

OVEREXPRESSION OF VEGF AND VEGFR2 IN CHRONIC HEPATITIS AND LIVER CIRRHOSIS

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ABSTRACT

VEGF (vascular endothelial growth factor) and the receptor for VEGF- Flk-1 (fetal liver kinase 1) are important players of normal and pathologic angiogenesis. Also, it was proved that they are involved in tumor progression and metastasis in many tumors types by overexpression in cancer cells. Liver malignances and premalignant lesions represent controversial issues concerning VEGF and VEGFR2 (vascular endothelial growth factor receptor 2) expression and their potential involvement in the progression of inflammatory and cirrhotic lesions and also in malignant transformation is virtually unknown. The aim of this work was to describe the differentiate expression and distribution of VEGF and VEGFR2 in chronic hepatitis and liver cirrhosis, and according to these findings to better characterize the molecular profiling of liver disease with malignant transformation potential. We investigated 20 cases with chronic hepatitis and cirrhosis on specimens taken during surgery. Immunohistochemistry was performed in all cases for VEGF, VEGFR2, and FVIII related antigen (Von Willebrand factor). We found significant correlation between HAI (histological activity index) value, VEGF and VEGFR2 expression and factor FVIII related antigen in central part of specimens with chronic hepatitis. Liver cirrhosis lacks this correlation. Our findings suggested that VEGF dependent angiogenesis is more active in chronic hepatitis in the center of the lesion compared with cirrhosis where MVD (microvessel density) is higher at the periphery of the nodules. We hypothesize that the involvement of VEGF and VEGFR2 complex in development of chronic hepatitis and liver cirrhosis could be considered for the use of anti VEGF antibodies as adjuvant therapy in early stages of these diseases.

Key Words: VEGF, VEGFR 2, liver cirrhosis, chronic hepatitis

INTRODUCTION

Vascular endothelial growth factor, also known as vascular permeability factor, is the most potent angiogenic factor. Cirrhotic livers had significantly higher MVD and VEGF expression compared with noncirrhotic livers. It has been shown that expression of the potent angiogenic factor, VEGF and its receptors, VEGFR-1 (vascular endothelial growth factor receptor 1) and VEGFR-2, increase during the development of liver fibrosis in murine model [1]. Although current treatments for fibrotic diseases such as idiopathic pulmonary fibrosis, liver cirrhosis, systemic sclerosis, progressive kidney disease, and cardiovascular fibrosis typically target the inflammatory response, there is accumulating evidences that the mechanisms driving fibrogenesis are distinct from those regulating inflammation [2]. These changes appear to be due in part to hypoxia produced by collapsed hepatic sinusoids following the liver regeneration processes.

An up-regulation of VEGF was found in the cirrhotic liver of patients with or without HCC (hepatocellular carcinoma), suggesting that this factor might be responsible for cirrhosis-associated angiogenesis [3] VEGF-positive expression was significantly higher

in surrounding cirrhotic liver tissues than in HCC, so VEGF may play an important role in the angiogenesis and prognosis of HCC, and in the angiogenesis of liver cirrhosis as well. Capsular infiltration, vascular invasion and hepatic metastasis were observed more frequently in patients with VEGF-positive expression than in those with VEGF negative expression [4].

Few data are available about expression of VEGF and VEGFR2 in chronic hepatitis and liver cirrhosis without malignant transformation. Most of the recent studies used animal models for VEGF/VEGFR2 expression in induced liver cirrhosis. The characterization of human liver tissue from liver cirrhosis and chronic hepatitis without associated hepatocellular carcinoma is a difficult approach due to the lack of biopsy material. A better understanding of VEGF/VEGFR2 expression in liver lesions without neoplastic transformation could explain some of the controversial issues concerning the involvement of these markers in tumor progression and malignant transformation and also their possible use in monitoring the effects of therapy.

We hypothesized that distribution and expression pattern of VEGF and VEGFR2 in hepatocytes are different between liver cirrhosis and chronic hepatitis.

Quantification of this particular features might be useful to explain the controversial results from the literature concerning the utility of VEGF and VEGFR2 as prognostic and follow up markers, and also might be a startpoint to introduce the anti VEGF therapies in liver lesions with malignant transformation potential.

MATERIAL AND METHODS

We included in our study 20 patients diagnosed with liver chronic diseases diagnosed by imagistic investigations and modified laboratory tests. All patients had history of alcoholic intake or C hepatitis. No specimens had associated hepatocellular carcinoma or any type of metastasis. Resected liver specimens obtained by open surgery during gallbladder ablation were fixed in 10 % buffered formalin for 48 hours and paraffin embedded. Sections from each case were stained with routine haematoxylin and eosin method for histopathologic evaluation. Immunohistochemical study included antibodies against VEGF, Flk 1 and FVIII antigens. Immunohistochemical technical details are summarized in Table 1.

We used normal renal parenchyma as positive control for VEGF and also the staining of blood vessels endothelium as positive control for VEGFR2.

Evaluation of the specimens included the intensity and distribution of positive reaction of VEGF and correlation with F VIII positive vessels density. Microscopic observation was performed by two independent observers using Nikon Eclipse E600.

RESULTS

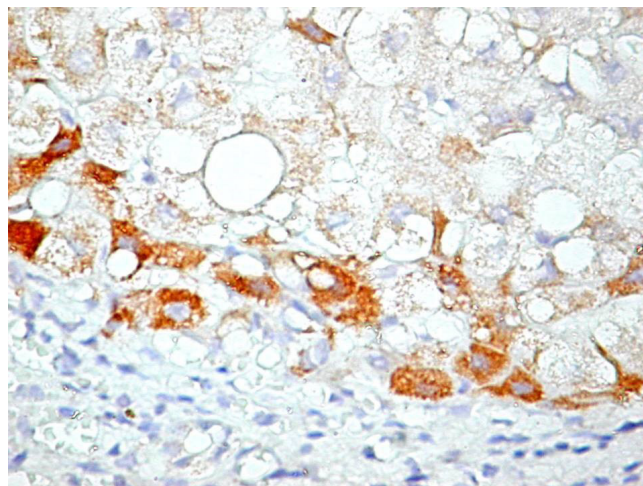
Microscopic examination of 20 specimens revealed 10 cases with chronic hepatitis and 10 cases with cirrhosis. Histopathology of liver cirrhosis included classic nodular pattern showing nodules of liver tissue surrounded by fibrous septae. Chronic hepatitis cases were microscopically characterised by classic hepatocyte changes and severe portal inflammation with lymphoid aggregates in some cases. Association of hepatic steatosis were observed in 4 patients with cirrhosis. Most of the

cases showed macrovesicular steatosis either for chronic hepatitis and cirrhosis.

The Histologic Activity Index (HAI) was evaluated for all cases with chronic hepatitis and liver cirrhosis. Nine from ten cases of liver cirrhosis had a HAI value over 14 and only one case was scored as 18.

Immunohistochemistry for VEGF revealed differences of expression between chronic hepatitis and cirrhosis. In chronic hepatitis we found a weak to moderate expression for VEGF with homogeneous distribution of positive reaction in the entire hepatic parenchyma. A particular aspect was found in hepatocytes disposed close to inflammatory infiltrate. Few cells in these area were intensely stained for VEGF with cytoplasmic granular pattern (Fig. 1). We noticed that VEGF expression correlated with VEGFR 2 expression without significant differences between chronic hepatitis and liver cirrhosis. No correlation was found between VEGF and VEGFR2 expression and HAI in chronic hepatitis. The lack of such correlation was also observed in liver cirrhosis ($p=0,134$).

Figure 1. VEGF expression in chronic hepatitis. Note the intense reaction for VEGF of hepatocytes close to the inflammatory zone. IHC, VEGF, x400.



Liver cirrhosis showed particular aspects of VEGF expression. The intensity of reaction increased with HAI. All hepatocytes from the nodular structures expressed VEGF but with heterogeneous pattern linked

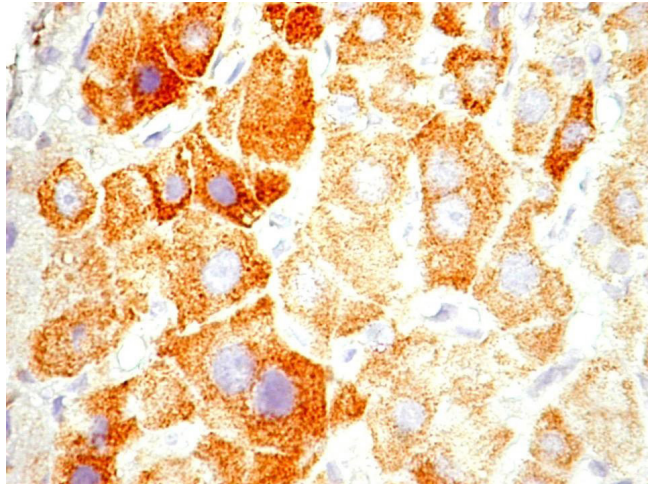
Table 1. Technical details of antibodies and working systems used for immunohistochemistry

Primary antibody	Source	Clone	Dilution	Incubation time	Antigen retrieval	Working system	Chromogen & counterstain
VEGF	Dako Glostrup Denmark	VG1	1:25	30 minutes, RT	MW,15' High pH	LSAB+, HRP	3,3' diaminobenzidine Modified Lille haematoxylin
F VIII related antigen	Dako Glostrup Denmark	Polyclonal	1:400	30 minutes, RT	Proteinase K, 20', RT	EnVision, HRP	3,3' diaminobenzidine Modified Lille haematoxylin
Flk1, VEGFR2	Neomarkers	Rabbit polyclonal	1: 2	30 minute, RT	MW, 15', High pH	Ultravision, HRP	3,3' diaminobenzidine Modified Lille haematoxylin

RT-room temperature, MW-microwave, HRP-horseradish peroxidase

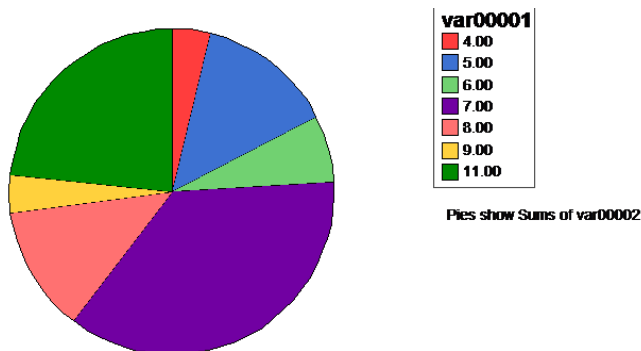
to the intensity and distribution of positive reaction. The strongest expression was noticed in at the periphery of liver nodules. In the nodules the reaction for VEGF was moderate in intensity with homogeneous distribution (Fig. 2).

Figure 2. Detailed view of intense cytoplasmic granular reaction for VEGF of the peripheral hepatocytes from cirrhotic nodules. IHC, VEGF, x400.



FVIII related antigen expression was restricted to the endothelium of blood vessels. We found a double FVIII-MVD value in the central area compared with periphery in 8 cases of chronic hepatitis (80 % of total cases). Cases with HAI value ranged between 6 and 8 had the highest microvessel density in central area (Fig. 3) which was correlated with FVII-MVD values (Fig. 4). We found a significant correlation between HAI and FVIII-MVD values in central area of chronic hepatitis lesions ($p=0.031$). In liver cirrhosis we report a higher MVD for FVIII in peripheric areas of the nodules. Globally, no significant correlation was found between FVIII-MVD and HAI values in liver cirrhosis ($p= 0,787$). VEGF expression in entire cirrhotic nodule found in only one case with highest HAI (18) was correlated with high FVIII positive microvessel density.

Figure 3. Distribution of cases according with HAI value.



Receptor 2 for VEGF (Flk 1) was also found in chronic hepatitis and cirrhotic nodules. Both lesions shared positive reaction for VEGFR 2 in most of the

cases. Some particular aspects were found concerning the distribution of positive reaction. In chronic hepatitis VEGFR 2 was present as weak to moderate staining with diffuse pattern and increased peripheral intensity in isolated cases. A particular feature of the positive reaction for VEGFR 2 in chronic hepatitis was represented by the presence of intense staining in the hepatocytes located around portal spaces. This aspect was found predominantly in chronic hepatitis with low HAI value. Isolated hepatocytes with intense staining was observed scattered in hepatic parenchyma. VEGFR 2 expression had moderate intensity in cirrhotic lesions with differences of reaction intensity in hepatocyte of periphery and central areas from cirrhotic nodules. In the case with highest value of HAI (18) all hepatocytes from nodular lesions intensely expressed VEGFR2 (Fig. 5).

Figure 4. Correlation between HAI and FVIII-MVD in center of nodular areas of chronic hepatitis.

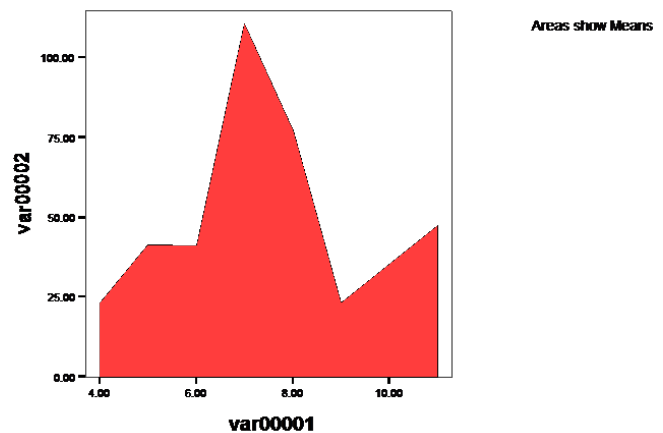
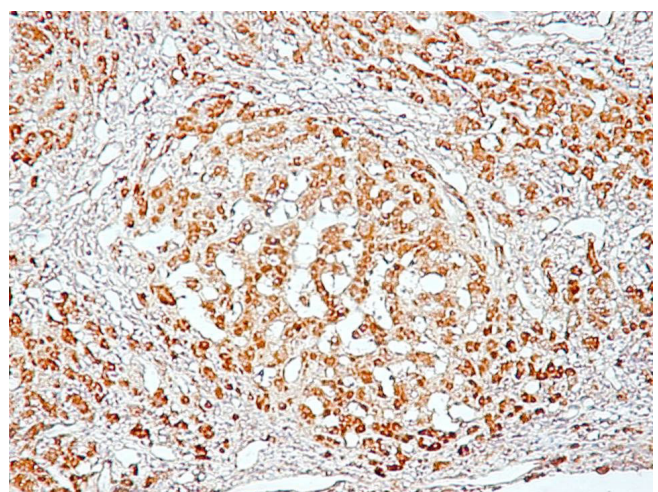


Figure 5. Particular aspect of VEGFR2 positive staining in cirrhotic nodules from high value HAI case. Note the lack of differential expression between periphery and center of the nodule. IHC, VEGFR2, x200.



DISCUSSION

Angiogenic factors represent important players involved in normal [5] and pathologic angiogenesis [6-8]. Vascular endothelial growth factor (VEGF) is a potent factor involved in vascular permeability and has

strong mitogenic properties for endothelial cells [9]. It acts through its specific receptors Flt1, Flk1, and Flt 4. Also, it was demonstrated that the complex VEGF-VEGFR2 is involved in tumorigenesis by the expression of ligand [10] and receptors [11-13] in many tumor types. VEGF and its receptors are intensely studied in hepatocellular carcinoma [14,15]. Few data are available concerning the expression of VEGF and VEGFR2 in hepatic lesions with premalignant potential, especially on animal models induced cirrhosis [16]. Most of these studies reflected the involvement of VEGF-VEGFR2 complex in vascular changes and angiogenesis process from cirrhosis and hepatocellular carcinoma [17] and did not explain the potential role of such complex in malignant transformation. Our data support the angiogenic effect of VEGF in liver lesions by correlation between the strong expression of VEGF and high micro vessel density for F VIII in cirrhosis. It was reported that serum level of VEGF tend to increases in acute and chronic hepatitis and decreases in liver cirrhosis [18] and that VEGF serum levels was not correlated with the progression of the disease. These divergent results could be explained in part by our observation concerning the distribution of VEGF and VEGFR2 in chronic hepatitis and liver cirrhosis. The disagreements concerning the positive rates of VEGF protein and mRNA level could be explained by microscopic distribution of VEGF positive hepatocytes in chronic hepatitis (close to inflammatory zones) and liver cirrhosis (limited to the peripheral hepatic cells of the nodules). Controversies could derive from the use of aleatory hepatic tissues specimens. The weak to moderate expression of VEGFR2 in chronic hepatitis with particular strong expression around portal spaces correlated with VEGF expression predominantly in hepatocytes close to inflammatory infiltrate suggested an autocrine and paracrine mechanism of VEGF action in this type of liver disease. In chronic hepatitis we can hypothesize that fibrosis could be generated by a crosstalk between VEGF secreting hepatocytes and stromal cells of the portal space connective tissue. This is in accord with many data which demonstrated the VEGF involvement in the progression of liver fibrosis and regeneration [19,20]. This autocrine and paracrine mechanisms seems to be kept in liver cirrhosis but with different patterns. The intense expression of VEGF in the periphery of the cirrhotic nodules suggested the existence of a particular subpopulation of VEGF secreting hepatocytes which are also positive for VEGFR2. These hepatocytes might have a neoplastic transformation potential. These findings are supported by a recent study of Coradinni et al [15] which compared the VEGF-A protein level in hepatocellular carcinoma (HCC), surrounding cirrhotic liver and cirrhosis at distance from the HCC. They reported a higher expression of VEGF-A protein in HCC and surrounding cirrhotic liver compared with cirrhosis lesions distant from carcinoma. In a murine model of hepatocellular carcinoma, Kornek et al [21] demonstrated

that the elevated status of VEGF-A and VEGFR2 accelerated tumor growth probably by a pro-angiogenic mechanism. Bockhorn et al [22] demonstrated that anti-VEGF almost completely suppressed and VEGF markedly enhanced hepatic proliferation in the first 24 h after partial liver resection. We hypothesize that in liver cirrhosis, overexpression of VEGF and VEGFR2 could induce a similar liver regeneration but possibly associated with a neoplastic transformation of hepatocytes. This hypothesis could be supported by the overexpression of VEGF and VEGFR 2 in other types of cancer cells.

Correlation between FVIII and HAI ($p=0,031$) suggests that angiogenic process is more active in chronic hepatitis compared with liver cirrhosis.

CONCLUSIONS

VEGF and VEGFR2 overexpression represent an early event in the development of the liver lesions with inflammatory or premalignant potential. Both markers are expressed in hepatocytes from chronic hepatitis and liver cirrhosis but the pattern and distribution of positive cells are different between these two lesions. We conclude that VEGF-VEGFR2 complex play an important role not only as angiogenic factors and promoters of liver fibrosis but also as a keyplayer of neoplastic transformation. These findings could be a evidences for the use of anti VEGF therapies as an adjuvant treatment for chronic hepatitis and cirrhosis in order to diminish the risk for malignant transformation.

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