-1, TNF-α and VEGF between model group (portal hypertensive rats) and control in the stomach (B) or colon (C). The mRNA levels in model group were relative to control. Values are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, Unpaired *t* test.

with this model. Therefore, this model is appropriate for the study of the pathogenesis of PHC.

In agreement with previous studies (20,21), the portal pressure of the rats in model group increased dramatically, companied with the dilation of paraesophageal vein plexus, abdominal collaterals and mesentery vessels, which mimics the histological changes of human PHC and PHG.

Yamakado et al reported that the HVPG was higher in the portal hypertensive patients with PHC than in the patients without PHC (12). Decompression shunt surgery was shown to be effective for PHC (5,13,19). Nevertheless, PHC is not an inevitably concomitant of portal hypertension (11). Therefore, a hypothesis that an elevated portal pressure triggers local colonic lesion and further promotes the development of PHC combining with other pathological factors was suggested, just as what was demonstrated in PHG (10). Among possible pathological factors, humoral factors, such as iNOS, ecNOS, ET-1, TNF-α and VEGF, have been shown playing an important role in the occurrence of PHG in portal hypertension (10,14-16). This is supported by our study.

Increased NOS activity and the consequently increased release of NO have been suggested responsible for the hyper-dynamic circulation of cirrhosis in portal hypertension (22-26). Elevated expression of iNOS mRNA was seen in both the colonic mucosa and gastric mucosa of portal hypertensive rats in the present study. Furthermore, the level of ecNOS mRNA was also upregulated in the portal hypertensive gastric and colonic mucosa, but to a different extent, i.e. more obvious upregulation in the gastric mucosa. This may indicate that the activation of iNOS in the colonic mucosa played a main role in the overproduction of NO. The differential expression of ecNOS observed in the colonic and gastric mucosa of portal hypertensive rats may resulted from the different effects of the elevated portal pressure on the stomach and colon.

Recent studies have shown that the level of ET-1 significantly increased in the serum of portal hypertensive rats (27-29) and thus impaired mucosal microcirculation resulting the occurrence of PHG (14). In our study, the over-expression of ET-1 was observed also in the colonic mucosa of portal hypertensive rats. To the best of our knowledge, this is the first report about the involvement of ET-1 in the occurrence of PHC. Overexpression of ET-1 may mediate vasoconstriction by inducing the overproduction of NO and prostacyclin in

PHG (30). Probably a similar role of highly expressed ET-1 plays in the pathogenesis of PHC, which is indicated by the correlation between the increase of the mRNA levels of ET-1 and iNOS in the colonic mucosa of portal hypertensive rats in the present study.

The overproduction of TNF-α has been implicated in vasodilatation by mediating the release of NO (31-33). Furthermore, TNF-α was suggested activating not only NOS but also ET-1 in portal hypertensive gastric mucosa of rats (15,34,35). Positive correlation between the increases of the mRNA levels of TNF-α and ET-1 in the portal hypertensive gastric and colonic mucosa in the present study may suggest that the over-expression of TNF-α activates the expression of ET-1 and subsequently results in the overproduction of NO in the gastric mucosa as well as colonic mucosa.

VEGF is involved in both physiological and pathological angiogenesis (36,37). The expression of VEGF significantly increased in portal hypertensive gastric mucosa (38-40) just as what we observed in the present study. VEGF may induce endothelial proliferation, angiogenesis and capillary hyper-permeability and congestion in the gastric mucosa of cirrhotic patients (41-43). No significant alterations of the mRNA levels of VEGF, however, were seen in the portal hypertensive colonic mucosa in our study. Further studies are required to rule out the roles of VEGF in the occurrence of PHC.

With the elevation of portal pressure, the increased resistance to backward blood stream may result in vascular congestion, oedema of colon and the injury of the barrier of colon subsequently. The impairment of the barrier of colon could lead to increased translocation of noxious agents, such as bacteria (44). Bacterial endotoxin and the impairment of the tissue induce the activation of iNOS (45) and TNF-α (46), and the increased TNF-α in turn induces the over-expression of iNOS and ET-1. Consequently, NO is overproduced in the colonic mucosa and induces vascular lesions possibly by several mechanisms ; (1) vasodilatation, which results in vascular congestion and hyper-permeability, (2) initiating membrane lipid peroxidation and cell injury through producing peroxynitrite (47), which results in enhanced permeability of colonic mucosa, (3) the hypokinesia of colon induced by increased NO (48) delays the contact time of the toxic substance with colonic mucosa, which aggravates the injury of colonic mucosa.

Taken together, our data suggest the involvement of the up-regulations of iNOS, ET-1 and TNF-α in the occurrence of PHC in portal hypertensive rats. However, whether they contribute alike to the pathogeneses of PHC and PHG as well as the mechanisms of the aberrant expression of these humoral factors in the occurrence of PHC need to be further clarified.

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