Increased Expression of Vascular Endothelial Growth Factor and Angiogenesis in the Early Stage of Multistep Hepatocarcinogenesis

Young Nyun Park, MD; Young-Bae Kim, MD; Kyung Moo Yang, MD; Chanil Park, MD

● ***Background.*—Hepatocellular carcinoma (HCC) is known to receive its blood supply principally from the hepatic arteries. Recent studies have reported differences in the vascular supply, especially arterial supply among low- and high-grade dysplastic nodules (DNs) (also referred to as *adenomatous hyperplasia* and *macroregenerative nodules*) and HCCs. Increased expression of vascular endothelial growth factor (VEGF) has been reported in HCC. In addition, VEGF may play an important role in the early phases of hepatocarcinogenesis.**

***Methods.*—We immunohistochemically stained 7 low-grade DNs, 8 high-grade DNs, 11 early HCCs, 17 small HCCs, and 21 advanced HCCs with antibodies against VEGF, α-smooth muscle actin (to identify unpaired arteries, ie, arteries not accompanied by bile ducts, indicative of angiogenesis), CD34 (as a marker of sinusoidal capillarization), and proliferation cell nuclear antigen.**

***Results.*—Expression of VEGF was found in the hepatocytes and HCC cells. The degree of VEGF expression increased gradually according to the stepwise development of hepatocarcinogenesis. It was higher in high-grade DNs and early HCCs than in low-grade DNs. The hepatocytes and HCC cells adjacent to peliosis and fibrous septa showed stronger VEGF expression. Angiogenesis, unpaired arteries, and sinusoidal capillarization developed from low-grade DNs and gradually increased. It was highest in HCCs. The proliferation cell nuclear antigen labeling indexes of hepatocytes and HCC cells also increased gradually as hepatocarcinogenesis progressed. Small HCCs showed a higher status of neoangiogenesis and cell proliferation activity than advanced HCCs. The degree of VEGF expression was correlated with angiogenesis and cell proliferation activity.**

***Conclusion.*—We conclude that VEGF plays a significant role in angiogenesis, growth, and development of HCC.**

(*Arch Pathol Lab Med.* 2000;124:1061–1065)

It has been demonstrated that angiogenesis is critical for tumor growth and that, without the ability to recruit new blood vessels, it is unlikely that most tumors would ever grow beyond a limited size.[1,2] It is, therefore, suggested that tumors may continuously produce certain mediators that induce angiogenesis, permitting tumor cell proliferation and expansion.

Vascular endothelial growth factor (VEGF), identical to vascular permeability factor,[3–5] was originally discovered as a tumor-secreted protein, and its role in tumor development has been investigated.[6,7] It is considered to play an important role in tumor biology in at least 2 ways: as a vascular permeability factor and/or endothelial growth factor. As a potent permeability factor, VEGF promotes extravasation of plasma fibrinogen, leading to the formation of a fibrin network that serves as a substratum for cell migration during angiogenesis. In addition, as an endothelial growth factor, VEGF stimulates endothelial cell proliferation and is likely to induce the formation of new blood vessels.[8]

Recently, distinctly hepatocellular nodules have been found in chronic liver disease and cirrhosis, and they have been considered to be premalignant lesions. These nodules are referred to variously as *dysplastic nodules* (DNs), *adenomatous hyperplasia*, or *macroregenerative nodules*.[9–12] The DNs might be the first step of hepatocarcinogenesis, subsequently developing into a hepatocellular carcinoma (HCC) nodule through low-grade DN, high-grade DN, and early HCC.

Since HCC is a distinctively hypervascular tumor and angiogenesis is important in hepatocarcinogenesis,[13–15] and also since increased expression of VEGF has been reported in HCC,[16–20] we studied the degree of VEGF expression and its relationship to angiogenesis and the proliferation activity of hepatocytes and HCC cells in low-grade DNs, high-grade DNs, early HCCs, small HCCs, and advanced HCCs to understand the role of VEGF in the early stage of hepatocarcinogenesis. To evaluate angiogenesis, we examined the development of unpaired arteries unaccompanied by bile duct and sinusoidal capillarization.

MATERIALS AND METHODS

We studied 7 low-grade DNs, 8 high-grade DNs, 11 early HCCs, 17 small HCCs, and 21 advanced HCCs, which were cho-

Accepted for publication December 29, 1999.

From the Department of Pathology, Institute of Genetic Science and Brain Korea 21 project for Medical Sciences, Yonsei University College of Medicine, Seoul, Korea (Drs Y. N. Park, Yang, and C. Park), and the Department of Pathology, Ajou University School of Medicine, Suwon, Korea (Dr Kim).

This study was supported by a faculty research grant from Yonsei University College of Medicine for 1997.

Reprints: Chanil Park, MD, Department of Pathology, Yonsei University College of Medicine, CPO Box 8044, Seoul, Korea.

sen from the resected liver pathology files of Yonsei University Medical Center between 1991 and 1997. Low-grade DN showed mild atypia, and in high-grade DN atypia was moderate but insufficient for the diagnosis of malignant neoplasm.[9] Early HCC was defined as high-grade DN with microscopic foci of well-differentiated HCC.[21] The HCCs that were less than 3 cm in diameter were classified as small HCCs, and the HCCs that were more than 3 cm in diameter were classified as advanced HCCs. The differentiation of HCC was evaluated by the Edmondson-Steiner grade system. All the early HCCs were grade I. Small HCCs included 13 cases of grade II and 4 cases of grade III. In advanced HCCs, 10 cases were grade II and 11 cases were grade III.

All these lesions were obtained from 53 patients (6 men and 1 woman; mean age, 51.4 years), and the number of lesions per patient varied from 1 to 4. The causes of chronic liver disease were hepatitis B virus in 39 cases and hepatitis C virus in 7 cases, whereas 7 cases showed negative reaction to a serologic test for both hepatitis virus B and C. The nonneoplastic liver showed cirrhosis in 36 cases.

All tissue sections were fixed in 10% buffered formaldehyde solution, processed by routine methods, and stained with hematoxylin-eosin. Immunohistochemical staining was carried out as described previously.[13] The primary antibodies used were polyclonal antibody to VEGF (Santa Cruz Biotechnology, Santa Cruz, Calif) at a dilution of 1:200, monoclonal antibody to alpha-smooth muscle actin (Dako Corporation, Carpenteria, Calif) at a dilution of 1:50, monoclonal antibody to CD34 (Q BEND 10) (Biogenex, San Ramon, Calif) at a dilution of 1:10, and proliferation cell nuclear antigen (PCNA) (Dako) at a dilution of 1:50.

The expression of VEGF was semiquantitatively evaluated as follows: those having positive staining in less than 5% were regarded as 1+, between 5% and 15% as 2+, between 15% and 50% as 3+, and greater than 50% as 4+. Arteries of angiogenesis, so-called unpaired arteries, were defined as those not accompanied by bile duct. The number of unpaired arteries was counted in 10 random fields of ×100 magnification, and it was semiquantitatively evaluated as follows: 1+, 1 to 5 unpaired arteries; 2+, 6 to 15 unpaired arteries; 3+, 16 to 50 unpaired arteries; and 4+, more than 50 unpaired arteries. Sinusoidal capillarization of angiogenesis was evaluated by the degree of CD34 expression as follows: 1+, staining of some endothelial cells occupying approximately less than 10% of the sinusoidal liver cell surface; 2+, staining of endothelial cells occupying approximately 10% to 30% of the sinusoidal liver cell surface; 3+, staining of endothelial cells occupying approximately 30% to 50% of the sinusoidal liver cell surface; and 4+, staining of endothelial cells occupying more than 50% of the sinusoidal liver cell surface. Areas of the Glisson capsule, fibrous septa, collapsed parenchyma, and necrotic area were excluded from evaluation of angiogenesis. The proliferation activity was evaluated as PCNA labeling indexes (LIs). At least 500 nuclei were selected randomly using a monoclonal eye-piece grid and a mechanical stage to avoid recounting. The PCNA-LIs were expressed as a percentage of positive nuclei per all nuclei counted.

Statistical analysis was performed using χ^2 test, Fisher exact test, and linear regression test as deemed appropriate.

RESULTS

Expression of VEGF in Multistep Hepatocarcinogenesis

Expression of VEGF was found in hepatocytes and tumor cells. It was semiquantitatively evaluated and summarized in Table 1.

The degree of VEGF expression increased gradually according to the stepwise development of hepatocarcinogenesis (Figure 1). All the low-grade DNs showed very weak (1+) expression of VEGF. High-grade DNs and early HCCs showed relatively higher VEGF expression than low-grade DNs. Two of 8 high-grade DNs and 2 of 11 early HCCs showed 3+ VEGF expression. The degree of VEGF expression was more intense in the hepatocytes adjacent to peliosis. In small and advanced HCCs, the degree of VEGF expression was relatively higher than in DNs. Four of 17 small HCCs and 4 of 21 advanced HCCs showed strong (4+) expression of VEGF. The degree of VEGF expression was more intense in the tumor cells adjacent to necrosis and fibrous septa. There was no significant difference of VEGF expression according to the differentiation of HCCs.

Table 1. Semiquantitative Assessment of Vascular Endothelial Growth Factor (VEGF) Expression*

Degree of VEGF Expression	Low-Grade DNs	High-Grade DNs	Early HCC	Small HCC	Advanced HCC
1	7	5	6	5	6
2	0	1	3	4	10
3	0	2	2	4	4
4	0	0	0	4	1

* DN indicates dysplastic nodule; HCC, hepatocellular carcinoma.

Expression of VEGF in the nonneoplastic portion of the liver was very weak (1+ or not appreciable), whereas the compressed and inflamed liver tissue adjacent to the tumor capsule showed 1+ or 2+ VEGF expression.

Angiogenesis of Development of Unpaired Arteries and Sinusoidal Capillarization and Its Relationship to VEGF Expression in Multistep Hepatocarcinogenesis

The development of unpaired arteries was semiquantitatively evaluated and is summarized in Table 2. The development of unpaired arteries started in low-grade DNs, and it gradually increased according to the stepwise progression of hepatocarcinogenesis (Figure 2). The number of unpaired arteries of high-grade DNs and early HCCs was significantly higher than those of low-grade DNs ($P = .01$). In HCCs, the number of unpaired arteries markedly increased, and small HCCs showed the highest number of unpaired arteries. Twelve of 17 small HCCs showed more than 50 unpaired arteries per 10 × 100 fields, whereas 8 of 21 advanced HCCs showed less than 6 unpaired arteries per 10 × 100 fields, and all of them showed a thick trabecular pattern with patent sinusoid.

The degree of VEGF expression increased as the unpaired arteries developed. The degree of VEGF expression and the number of unpaired arteries showed a significant correlation ($P = .0103$, $r = 0.3209$).

To assess the sinusoidal capillarization, CD34 expression of sinusoidal endothelial cells was evaluated (Table 3). CD34 expression gradually increased as multistep hepatocarcinogenesis progressed (Figure 3). The degree of CD34 expression was significantly higher in high-grade DNs than in low-grade DNs ($P = .004$). In low-grade DNs, CD34 expression was confined mainly to the outer region with periportal and peripheral pattern. CD34 expression became more diffuse, and this pattern was not retained in high-grade DNs and more advanced lesions of multistep hepatocarcinogenesis. Both small and advanced HCCs showed a significantly higher degree of CD34 expression than in high-grade DNs and early HCCs ($P = .018$). In advanced HCCs, all 8 cases with thick trabecular and patent sinusoid pattern showed 4+ CD34 expression. Their VEGF expression was 1+ in 3 cases and 2+ in 5 cases. Sinusoidal capillarization did not show a significant difference according to the differentiation of HCC.

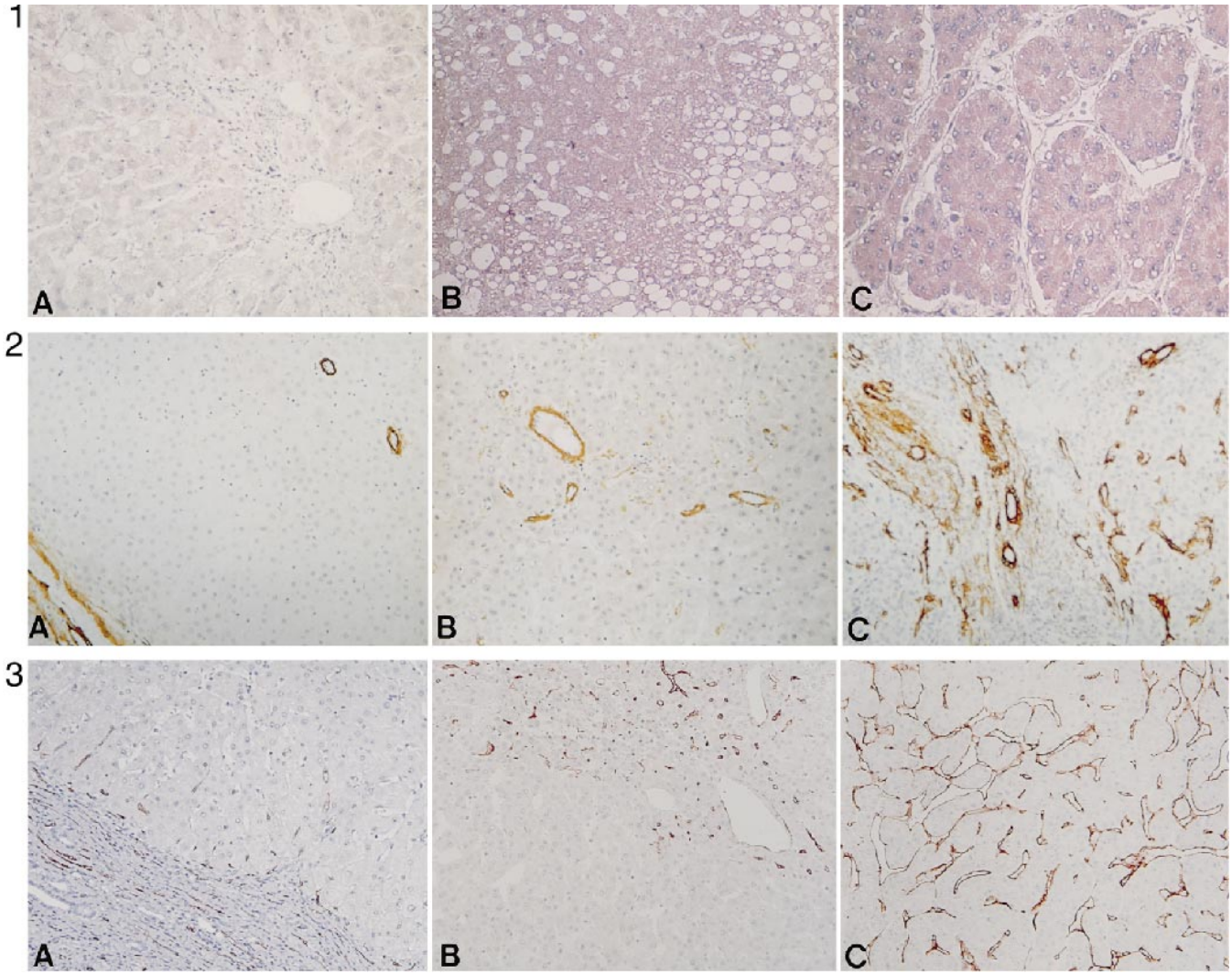

Figure 1. *Vascular endothelial growth factor (VEGF) expression in multistep hepatocarcinogenesis. Expression of VEGF is very weak in low-grade dysplastic nodules (A, ×200) and intermediate in high-grade dysplastic nodules (B, ×100) and diffuse and strong in hepatocellular carcinoma (C, ×200).*

Figure 2. *Development of unpaired arteries in multistep hepatocarcinogeneis. Immunohistochemical stain for α smooth muscle actin showing 2 unpaired arteries in low-grade dysplastic nodules (A), more developed status of unpaired arteries in high-grade dysplastic nodules (B), and numerous unpaired arteries in hepatocellular carcinoma (C) (×100).*

Figure 3. *Sinusoidal capillarization in multistep hepatocarcinogenesis. Immunohistochemical stain for CD34 showing peripheral and periportal sinusoidal capillarization in low-grade dysplastic nodules (A), more diffuse sinusoidal capillarization in high-grade dysplastic nodules (B), and widespread sinusoidal capillarization in hepatocellular carcinoma (C) (×100).*

The degree of VEGF expression increased as sinusoidal capillarization developed in multistep hepatocarcinogenesis, and there was a significant correlation between them ($P < .0001$, $r = 0.4932$).

Cell Proliferation Activity and Its Relationship to VEGF Expression in Multistep Hepatocarcinogenesis

The range of PCNA-LI was 1 to 9 with a mean of 4.2 in low-grade DNs, 4 to 12 with a mean of 7.9 in high-grade DNs, 3 to 26 with a mean of 9.3 in early HCCs, 18 to 80 with a mean of 39.9 in small HCCs, and 7 to 70 with a mean of 27.6 in advanced HCCs. The PCNA-LI increased gradually according to stepwise development of hepatocarcinogenesis. The PCNA-LIs of small HCCs were significantly higher than those of early HCCs ($P = .0001$) and advanced HCCs ($P = .015$). There was no significant difference of PCNA-LI according to the differentiation of HCCs.

Table 2. Number of Unpaired Arteries in Multistep Hepatocarcinogenesis*

No. of Unpaired Arteries	Low-Grade DNs	High-Grade DNs	Early HCC	Small HCC	Advanced HCC
0–5	2	1	0	1	8
6–15	5	2	2	0	3
16–50	0	3	3	4	6
>51	0	2	6	12	4

* DN indicates dysplastic nodule; HCC, hepatocellular carcinoma.

The PCNA-LI showed a significant correlation to the degree of VEGF expression ($P = .0054$, $r = 0.3671$). In ad-

Table 3. Semiquantitative Assessment of CD34+ Sinusoidal Endothelial Cells in Multistep Hepatocarcinogenesis*

Degree of Sinusoidal Capillarization	Low-Grade DNs	High-Grade DNs	Early HCC	Small HCC	Advanced HCC
1	7	2	2	1	1
2	0	2	2	1	1
3	0	2	4	4	5
4	0	2	3	11	14

* DN indicates dysplastic nodule; HCC, hepatocellular carcinoma.

dition, PCNA-LI showed a significant correlation to the degree of angiogenesis, the development of unpaired artery ($P = .0034$, $r = 0.3853$), and sinusoidal capillarization ($P < .0001$, $r = 0.5240$).

COMMENT

In recent years, many studies have been performed to find premalignant lesions in the human liver, and DNs are considered likely candidates.[9–12] It is suggested that DNs may be the first step of multistep hepatocarcinogenesis and that they develop into HCC nodules probably through high-grade DNs and subsequently into early HCCs, at least in HCC cases associated with chronic liver disease.[21]

In physiologically normal tissue, angiogenesis is unremarkable. Whereas neoplasms require the development of neovascularization,[12,22] angiogenesis is suggested to be induced by the local activation of genes encoding angiogenic factors such as VEGF.[3–6] It has been demonstrated that messenger RNA and the protein of VEGF are expressed and produced in a wide variety of human neoplasms and are strongly correlated with tumor-associated angiogenesis.[6,7] Also, VEGF is reported to be important in angiogenesis of HCCs, and the VEGF gene is reported to be transcribed, expressed, and secreted by HCC cells.[16–20] This study demonstrated that VEGF expression and angiogenesis had already occurred in the preneoplastic lesion of DNs. The degree of VEGF expression and angiogenesis gradually increased according to the progression of multistep hepatocarcinogenesis, and there was a significant correlation between them. This finding suggests that VEGF plays an important role in angiogenesis of multistep hepatocarcinogenesis starting from an early stage.

Immunohistochemical stain in this study showed that VEGF is mainly expressed by hepatocytes and HCC cells. It, therefore, seems reasonable to suggest that the excessive VEGF produced and secreted by hepatocytes and HCC cells may subsequently act on endothelial cells, resulting in the growth of new blood vessels and the capillarization of sinusoidal endothelial cells. Such paracrine communication between hepatocytes and endothelial cells in the liver via VEGF and its receptor has previously been reported.[23]

The tumor cells adjacent to peliosis or necrosis showed a stronger expression of VEGF in this study, and hypoxia is considered to enhance the expression of VEGF.[24] Expression of VEGF messenger RNA has been reported to be locally elevated in the area adjacent to tumor necrosis.[18,25,26] In this study, VEGF expression was also found in compressed and inflamed nonneoplastic liver tissue adjacent to the tumor capsule as previously reported.[16–18] This suggests that VEGF expression is regulated by inflammatory cytokines released from infiltrating inflammatory cells. Several cytokines, such as basic fibroblast growth factor, transforming growth factor α, epidermal growth factor, transforming growth factor β, and platelet-derived growth factor, have been reported to act cooperatively and could enhance VEGF expression.[27–29]

Cell proliferation activities in this study increased according to the progression of multistep hepatocarcinogenesis, as in previous reports.[30,31] They also showed significant correlation to the degree of VEGF expression and angiogenesis. Thus, VEGF expression is considered to contribute to the growth advantage in hepatocarcinogenesis through angiogenesis. The association of VEGF with tumor growth essentially supports the biological importance of angiogenic peptide in hepatocarcinogenesis as shown by in vitro culture studies.[20,22]

Small HCCs had the highest number of unpaired arteries and the highest PCNA-LI. Expression of VEGF was 4+ in 4 of 17 small HCCs in contrast to 1 of 21 advanced HCCs. Expression of VEGF has been reported to be an important angiogenic factor for the growth of small breast carcinoma in vivo; however, it is not essential after the tumor size becomes larger.[32] Usually, the turnover time of endothelial cells is 50 to 60 hours, whereas that of neoplastic cells is 22 hours. Therefore, the different rates of endothelial cell and neoplastic cell turnover seem to make the intercapillary distance increase as the tumor size increases.[33] Thus, small HCC is considered to be the highest developed stage of angiogenesis in multistep hepatocarcinogenesis.

Many consider VEGF one of the key factors promoting the production of tumor vessels in hepatocarcinogenesis. However, the degree of VEGF expression was variable in the cases of HCCs, and 30% of HCCs showed VEGF expression in less than 5% of the tumor cells. As a result, VEGF may not be the only factor involved in the angiogenesis of hepatocarcinogenesis in these cases, and other angiogenic factors may contribute to the development of tumor angiogenesis.

At present, our knowledge about the mechanism of VEGF overexpression and its participation in multistep hepatocarcinogenesis remains limited. However, the data presented herein suggest that VEGF is strongly involved in the development of angiogenesis of multistep hepatocarcinogenesis starting from the early stage and that it contributes to the growth advantage of hepatocytes via angiogenesis.

References

1. Folkman J, Watson K, Ingber D, Hanahan D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature.* 1989;339:58–61.
2. Folkman J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst.* 1990;82:4–6.
3. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science.* 1989;246:1306–1309.
4. Keck PJ, Hauser SD, Krivi G, et al. Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science.* 1989;246:1309–1312.
5. Gospodarowicz D, Abraham JA, Schilling J. Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. *Proc Natl Acad Sci U S A.* 1989;86:7311–7315.
6. Conn G, Soderman DD, Schaeffer MT, Wile M, Hatcher VB, Thomas KA. Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line. *Proc Natl Acad Sci U S A.* 1990;87:1323–1327.
7. Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumor angiogenesis factor in human gliomas in vivo. *Nature.* 1992;359:845–848.
8. Senger DR, Van De Water L, Brown LF, et al. Vascular permeability factor (VPF, VEGF) in tumor biology. *Cancer Metastasis Rev.* 1993;12:303–324.

9. International Working Party. Terminology of nodular hepatocellular lesion. *Hepatology.* 1995;22:983–993.
10. Nakanuma Y, Terada T, Ueda K, Terasaki S, Nonomura A, Matsui O. Adenomatous hyperplasia of the liver as a precancerous lesion. *Liver.* 1993;13:1–9.
11. Ferrell L, Wright T, Lake J, Roberts J, Ascher N. Incidence and diagnostic features of macroregenerative nodules vs. small hepatocellular carcinoma in cirrhotic livers. *Hepatology.* 1992;16:1372–1381.
12. Hytiroglou P, Theise ND, Schwartz M, Mor E, Miller C, Thung SN. Macroregenerative nodules in a series of adult cirrhotic liver explants: issues of classification and nomenclature. *Hepatology.* 1995;21:703–708.
13. Park YN, Yang CP, Fernandez GJ, Cubukcu O, Thung SN, Theise ND. Neoangiogenesis and sinusoidal "capillarization" in dysplastic nodules of the liver. *Am J Surg Pathol.* 1998;22:656–662.
14. Dhillon AP, Colombari R, Savage K, Scheuer PJ. An immunohistochemical study of the blood vessels within primary hepatocellular tumours. *Liver.* 1992;12:311–318.
15. Kimua H, Nakajima T, Kagawa K, et al. Angiogenesis in hepatocellular carcinoma as evaluated by CD34 immunohistochemistry. *Liver.* 1998;18:14–19.
16. Miura H, Miyazaki T, Kuroda M, et al. Increased expression of vascular endothelial growth factor in human hepatocellular carcinoma. *J Hepatol.* 1997;27:854–861.
17. Mise M, Arii S, Higashituji H, et al. Clinical significance of vascular endothelial growth factor and basic fibroblast growth factor gene expression in liver tumor. *Hepatology.* 1996;23:455–464.
18. Suzuki K, Hayashi N, Miyamoto Y, et al. Expression of vascular permeability factor/vascular endothelial growth factor in human hepatocellular carcinoma. *Cancer Res.* 1996;56:3004–3009.
19. Yamaguchi R, Yano H, Iemura A, Ogasaara S, Haramaki M, Kojiro M. Expression of vascular endothelial growth factor in human hepatocellular carcinoma. *Hepatology.* 1998;28:68–77.
20. Chow NH, Hsu PI, Lin XZ, et al. Expression of vascular endothelial growth factor in normal liver and hepatocellular carcinoma: an immunohistochemical study. *Hum Pathol.* 1997;28:698–703.
21. Sakamoto M, Hirohashi S, Shimosato Y. Early stages of multistep hepatocarcinogenesis: adenomatous hyperplasia and early hepatocellular carcinoma. *Hum Pathol.* 1991;22:172–178.
22. Ribatti D, Vacca A, Roncali L, Dammacco F. Angiogenesis under normal and pathological conditions. *Haematologica.* 1991;76:311–320.
23. Yamane A, Seetharam L, Yamaguchi S, et al. A new communication system between hepatocytes and sinusoidal endothelial cells in liver through vascular endothelial growth factor and Flt tyrosine kinase receptor family (Flt-1 and KDR/Flk-1). *Oncogene.* 1994;9:2683–2690.
24. Minchenko A, Bauer T, Salceda S, Caro J. Hypoxia stimulation of vascular endothelial growth factor expression in vitro and in vivo. *Lab Invest.* 1994;71:374–379.
25. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature.* 1992;359:843–845.
26. Vaupel P. Hypoxia in neoplastic tissue. *Microvasc Res.* 1977;13:399–408.
27. Pepper MS, Ferrara N, Orci L, Montesano R. Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. *Biochem Biophys Res Commun.* 1992;189:824–831.
28. Pertovaara L, Kaipainen A, Mustonen T, et al. Vascular endothelial growth factor is induced in response to transforming growth factor-β in fibroblastic and epithelial cells. *J Biol Chem.* 1994;269:6271–6274.
29. Finkenzeller G, Marme D, Weich HA, Hug H. Platelet-derived growth factor-induced transcription of the vascular endothelial growth factor gene is mediated by protein kinase C. *Cancer Res.* 1992;52:4821–4823.
30. Terada T, Nakanuma Y. Cell proliferative activity in adenomatous hyperplasia of the liver and small hepatocellular carcinoma: an immunohistochemical study demonstrating proliferating cell nuclear antigen. *Cancer.* 1992;70:591–598.
31. Tiniakos DG, Brunt EM. Proliferating cell nuclear antigen and Ki-67 labeling in hepatocellular nodules: a comparative study. *Liver.* 1999;19:58–68.
32. Yoshiji H, Harris SR, Thorgeirsson UP. Vascular endothelial growth factor is essential for initial but not continued in vivo growth of human breast carcinoma cells. *Cancer Res.* 1997;57:3924–3928.
33. Tannock IF, Hayashi S. The proliferation of capillary endothelial cells. *Cancer Res.* 1972;32:77–82.