Vascular Endothelial Growth Factor and Its Type 2 Receptor in Hepatocellular Carcinoma

E. S. Gershtein, E. A. Dubova*, A. I. Shchegolev*, and N. E. Kushkinskii

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Histological analysis demonstrated an increase in the number of blood vessels and expression of CD34 and vascular endothelial growth factor in hepatocellular carcinoma tissue compared to adjacent liver tissue. Increased immunohistochemical expression of vascular endothelial growth factor and the content of this factor and its type 2 receptor correlated with the degree of histological differentiation and stage of the tumor.

Key Words: *angiogenesis; hepatocellular carcinoma; vascular endothelial growth factor; type 2 vascular endothelial growth factor receptor*

Hepatocellular carcinoma (HCC) is the most prevalent malignant tumor of the liver; it constitutes about 80% of all malignant neoplasms of the liver. About 500,000 new cases of this disease are recorded annually [2].

HCC is a highly vascularized tumor. Angiogenesis in HCC tissue promotes rapid tumor growth, invasion, and metastasizing [14]. Vascular endothelial growth factor (VEGF) stimulating proliferation of endothelial cells and increasing vascular permeability is the leading and directly acting angiogenic factor [6]. Biological effects of VEGF are mediated by specific membrane receptors, representatives of the receptor tyrosine kinase family. Type 2 VEGF receptor (VEGFR2) is essential for differentiation, proliferation, and migration of endothelial cells.

VEGF is secreted by many tumor cells, including HCC [15]. Published data suggest [5] that the expression of this factor correlated with tumor progression and prognosis of the disease. Moreover, serum level of VEGF above 500 pg/ml is an indicator of vascular invasion of HCC and early relapse of the disease after liver resection [9]. In light of this, neoadjuvant therapy in the pre-operation period was proposed for patients with HCC for suppressing tumor growth and prevention of metastasizing [4]. In experimental studies, antiangiogenic preparations also suppressed the development of metastases [13]. Therefore, measurement of serum VEGF level can be used for evaluation of treatment efficiency and as a marker of tumor relapse.

Here we compared the levels of VEGF in tumors of different differentiation degree by immunohistochemical method and by enzyme immunoassay.

MATERIALS AND METHODS

We analyzed operation material from 28 patients treated at A. V. Vishnevskii Institute of Surgery in 2007-2009. Complex morphological examination revealed HCC in 22 patients (15 men and 7 women aging 17-72 years) and focal nodular hyperplasia (FNH) in 6 patients (women aging 24-45 years).

According to recommendations of International Histogenetic Classification of Tumors [8], highly differentiated (5 patients), moderately differentiated (11 patients), low differentiated (4 patients), and non-differentiated (2 patients) HCC were identified. According to clinical and morphological classification of tumors (TNM), 4 patients had stage II, 16 patients had stage III, and 2 patients had stage IV HCC.

N. N. Blokhin Cancer Research Center, Russian Academy of Medical Sciences; *V. I. Kulakov Research Center of Obstetrics, Gynecology, and Perinatology, Federal Agency of Medical Technologies, Moscow, Russia. ***Address for correspondence:*** esgershtein@gmail.com. E. S. Gershtein

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Tissue samples were fixed in 10% neutral formalin. Histological study was performed on paraffin sections (5 μ) stained with hematoxylin and eosin. Immunohistochemical analysis of VEGF (Novocastra) and CD34 (BioGenix) was performed routinely on deparaffinized sections. The samples were boiled in citrate buffer (pH 6.0) for primary demasking of the antigen and incubated for 15 min with 0.3% hydrogen peroxide for blockade of endogenous peroxidase. Hematoxylin was used for background staining. Immunohistochemical reaction to VEGF was evaluated visually and expressed as the sum of scores by the intensity of staining (0: absence of the reaction; 1: weak reaction; 2: moderate reaction; and 3: pronounced reaction) and the percent of stained cells (1: <10% stained cells; 2: 10-25% stained cells; 3: 25-50% stained cells; and 4: >50% stained cells). On CD34-positive preparations, the number of blood vessels per area unit was determined.

For enzyme immunoassay, fragments of the tumor and peritumoral tissue (without signs of tumor growth) were stored at -70°C until the assay. Tissue samples were lysed in a buffer containing 20 mM tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 1 μg/ml leupeptin (1:3 tissue-buffer ratio). The lysates were centrifuged for 30 min at 20,000 rpm and 4°C (OptimaTM TLX centrifuge, Beckman) for obtaining low-speed cytosol. The content of VEGF and VEGFR2 in tissue cytosols was measured using Quantikine Human VEGF Immunoassay and Quantikine Human VEGFR2 Immunoassay kits (R&D systems) according to manufacturer's instructions. The concentration of these factors in tissues was expressed in pg per 1 mg protein; protein content was measured by the method of Lowry.

The data were processed using Statistica 6.0 software.

RESULTS

Histological examination of hematoxylin-eosin-stained preparations revealed thick-walled arteries and clear-cut sinusoids in FNH samples. In HCC tissue, the vessels and sinusoids were poorly visualized due to more compact arrangement of the tumor cells. They were better seen after immunohistological staining for CD34 allowing quantitative evaluation of the number of vessels (Table 1). In FNH samples, the number of vessels (sinusoids) per tissue unit area was 2-fold higher than in normal liver tissue ($p<0.05$). In HCC samples, the number of vessels was also higher then in the adjacent non-tumor tissue. Their content was maximum in moderately differentiated HCC and somewhat lower in low- and non-differentiated tumors ($p>0.05$).

Immunohistochemical analysis showed that non-tumor tissue was characterized by minimum level of VEGF (Table 1) and cytoplasmic staining pattern. In FNH, the expression was by 83.3% higher than in normal tissue ($p<0.05$); it was more intensive and was detected in greater number of cells. VEGF expression in HCC tissue was above the normal level, the elevation directly correlated with the degree of tumor differentiation. The maximum values (by 2.4 times higher than in the normal tissue, $p<0.05$) were noted in non-differentiated tumors. It should be noted that VEGF expression in highly and moderately differentiated HCC was lower than in FNH, hence, this parameter cannot be used as a diagnostic criterion.

Enzyme immunoassay revealed different concentrations of VEGF in cytosols of normal and peritumoral normal liver tissue (Table 1). The concentration of VEGF surpassed the normal values in the liver tissue surrounding FNH and low and non-differentiated HCC forms and surpassed the normal values in the tissue surrounding highly and moderately differentiated HCC. These differences indicate the necessity of comparative analysis of VEGF content in the tumor and surrounding unchanged liver tissue for correct conclusions.

In FNH tissue, the level of VEGF was below the corresponding values in the adjacent liver tissue ($p<0.05$), which also attests to slow and benign growth of FNH. In HCC tissue, VEGF content usually surpassed the corresponding parameter in the liver tissue. The maximum VEGF concentration was noted in low-differentiated HCC ($p<0.05$). However, the level of VEGF in the tissue of highly differentiated HCC practically did not differ from the corresponding parameter in the surrounding liver tissue and from normal values.

The concentrations of VEGFR2 in cytosols of normal and peritumoral unchanged liver tissue were also different. The maximum level of VEGF in peritumoral tissue surpassing the control values by 55% ($p<0.05$) was observed in patients with non-differentiated HCC. In FNH tissue, VEGFR2 concentration was lower than in normal liver tissue (by 24.3%) and peritumoral unchanged liver tissue (by 27.4-51.2%). The levels of this receptor in HCC tissue increased with reducing the level of histological differentiation of the tumor. The maximum values surpassing the control level by 43.9% ($p<0.05$) and the levels in surrounding liver tissue by 9.2% ($p>0.05$) were observed in low-differentiated HCC.

The life prognosis for patients with malignant neoplasms largely depends on the stage of the disease, which is primarily determined by the degree of tumor differentiation, size of primary tumor, and the

TABLE 1. Results of Immunohistochemical Analysis and Enzyme Immunoassay of HCC of Different Differentiation Degree (*M*±*m*)

Nosology	Immunohistochemistry		VEGF, pg/mg protein		VEGFR2, pg/mg protein	
	CD34	VEGF	liver	tumor	liver	tumor
Liver	22.0±1.5	3.0±0.2	191±9		469±26	
FNH	40.0±2.6	5.5±0.4	218±15	158±7	489±28	355±19
Highly differentiated HCC	27.0±1.8	4.0±0.3	172±12	178±13	522±29	543±32
Moderately differentiated HCC	31±2	5.0±0.4	177±14	740±38	507±29	591±33
Low differentiated HCC	30.0±2.1	6.3±0.5	225±12	856±46	618±32	675±37
Non-differentiated HCC	30.0±2.2	7.0±0.6	242±14	720±39	727±37	620±35

TABLE 2. Results of Immunohistochemical Analysis and Enzyme Immunoassay of HCC of Different Stages (*M*±*m*)

Nosology	Immunohistochemistry		VEGF, pg/mg protein		VEGFR2, pg/mg protein	
	CD34	VEGF	liver	tumor	liver	tumor
Liver	22.0±1.5	3.0±0.2	191±9		469±26	
Stage II HCC	27.0±1.7	5.0±0.4	176±14	320±21	541±29	553±32
Stage III HCC	26.0±2.2	4.5±0.3	238±14	660±35	582±31	684±37
Stage IV HCC	32.0±2.3	6.3±0.5	229±12	742±39	674±34	662±35

presence of regional and distant metastases. In turn, the mechanisms of invasive growth and metastasizing largely depend on the level of tumor neovascularization mediated by VEGF.

In HCC samples with positive staining for CD34, the number of vessels was higher then in the adjacent non-tumor tissue. Their content was maximum in stage IV HCC ($p<0.05$). Immunohistochemically detected expression of VEGF underwent similar changes; the maximum values were also noted in stage IV HCC. The reaction to CD34 and VEGF in stage III HCC was lower than in stage II HCC ($p>0.05$; Table 2).

The level of VEGF in HCC cytosol directly correlated with the stage of the disease. The maximum concentration (almost 4-fold surpassing the control values) was noted in stage IV HCC. At the same time, VEGFR2 levels were maximum (by 45.8% above the control level, $p>0.05$) in stage III HCC tissue.

Thus, we observed pronounced changes in the content of VEGF and VEGFR2 in HCC tissue. It can be hypothesized that VEGF not only exhibit proangiogenic activity, but also directly participates in the regulation of tumor cell proliferation.

According to some reports [12], processes of angiogenesis are an important factor of HCC progression. Immunohistochemical and FISH assays demonstrated enhanced expression of VEGF in HCC cells compared to that in normal hepatocytes and in cirrhosis of the liver [3]. Higher activity was observed in cells at the periphery of tumor necrosis foci, which drove the authors to a conclusion that hypoxia is the leading factor of angiogenesis stimulation [7]. Immunohistochemical analysis of HCC tissue revealed more pronounced VEGFR2 expression in low-differentiated tumor sites [1]. A strong correlation between serum VEGF level and the degree of tumor invasion and the presence of metastases in HCC patients was previously reported [10,11]. According to previous reports [9], serum level of VEGF in patients with stage I and stage II HCC little differed from normal, but it was higher in patients with aggressive course of the disease, which agrees with our findings on the level of VEGF expression and concentration in the HCC tissue.

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