Sinusoidal Capillarization and Arterial Blood Supply Continuously Proceed with the Advance of the Stages of Hepatocarcinogenesis in the Rat

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Hepatocellular carcinoma (HCC) is supplied only with arterial blood and has capillaries instead of sinusoids, unlike the normal hepatic tissue. In order to reveal the sequential changes of sinusoidal structure and blood supply during hepatocarcinogenesis, we examined hyperplastic nodules (HPN), atypical hyperplastic nodules (AHPN) and HCC in F344 rats fed with 2-acetylaminofluorene for 25-35 weeks. The extent of arterial blood supply and the degree of sinusoidal capillarization were assessed by the staining of hepatic nodules with arterially infused ink and by immunohistochemical and ultrastructural analyses of the sinusoids, respectively. The proportion of arterialized nodules was 47% in HPN, 57% in AHPN and 85% in HCC, which indicates that arterIALIZATION begins at the stage of HPN and a few HCC still receive portal venous blood. The proportion of Factor-VIII-related antigen (F-VIII)-positive nodules was 25% in HPN, 40% in AHPN and 100% in HCC. Non-arterialized nodules did not express F-VIII, while some F-VIII-negative nodules were already arterIALIZED. Electron microscopically, non-arterIALIZED HPN exhibited normal features of sinusoids, while arterIALIZED HPN showed disappearance of Kupffer cells and partial defenestration of sinusoidal endothelial cells. In non-arterIALIZED and arterIALIZED AHPN, basement membrane became continuous and lipid droplet-containing stellate cells were no longer seen. In HCC, endothelial fenestrae were diminished and basement membrane became thick. The present study has demonstrated that sinusoids are altered in the following order as hepatocarcinogenesis advances from HPN to HCC; onset of arterIALIZATION and decrease of endothelial fenestrae, expression of F-VIII by endothelial cells, disappearance of Kupffer cells, diminution of lipid droplets in stellate cells and thickening of basement membrane.

Key words: Hepatocarcinogenesis — Hyperplastic nodule — Hepatocellular carcinoma — Sinusoid — Blood supply

Human and murine hepatocellular carcinoma (HCC) has microvasculature that is structurally different from the sinusoids of normal hepatic tissue,¹⁻³ i.e., the former has few intraluminal Kupffer cells, non-fenestrated endothelial cells with underlying continuous basement membrane and pericytes with no vitamin A lipid droplets. These morphologic characteristics resemble those of capillaries. The blood supply of HCC is also different from that of the normal liver. HCC receives only arterial blood, as demonstrated by positive staining in trans-arterial angiography⁴ and the selective necrosis of tumors after arterial embolization. It has therefore been considered that rapid growth of HCC cells may induce formation of new capillaries from the hepatic artery. Another possibility is the transformation of hepatic sinusoids into capillaries during hepatocarcinogenesis. Such "capillarization of hepatic sinusoids"⁵ has been reported in several hepatic disorders, such as liver fibrosis^{6,7} and

nodular regenerative hyperplasia,⁸ but has not yet been demonstrated in hepatocarcinogenesis.

Recently, it has been shown that small-sized human HCC before encapsulation often exhibit negative staining in arteriography⁹ and show the normal features of sinusoidal structure under an electron microscope (our unpublished data). These facts suggest that the microvasculature of HCC, at least at its early stage, may derive directly from the sinusoids.

Most human HCC are considered to develop in a multi-step fashion.¹⁰ Chronic active hepatitis leads to liver cirrhosis, which produces adenomatous hyperplasia (AH), and finally HCC appears within the nodule of AH.¹¹ Human precancerous lesions such as AH are, however, rather difficult to obtain because of their small size and lack of appropriate clinical diagnostic procedures.

In this study, in order to reveal the sequential changes of the sinusoidal structure and blood supply from precancerous hyperplastic lesions to HCC, we analyzed a rat model of chemical hepatocarcinogenesis¹² which in-

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cludes three consecutive stages, i.e., hyperplastic nodules (HPN), atypical hyperplastic nodules (AHPN) and HCC. We examined the Factor VIII-related antigen (F-VIII) expression and ultrastructural changes in order to detect sinusoidal capillarization, and also the staining of nodules with arterially infused carbon particles as a marker of predominantly arterial blood supply to the tumor.

MATERIALS AND METHODS

Animals Twenty-one specific-pathogen-free F344 rats (male, 6 weeks old) were purchased from Charles River Japan Inc. (Kanagawa).

Hepatocarcinogenesis Experimental hepatocarcinogenesis was performed by a modification of the Solt and Farber procedure.¹²⁾ Briefly, a single dose of 200 mg/kg b.w. of diethylnitrosamine (Wako, Osaka) was intraperitoneally injected into animals at 6 weeks after birth (Fig. 1). Rats were then subjected to 2/3 partial hepatectomy at 9 weeks under ether anesthesia. They were fed basal diet (Oriental EA, Tokyo) containing 0.02% 2-acetylaminofluorene (Tokyo Kasei, Tokyo) during 8–14 weeks and killed between 31 and 41 weeks when HPN, AHPN and HCC occurred and co-existed in the liver.

Histology Under ether anesthesia, an 18G stainless steel needle was inserted into the portal vein and the liver was perfused with phosphate-buffered saline, pH 7.4, for 40 s at 10 ml/min with a pump to wash out the intrahepatic blood (Fig. 2). The chest was then opened and an 18G stainless steel needle that was connected to a plastic syringe was inserted into the thoracic aorta until proximal to the celiac artery. The abdominal portion of the aorta was ligated beyond the celiac artery to lead the perfusate efficiently into the hepatic artery. The liver was then perfused with 10% formalin or 1.5% glutaraldehyde in 0.062 M cacodylate buffer, pH 7.4, plus 1% sucrose via the portal vein for 2 min at 10 ml/min. At the same time, the same fixative containing India ink (Pelikan, Hannover, Germany) was gradually infused via the aorta by hand for 20 s.

After the perfusion-fixation, the liver was cut into slices 2–3 mm thick. We counted macroscopically the number of ink-stained and non-stained nodules. A part of the formalin- or glutaraldehyde-fixed nodules was dehydrated in an ethanol series and embedded in paraffin. Sections were stained with hematoxylin and eosin (H-E). These nodules were histologically classified into HPN, AHPN and HCC.

A part of the formalin-fixed nodules was used for immunohistochemistry. Paraffin sections were stained with a polyclonal antibody against human F-VIII (Dako, Tokyo) by means of the avidin-biotin-complex method. This antigen is reported to be negative in sinusoidal

Fig. 1. Protocol for chemical carcinogen-induced hepatocarcinogenesis in F344 rats.

Fig. 2. Procedure of perfusion-fixation. Cannulae were inserted into the portal vein (PV) and the thoracic aorta (Ao). The abdominal aorta was ligated beyond the hepatic artery (HA). A. India ink-containing fixative was perfused via the thoracic aorta and ink-free fixative via the portal vein. B. India ink-containing fixative was perfused via the portal vein and ink-free fixative via the thoracic aorta.

endothelial cells but to become positive after capillarization.¹³⁾

A part of the glutaraldehyde-fixed nodules was post-fixed in 1% OsO₄ in 0.1 M phosphate buffer, pH 7.4, dehydrated in ethanol and embedded in Epon 812 resin (TAAB, Reading, England). Thin sections were stained with saturated uranyl acetate and lead citrate and observed under a JEM-1200EXII electron microscope (JEOL, Tokyo) at 100 kV.

RESULTS

Histological classification of hepatic nodules We observed several different histopathologic types of hepatic nodules coexisting in the rat liver at 31–41 weeks (Fig. 3). Rat hepatic tumors induced by chemical carcinogens

Fig. 3. Light microscopy of three stages of hepatocarcinogenesis in F344 rats. a, hyperplastic nodule; b, atypical hyperplastic nodule; c, hepatocellular carcinoma. H-E staining. Bar: 0.25 mm.

Table I. Proportion of Nodules Positively Stained with India Ink or with F-VIII in HPN, AHPN and HCC (Number of Stained Nodules/Number of Total Nodules)

	India ink		F-III
	via the hepatic artery	via the portal vein	
HPN	21/45 (47%)	5/5 (100%)	1/4 (25%)
AHPN	13/23 (57%)	3/5 (60%)	2/5 (40%)
HCC	11/13 (85%)	1/4 (25%)	5/5 (100%)

* $P < 0.05$.

have been so far classified into HPN and HCC. In the present study, we further divided the original HPN into HPN and AHPN for detailed analyses. These groups of hepatic nodules had the following light microscopic characteristics.

HPN⁽⁴⁾: They were larger than a hepatic lobule but did not include Glisson's sheath within them. Hepatocytes were pleomorphic but showed neither hypercellularity nor structural atypism. The nucleus was usually round and the nucleolus was not prominent. Such histology resembles that of human adenomatous hyperplasia.

AHPN: They were similar in size to HPN and showed both hypercellularity and nuclear atypism. Structural atypism such as a pseudoglandular arrangement of the hepatocytes was often observed. AHPN was histologically compatible with human well differentiated HCC.

HCC: They showed a high degree of structural and nuclear atypism. Tumor cells were either basophilic or eosinophilic and invaded into the surrounding normal hepatic tissue. They were arranged in a pseudoglandular or a thick trabecular pattern. Histologically, HCC of the rat was compatible with moderately or poorly differentiated HCC of human beings.

Correlation of arterial blood supply with the stage of hepatocarcinogenesis By simultaneous perfusion with ink-containing fixative via the hepatic artery and ink-free fixative via the portal vein, hepatic nodules which mainly received arterial blood were stained black, whereas those supplied with both portal venous blood and arterial blood were not stained because ink particles that entered the sinusoid via the artery were flushed out by the ink-free perfusate coming from the portal vein (Figs. 2 and 4).

Fig. 4. A macroscopic view of hepatic nodules on a slice of the liver at 40 weeks (a) and its schema (b). India ink-containing fixative was perfused via the hepatic artery and ink-free fixative via the portal vein. Carbon-stained black nodules are supplied predominantly with arterial blood, while non-stained whitish nodules are supplied predominantly with portal venous blood. Histological types of the nodules (HPN, AHPN and HCC) shown in the schema were determined by the observation of H-E-stained sections.

Eighty-one nodules (45 HPN, 23 AHPN and 13 HCC) were macroscopically examined. The proportion of ink-stained nodules was 47% in HPN, 57% in AHPN and 85% in HCC (Table I). Most of the nodules were stained homogeneously with ink and sharply demarcated from the surrounding tissue. Ink-positive HCC, however, often included an ink-free portion which was identified as post-necrotic fibrosis by light microscopy (Fig. 4).

We also performed the perfusion of India ink in a reversed manner, i.e., ink-containing fixative via the portal vein and ink-free fixative via the hepatic artery (Fig. 2). The normal liver area was stained black because of the predominantly portal venous blood flow. Fourteen nodules (5 HPN, 5 AHPN and 4 HCC) were examined. The proportion of stained nodules was 100% in HPN, 60% in AHPN and 25% in HCC (Table I).

Correlation of capillarization with the stage of hepatocarcinogenesis Expression of F-VIII by endothelial cells: In the normal liver, F-VIII was expressed by the endothelial cells of interlobular blood vessels but not by those of the sinusoids. However, sinusoidal endothelial cells became positive for F-VIII in some neoplastic nodules (Fig. 5).

Table II. Relationship of India Ink-staining of Nodules with Their Expression of F-VIII (Number of Nodules)

F-VIII	India ink-staining via the artery	
	+	-
+	8 (HCC:5 AHPN:2 HPN:1)	0
-	4 (AHPN:3 HPN:1)	2 (AHPN:2)

Fig. 5. Immunohistochemistry for F-VIII. a. The normal rat liver shows negative staining of sinusoidal endothelial cells. b. India ink-stained HPN shows positive staining of sinusoidal endothelial cells. Avidin-biotin-complex method. Bar: 0.1 mm.

Fig. 6. Electron micrographs of the rat liver at 40 weeks. a. An India ink-negative HPN. The architecture of hepatocytic plates and the sinusoidal structure are almost normal. A Kupffer cell (K) exists in the sinusoidal lumen (SN) and a stellate cell (SC) contains lipid droplets. b. An India ink-positive HPN. Although the architecture of hepatocytic plates is almost normal, there are no Kupffer cells inside the sinusoid. c. An India ink-negative AHPN. Hepatocytes are arranged in a thick trabecular pattern. There are no Kupffer cells in the sinusoid. d. An India ink-positive AHPN. Hepatocytes (H) are arranged in a pseudo-glandular manner and enclose a wide lumen (asterisk). Sinusoidal lumen is often occluded (arrowhead) by the expanding hepatocytic plates. E, sinusoidal endothelial cells. Bar: 10 μ m.

Fig. 7. Ultrastructural changes of endothelial lining of the sinusoid in the course of hepatocarcinogenesis. a. An India ink-negative HPN. Endothelial fenestration (arrowheads) is frequent and basement membrane (arrows) is incomplete. Hepatocytes bear microvilli. b. An India ink-positive HPN. Endothelial fenestration is decreased in frequency and bridged by a thin membrane (arrowheads). The basement membrane (arrows) is continuous. c. An India ink-positive AHPN. The fenestration is closed by a thin diaphragm (arrowheads). Basement membrane (arrows) becomes thicker than that of an India ink-positive HPN. d. An India ink-positive HCC. Endothelial cells are devoid of sieve plates and basement membrane (arrows) is thick. Bar: 10 μ m.

Among 14 nodules (4 HPN, 5 AHPN and 5 HCC) examined, F-VIII was expressed in 25% of HPN, 40% of AHPN and 100% of HCC (Table I), indicating that the frequency of F-VIII expression increased as the stage of hepatocarcinogenesis advanced. Immunohistological analysis of arterially ink-infused livers demonstrated the correlation between F-VIII expression and predominantly arterial blood supply (Table II). F-VIII-positive nodules (1 HPN, 2 AHPN and 5 HCC) were all stained with ink, while F-VIII-negative ones consisted of both

ink-stained (3 AHPN and 1 HPN) and non-stained ones (2 AHPN). Conversely, non-ink-stained nodules were all F-VIII-negative. These data indicate that arterIALIZATION preceded the F-VIII expression by sinusoidal endothelial cells.

Ultrastructure of the microvasculature: The architecture of the hepatocytic plates was almost normal in HPN, while in AHPN and HCC hepatocytes were arranged in a thick trabecular or pseudoglandular manner (Fig. 6). Some hepatic sinusoids were occluded in ink-stained AHPN due to the expanded growth of the hepatocytes, while some were compensatorily dilated (Fig. 6d).

Morphological characteristics of the sinusoids were well preserved in non-ink-stained HPN (Fig. 6a); endothelial cells had normal fenestration and incomplete basement membrane (Fig. 7a), Kupffer cells existed inside the lumen (Fig. 6a), and stellate cells contained several lipid droplets. In ink-stained HPN, endothelial fenestration was reduced in frequency and usually closed by a thin single-layered membrane (Fig. 7b). Thick continuous basement membrane was present. Kupffer cells significantly decreased in number in the sinusoidal lumen. Stellate cells still contained several lipid droplets in the cytoplasm (Fig. 8a).

In AHPN, no significant difference was recognized between the sinusoidal structure of ink-stained nodules and that of non-stained nodules. Endothelial fenestration was further decreased and bridged by a thin membrane (Fig. 7c). Basement membrane was continuous, but there was no significant increase of collagen fibers in the space of Disse. Hepatocytes tended to lose microvilli (Fig. 7c). Kupffer cells were rare. Stellate cells had no lipid droplets (Fig. 8b).

Most HCC were stained with ink. In these nodules, endothelial cells did not show fenestration. Basement membrane became thick (Fig. 7d) and often multi-layered. There were no Kupffer cells in the lumen. Stellate cells did not contain lipid droplets.

Morphologic changes of the sinusoidal structure during hepatocarcinogenesis observed in this study are summarized in Fig. 9.

DISCUSSION

Normal hepatic tissue is supplied with portal venous blood plus arterial blood and possesses the sinusoidal structure, while HCC at an advanced stage is absolutely fed by the hepatic artery and has typical features of the capillaries.¹⁻³ On the other hand, precancerous lesions or hyperplastic nodules retain several ultrastructural characteristics of the sinusoids.³ The relationship of the sinusoids of hyperplastic nodules with the capillaries of HCC has, however, not yet been clarified. The present study was designed to demonstrate the sequential

Fig. 8. Ultrastructure of the hepatic stellate cells (SC). a. An India ink-positive HPN. Stellate cells contain several lipid droplets (asterisks). b. An India ink-positive AHPN. There are no lipid droplets in the stellate cells. E, sinusoidal endothelial cells; SN, sinusoidal lumen; L, lymphocytes; H, hepatocytes. Bar: 10 μ m.

changes of blood supply and sinusoidal structure during the development of hyperplasia into HCC. For this purpose, we histologically divided neoplastic nodules into three groups, i.e., HPN, AHPN and HCC, rather than two as has been done in the past, and we examined F-VIII expression by sinusoidal endothelial cells, ultrastructural changes of the sinusoid and the predominancy of arterial blood supply.

The present finding that about 50% of HPN were stained with arterially infused ink indicates that predominantly arterial blood supply starts as early as in HPN. These nodules were, however, considered not to be absolutely supplied by arterial blood, but still fed with some portal venous blood because 100% of HPN were stained with ink via the portal vein. Ultrastructurally, HPN with predominantly portal venous blood supply had almost normal architecture of the sinusoid, while those with predominantly arterial blood supply exhibited alterations

Fig. 9. Schema of the sequential changes of the sinusoidal structure of India non-ink-stained HPN (A), India ink-stained HPN (B), ink-stained and non-stained AHPN (C) and HCC (D). H, hepatocytes; E, endothelial cells; K, Kupffer cells; S, stellate cells; BM, basement membrane.

of the sinusoidal structure, i.e., lower frequency of endothelial fenestration and Kupffer cells. The degree of sinusoidal capillarization advanced as arterialization proceeded from partial in HPN to absolute in HCC (Fig. 9). This suggests that transition of the blood supply from the portal venous blood to the arterial blood which lacks intestine-derived foreign bodies and has relatively high blood pressure may be an important factor to induce sinusoidal capillarization. We previously observed that arterialization followed by portal branch ligation induced endothelial defenestration and decrease of Kupffer cells (K. Ikeda *et al.*, unpublished data). Tsuda *et al.*¹⁵⁾ considered that acquisition of arterial blood supply by hyperplastic nodules might relate to their development into HCC.¹⁵⁾ As for the mechanism of the shift of blood supply from portal venous to arterial, a scanning electron microscopic study using vascular casts has demonstrated that terminal branches of arterioles that are thicker than normal merge into sinusoids of arterialized hyperplastic nodules.¹⁵⁾

In the present study, 40% of AHPN were still fed by the portal vein and showed decreased endothelial fenestration and continuous basement membrane (Table I). This fact suggests that extrasinusoidal factors may also affect the sinusoidal structure, besides arterialization. Sinusoidal capillarization often occurs in various types of hepatic fibrosis.⁵⁻⁷⁾ In the present experiment, however, AHPN was accompanied with no significant perisinusoidal fibrosis. Recently, sinusoidal capillarization without fibrosis has been reported to occur in nodular regenerative hyperplasia,⁸⁾ where deposits of laminin and type IV collagen in the space of Disse play important roles both in capillarization and angiogenesis.⁸⁾ Since endothelial fenestrae facilitate the exchange of substances between sinusoidal blood and hepatocytes,¹³⁾ formation of thick basement membrane and defenestration of endothelial cells will tend to degrade the function of hepatocytes. We observed here that hepatocytes in AHPN and HCC

were usually devoid of microvilli. Such features suggest that hepatocytes in these nodules might undergo functional alterations and might not require such frequent exchange of substances. As for the loss of lipid droplets in the stellate cells in advanced stages of hepatocarcinogenesis, this should be related either to the functional alteration of the hyperplastic hepatocytes or the impaired uptake of retinoids from the sinusoidal blood flow due to the capillarization.

A small proportion of HCC did not show positive carbon staining via the hepatic artery. This fact suggests that the blood flow from the portal branch to the sinusoids (or capillarized sinusoids) remained to some extent in these HCC. This finding, which is consistent with previous data that human HCC at an early stage showed no staining by arteriography⁹⁾ and often possessed sinusoidal structure (our unpublished data), suggests that capillaries in HCC, at least at the early stage, are derived from sinusoids. Conway *et al.*¹⁶⁾ have shown that carcinogen-induced rat hepatic tumors less than 2 mm in diameter receive the portal flow, while those more than 2 mm in diameter do not. Comparison of the arterialization of HCC between human beings and rats is difficult, however, because the former is usually accompanied with fibrous encapsulation, while the latter is not.

In conclusion, the present study has demonstrated that arterial predominancy and sinusoidal capillarization continuously proceed as hepatocarcinogenesis advances toward HCC.

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REFERENCES

- 1) Haratake, J., Hisaoka, M., Yamamoto, O. and Horie, A. An ultrastructural comparison of sinusoids in hepatocellular carcinoma, adenomatous hyperplasia, and fetal liver. *Arch. Pathol. Lab. Med.*, **116**, 67-70 (1992).
- 2) Ichida, T., Hata, K., Yamada, S., Hatano, T., Miyagiwa, M., Miyabayashi, C., Matsui, S. and Wisse, E. Subcellular abnormalities of liver sinusoidal lesions in human hepatocellular carcinoma. *J. Submicrosc. Cytol. Pathol.*, **22**, 221-229 (1990).
- 3) Shoji, Y., Kaneda, K., Wake, K. and Mishima, Y. Light and electron microscopic analysis of liver sinusoids during hepatocarcinogenesis with 2-acetylaminofluorene in rats. *Jpn. J. Cancer Res.*, **85**, 491-498 (1994).
- 4) Breedis, C. and Young, G. The blood supply of neoplasm in the liver. *Am. J. Pathol.*, **30**, 969-985 (1954).
- 5) Schaffner, F. and Popper, H. Capillarization of hepatic sinusoids in man. *Gastroenterology*, **44**, 239-242 (1983).
- 6) Martinez-Hernandez, A. The hepatic extracellular matrix. II. Electron immunohistochemical studies in rats with CCl₄-induced cirrhosis. *Lab. Invest.*, **53**, 166-186 (1985).
- 7) Bhunchet, E. and Fujieda, K. Capillarization and venularization of hepatic sinusoids in porcine serum-induced rat liver fibrosis: a mechanism to maintain liver blood flow. *Hepatology*, **18**, 1450-1458 (1993).
- 8) Dubuisson, L., Boussarie, L., Bedin, C.-A., Balabaud, C. and Bioulac-Sage, P. Transformation of sinusoids into

- capillaries in a rat model of selenium-induced nodular regenerative hyperplasia. An immunolight and immunoelectron microscopic study. *Hepatology*, **21**, 805-814 (1995).
- 9) Sonoda, T., Shirabe, K., Takenaka, K., Kanematsu, T., Yasumori, K. and Sugimachi, K. Angiographically undetected small hepatocellular carcinoma: clinicopathological characteristics, follow-up and treatment. *Hepatology*, **10**, 1003-1007 (1989).
 - 10) Okita, K., Yamashita, A. and Kurokawa, F. Multistep hepatocarcinogenesis in man. *Gastroenterol. Surg.*, **16**, 23-31 (1993).
 - 11) Sakurai, M., Wakasa, K., Monden, M., Yamada, T., Kuroda, C. and Marukawa, T. Hepatocellular carcinoma in adenomatous hyperplasia of the liver. *Cancer Chemother. Pharmacol.*, **23**, 110-113 (1989).
 - 12) Solt, D. and Farber, E. New principle for the analysis of chemical carcinogenesis. *Nature*, **263**, 701-703 (1976).
 - 13) Fukuda, Y., Nagura, H., Imoto, M. and Kayama, Y. Immunohistochemical studies on structural changes of the hepatic lobules in chronic liver disease. *Am. J. Gastroenterol.*, **81**, 1149-1155 (1986).
 - 14) Reuber, M. D. Development of preneoplastic and neoplastic lesions of the liver in male rats given 0.025 percent N-2-fluorenyldiacetamide. *J. Natl. Cancer Inst.*, **34**, 697-723 (1965).
 - 15) Tsuda, H., Tamano, S., Imaida, K., Ohshima, M., Kitahori, Y. and Ito, N. Three-dimensional observations by scanning electron microscopy on the blood supply and organization of vasculature during hepatocarcinogenesis in rats. *Acta Pathol. Jpn.*, **34**, 957-970 (1984).
 - 16) Conway, J. G., Popp, J. A. and Thurman, R. G. Microcirculation of hepatic nodules from diethylnitrosamine-treated rats. *Cancer Res.*, **45**, 3620-3625 (1985).