NITRIC OXIDE SYNTHASE AND VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION IN HEPATOCELLULAR CARCINOMA AND THE CORRELATION WITH ANGIOGENESIS

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ABSTRACT

Objective: To analyze the expression of inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF) in hepatocellular carcinoma (HCC) and its relation to angiogenesis. Methods: Tissue sections from 71 HCC patients were examined immunohistochemically for protein expression of iNOS, eNOS, and VEGF. Microvessel density (MVD) was counted by endothelial cells immunostained by anti-CD34 antibody. Results: Positive immunostaining for iNOS, eNOS was detected in 83.1% and 85.9% of HCC respectively. INOS and eNOS were not detected in normal hepatic tissue. MVD was 34.3±1.5/HP and 38.6±1.6/HP in HCC with positive staining for iNOS and VEGF while it was 31.2±2.8/HP, and 22.4±2.0/HP in HCC with negative staining for iNOS and VEGF (P<0.01). A correlation between NOS expression and VEGF in HCC was not observed. Conclusion: iNOS and eNOS may play a role in malignant transformation f post-hepatic cirrhosis. The expression of iNOS and VEGF favors angiogenesis of HCC.

Key words: Liver carcinoma, Nitric oxide synthase, vascular endothelial growth factor, Angiogenesis

It is now well established that tumor growth and metastasis are angiogenesis dependent[1]. Since Weidner reported microvessel density (MVD) was useful in prognosis of patients with breast cancer[2], the prognostic value of MVD have been certified in several tumors including primary liver cancer[3]. The increasing activity of Nitric oxide synthase (NOS) correlated with tumor angiogenesis[4]. We examined HCC tissue sections immunohistochemically for expression of iNOS, eNOS, VEGF, and CD34 to investigate the correlation of their expression with HCC angiogenesis and the role of nitric oxide (NO) in the signal transduction pathway of VEGF induced HCC angiogenesis.

MATERIALS AND METHODS

Patients

Samples were collected from 71 HCC patients who received curative resection in the Liver Cancer Institute, Zhongshan Hospital, Shanghai Medical University, from January 1992 to May 1993. The median age was 49.6 years (29-78 years). In 65 of these patients (91.5%) disease was associated with liver cirrhosis, and 64 patients were positive for serum HbsAg. According to pathological classification criteria of WHO, all patients were hepatocellular carcinoma.

Immunohistochemical Study

Formalin-fixed, paraffin-embedded, pathological-
ly proven HCC tissue blocks were collected from the tissue bank of the Department of pathology in Zhongshan Hospital. Immunohistochemical studies were performed on consecutive sections (4 μm thick) by two-step immunohistochemical method. Briefly, these sections were dewaxed in xylene and dehydrated in ethanol. Tissue antigen was renovated by microwave. After washing with tris (hydroxymethyl) aminomethane-buffered saline (TBS, 0.01mol/L, pH 7.4), the sections were then incubated with primary antibody at 37°C for one hour. After three times rinses with TBS, the sections were incubated with EnVision monoclonal antibody at room temperature for 30 rain. Finally, Slides were stained with 3,3- diaminobenzidine for 10 min, the sections were counterstained with haematoxylin. Controls included sections stained with TBS substituted for the primary or the second antibody.

Result Determination

VEGF and NOS were expressed in the cytosol of tumor cells. Results of immunohistochemical staining were judged according to staining density and positive cells frequency of. Positive staining was defined as more than 10-30% of the cell with weak or middle staining density. MVD was determined as follows: The slides were examined under 100 × magnification to identify the highest vascular density area within the tumor, and five such areas were selected to count the number of stained vascular under 200 × magnification (0.708 mm²/field). The average of the five areas was recorded as the MVD level of this case. Every single brown-stained cell and cell cluster was calculated as a blood vessel, no matter with or without the vessel lumen structure[6].

Statistical Analysis

Data analysis was performed using Epinfo 6 program for Windows. A two-sample t-test was used to compare the mean MVD counts of groups of patients. The χ²-test was used to analyze the relationship between immunohistocemical results and HCC recurrence.

RESULTS

Immunohistochemistry

General characteristics of immunohistochemical staining: Both NOS and VEGF were expressed within the cell cytoplasm. 59 out of 71 HCC (83.1%) showed positive staining for iNOS and in 61 (85.9%) for eNOS. ENOS expression was also occasionally detectable in tumor blood vessel endothelial cells or small bile duct epithelial cells. 50 HCC (70.4%) were positive for VEGF staining VEGF, also faint staining in tumor blood vessel and sinusoid endothelial cells. In some cases iNOS and VEGF showed stronger staining in the surrounding of tumor than intra-tumor. Anti-CD34 monoclonal antibody specifically binds to an endothelial cell in tumor blood vessels but not hepatic sinusoid endothelium, so brown staining endothelial cells were only detectable in tumor tissues (Figure 1-4).

Fig 1. Positive staining for iNOS in HCC.

Fig 2. Positive staining for eNOS in HCC

iNOS, eNOS, and VEGF Expression in HCC and Their Relationship to MVD and Prognosis

In iNOS and VEGF positive HCC MVD was 34.3±1.5/HP and 38.6±1.6/HP while in HCC negative for iNOS and VEGF MVD was 31.2±2.8/HP, and 22.4±2.0/HP in HCC negative for iNOS and VEGF (P<0.01). A correlation between NOS expression and VEGF in HCC was not observed. Although the recurrent rate of HCC with positive expression of VEGF (46%) were higher than with negative expression of VEGF (23.8%), the difference did not show statistics significance (P=0.06) (Table 1).