Vascular Density and VEGF Expression in Hepatic Lesions

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Abstract

Background. Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor worldwide and the third most common cause of cancer-related death. HCC is a hypervascular tumor expressing several angiogenic factors. The correlation between plasma levels of Vascular Endothelial Growth Factor (VEGF) and the survival of patients with HCC was demonstrated earlier. However, a relationship between the expression of VEGF and vascular density in the HCC and non-malignant hepatic parenchyma has not been investigated. **Material and methods**. We studied the expression of VEGF and vascular density in normal hepatic parenchyma, cirrhosis and HCC using a computer-based analysis of immunohistochemical stainings and confirmed it by Western Blot. **Results.** The vascular density in the areas of HCC and internodular fibrotic tissue in cirrhotic liver was significantly higher (185%; 146% respectively) than in non-neoplastic hepatic parenchyma. Additionally, cirrhotic nodules were characterized by significantly lower vascularization (71%) compared with normal liver. There was a strong correlation between the levels of VEGF expression in tissue and the number of vessels (r=0.98, R2=0.9696). **Conclusion.** Cirrhosis and HCC are characterized by different degrees of vascularization, which has been quantitated by a novel computer-based analysis of immunohistochemical stainings. One of the major stimuli for angiogenesis in these liver diseases could be VEGF, as the VEGF expression was higher in HCC and diminished in cirrhotic nodules, thus strongly correlating with the degree of vascularization. Our findings demonstrate that angiogenesis may play an important role in the pathogenesis of these conditions.

Key words

Angiogenesis - hepatocellular carcinoma - liver cirrhosis - vascular endothelial growth factor

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm worldwide and the third cause of a cancer-related death (1). Angiogenesis plays a key role in normal development and maturation of tissues and organs (2,3) and in the pathogenesis of many cancers (3,4). HCC is a hypervascular tumor mainly supplied by hepatic arteries, whereas normal liver parenchyma and dysplastic nodules are mainly supplied by the portal vein (1,5). It has been demonstrated that HCC expresses many angiogenic factors, including VEGF (23,11) and angiogenin (7). Moreover, VEGF expression by the tumor and VEGF levels in the blood of patients has been correlated with the size, invasiveness, metastases and prognosis of HCC (8, 9). Increased expression of VEGF receptors in HCC has been demonstrated on different levels, including mRNA and proteins (10,12). Different levels of VEGF expression in HCC and surrounding cirrhotic liver parenchyma has also been reported (13,14). VEGF expression has been found to be elevated in cirrhotic liver parenchyma surrounding HCC. However, no correlation between this data and the degree of vascularization has been demonstrated.

In the current study, we investigated the degree of vascularization as a surrogate marker of angiogenesis, as well as the levels of VEGF expression in unremarkable liver, cirrhotic liver and HCC. This was investigated using immunohistochemical methods and a computer-based quantitating analysis of protein expressions.

Material and method

Samples of hepatic tissue were selected from the Westchester Medical Center pathology database, and included viral C cirrhosis patients with (n=7) and without (n=7) HCC, and healthy sex- and age-matched subjects (n=7). The study was conducted using tissue sections obtained from total hepatectomy specimens from the patients who had underwent liver transplant surgery. No biopsy material was used in the study. Control tissue was obtained from autopsies of the sex- and age-matched

patients without clinical and histological evidence of liver diseases. Patients who were involved in the study had single or multiple nodules of HCC varying in size from 3.0 to 6.5 cm. Histological size of the tumor was calculated as the sum of all tumor nodules identified macro- and microscopically in the explanted liver. All patients selected did not have chemotherapy prior to the surgery. Histologically, tumor nodules had no or minimal necrosis and were classified as moderately to well differentiated hepatocellular carcinoma, grade 2-3. Patients with viral C cirrhosis alone had grade 2-3 stage 3-4 lesions based on the Modified Histological Activity Index (28).

All clinical diagnoses were first confirmed by a conventional H&E histology. The 5-mm histologic sections were stained with antibodies to PECAM (CD31) (Cell Marque Corp., Hot Springs, AR) and VEGF-A (ZYMED Labs, San Francisco, CA). Random sections were taken from the tumor nodules and at least 20 random fields were photographed and analyzed from each slide using an Olympus microscope, equipped with a digital camera. A computer-based quantitating analysis of immunohistochemical staining was performed (15,24). Briefly, the CD31-positive areas were extracted from the photographs using Adobe PhotoShop software. Vascular density was measured as the area of CD31-positive cells per area unit. Separately, the tissue samples from the same patients were homogenized and analyzed by Western Blot, using antibodies to the same proteins. The same technique was used to assess the expression of VEGF in different hepatic lesions.

All observations were completed by two independent observers, who were blinded to the origin of the data. All data are presented as mean ± SEM, unless specified. The means of two groups were compared using the two-tailed Student *t*-test.

Results

Immunostaining of hepatic tissue with CD31 antibodies highlighted endothelial cells in all vascular beds, including sinusoid capillaries. Computer-based analysis of CD31-positively stained areas (Fig.1 C) demonstrated that the number of vessels was significantly increased in the areas of HCC (185%) compared with control hepatic parenchyma without morphological abnormalities (Fig.1 A,E). In contrast, cirrhotic nodules showed a significant decrease in the number of vessels (71% compared with control). Interestingly, the number of vessels was significantly increased in the fibrotic stroma surrounding cirrhotic nodules not only compared with the cirrhotic areas (226%), but the control tissue as well (146%) (Fig.1 B,D). Western blot of fresh hepatic tissue from grossly recognizable tumors in the same patients revealed that the expression of CD31 protein was significantly higher in HCC compared to that of the control liver. On the other hand, tissue from the cirrhotic liver had an increased level of CD31 expression in comparison to the control liver also (Fig.1 F). This discordance with histological data is attributed to the mixture of stroma and cirrhotic nodules in the samples prepared for Western blot. Nevertheless, the degree of CD31 expression in cirrhotic liver was less when compared to that of HCC.

In order to investigate possible pathophysiological mechanisms of these differences in the number of vessels in different hepatic lesions, immunohistochemical staining with anti-VEGF-A antibodies was performed. VEGF expression proved to be directly correlated with the number of vessels as measured by CD31 expression – the highest degree of VEGF expression was identified in HCC (2.5-fold higher), whereas cirrhotic nodules expressed a decreased amount of VEGF as compared to that of the normal liver parenchyma (Fig.2 A,B,E). In addition, the number of VEGF-producing cells in the internodular fibrotic stroma in cirrhotic liver was significantly higher than in the cirrhotic nodules or control liver (235% and 211%, respectively) (Fig.2 D). Computer-based analysis of VEGF-positive areas verified these tendencies (Fig.2 C). Western Blot of a fresh tissue obtained from the same patients revealed that VEGF protein expression was significantly higher in HCC as compared with that of the control liver, whereas the cirrhotic liver showed an amount of VEGF expressed similar to controls (Fig.2 F). There was a strong correlation between the levels of VEGF expression in tissue and the number of vessels present ($r=0.98$, $R2=0.9696$).

Discussion

In the current study, using a novel computer-based analysis of the immunohistochemical stainings, we demonstrate that different pathological processes in the liver are characterized by different degrees of vascularization. This is the first attempt to quantitate the differences in the vascular density and VEGF expression in different liver lesions. HCC has the highest number of vessels present, whereas cirrhotic nodules have the lowest degree of vascularization. Interestingly, fibrotic stroma surrounding the cirrhotic nodules had a significantly increased number of vessels.

HCC is a hypervascular tumor (1,16), with an increased angiogenesis, which correlates with the risk of vascular invasion (17) and, thus, metastases (18). Sinusoidal endothelial cells in HCC are characterized by immunoreactivity with CD31, CD34 and BNH9, and they are phenotypically different from endothelial cells in normal liver, losing fenestration and accruing basement membrane (19,20). This may be due to changes in hemodynamics and blood supply of HCC, which loses the dual hepatic arterial and portal venous supply of the normal hepatic parenchyma and receives blood predominantly from the arterial system (5).

The formation of granulation-like tissue in the fibromuscular stroma has been demonstrated earlier (30). The important role of VEGF in neovascularization and angiogenesis in granulation tissue formation and wound healing has been demonstrated earlier by numerous studies (29). We quantitated the number of vessels in the para-cirrhotic stroma and compared it with the vascular density

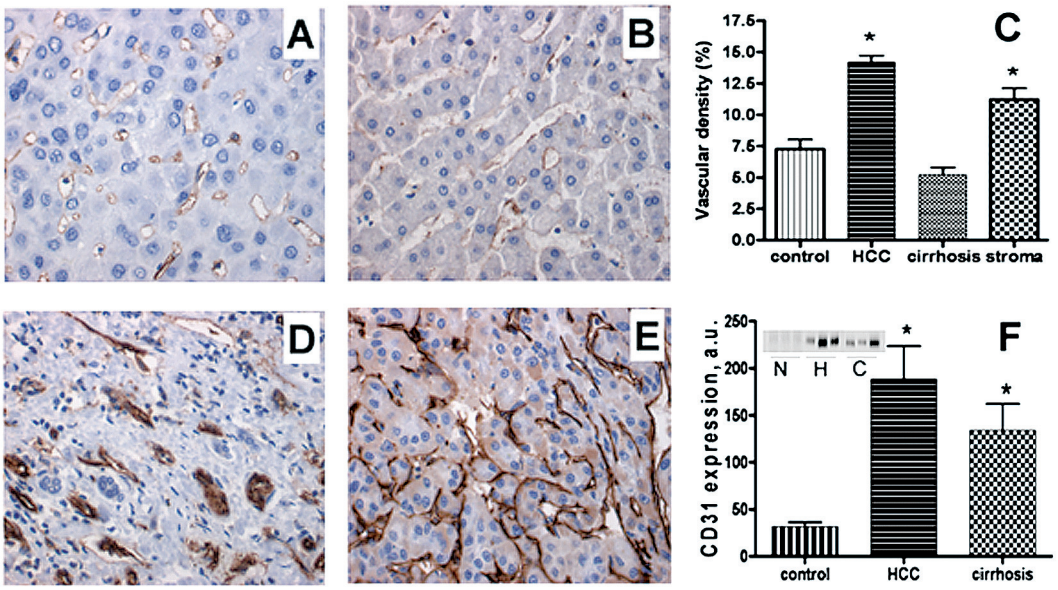

Fig.1 Vascular density in HCC, cirrhosis and para-cirrhotic stroma. Sections of liver tissue obtained from viral C cirrhosis patients with (n=7) and without (n=7) HCC were stained with CD31 antibodies. A – control liver; B – cirrhotic nodules; D – para-cirrhotic stroma; E – HCC. C - The quantitated analysis of the vascular density calculated by CD31 staining. F – Western Blot was performed on hepatic tissue obtained from the same patients (n=5) (insert: N- control, H – HCC, C – cirrhotic liver tissue). Densitometry of Western Blots stained with anti-CD31 antibodies.

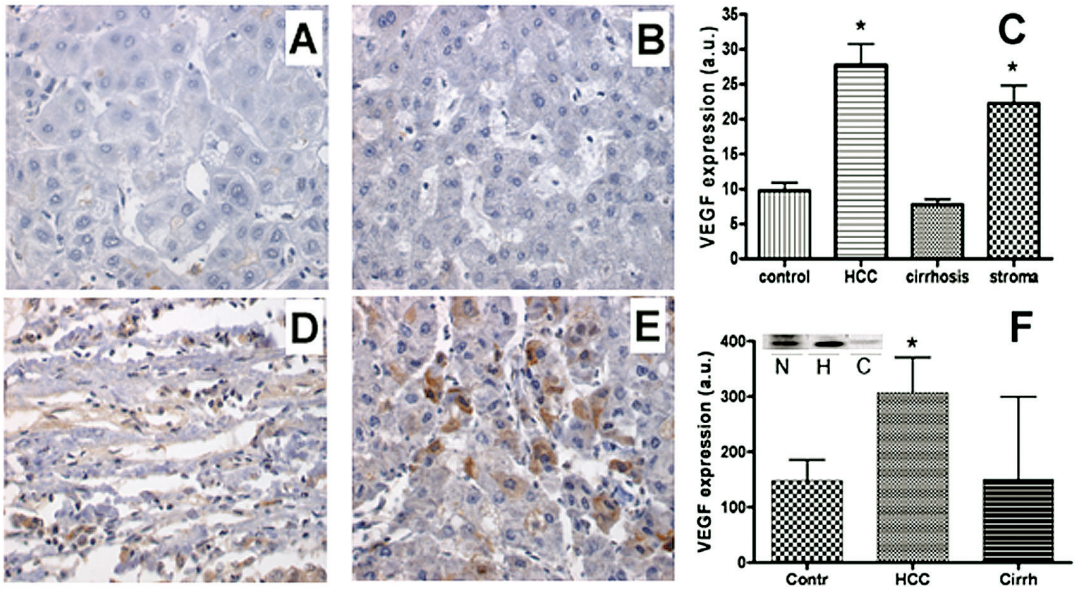

Fig.2 VEGF expression in HCC, cirrhosis and para-cirrhotic stroma. Sections of liver tissue obtained from viral C cirrhosis patients with (n=5) and without (n=5) HCC were stained with anti-VEGF-A antibodies. A – control liver; B – cirrhotic nodules; D – para-cirrhotic stroma; E – HCC. C - The quantitated analysis of the vascular density calculated by VEGF staining. F – Western Blot was performed on hepatic tissue obtained from the same patients (insert: N- control, H – HCC, C – cirrhotic liver tissue). Densitometry of Western Blots stained with anti-VEGF-A antibodies.

in cirrhotic nodules. Our data revealed significant differences in the degree of vascularization in cirrhotic nodules and surrounding stroma. In the early stages of cirrhosis, the production of VEGF and the neovascularization increases (21), whereas in the later stages, cirrhotic nodules in Hepatitis C patients are characterized by a decreased density of microvasculature (22), correlating with our findings. However, the distinction in the degree of vascularization within cirrhotic nodules versus surrounding cirrhotic stroma, found by us, has not been previously reported. We found that fibrotic stroma surrounding cirrhotic nodules has an increased number of vessels not only compared to the cirrhotic nodules, but to the normal liver parenchyma as well. This may result in the detection of an increased amount

of endothelial cell markers by different methods, which are not able to distinguish between different tissue types, such as Western Blot (Figs.1 F, 2 F) or PCR (14).

One of the major stimuli for angiogenesis in liver may be VEGF. As demonstrated here, VEGF expression was the highest in HCC and minimal in cirrhosis, which strongly correlates with the degree of vascularization. Indeed, HCC is characterized by an increased expression of VEGF receptors, demonstrated by both mRNA and protein expression levels (12). Deli et al (13) and Mathonnet et al (14) reported earlier that cirrhotic liver, surrounding HCC, expresses higher levels of VEGF compared to that of the tumor. In the current study we not only provided the quantitative analysis of VEGF expression, but also divided cirrhotic liver into two components: 1) cirrhotic nodules where the levels of vascularization and VEGF expression were decreased, and 2) fibrotic stroma, surrounding these nodules, where both the number of vessels and VEGF expression were significantly increased.

It has been previously demonstrated that the expression of VEGF in HCC is significantly associated with a higher proliferative index of tumor cells. Also, VEGF is expressed in the fibrovascular stroma and pericellular matrix of HCC (25). The expression of VEGF by hepatocytes in the cirrhotic liver has recently been demonstrated (26). One of the sources of VEGF in the fibromuscular stroma may be fibroblasts, since the ability of these cells to produce VEGF was demonstrated earlier (27) .

Our data suggest new pathophysiological mechanisms of cirrhosis formation, i.e. the decreased number of vessels in cirrhotic nodules may lead to diminished cell function, whereas increased vascularization of the surrounding stroma may be either a part of the formation of liver fibrosis and granulation tissue or be a compensatory response to the decreased blood supply in the cirrhotic nodules.

Conclusion

Earlier reports did not provide data on the distribution of VEGF expression within areas of cirrhosis and neoplastic tissue as compared with normal liver. In the current study, we quantitated not only the degree of vascularization in the HCC and cirrhotic liver, but in the non-diseased liver parenchyma as well. Our findings demonstrate that cirrhosis and HCC are characterized by different degrees of vascularization, suggesting an important role of angiogenesis in the pathogenesis of these conditions. One of the major stimuli for angiogenesis in these pathological processes could be VEGF, since its expression was directly correlated with the degree of vascularization.

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Conflicts of interest

None to declare.

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