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Role of Increased DNA Synthesis Activity of Hepatocytes in Multicentric Hepatocarcinogenesis in Residual Liver of Hepatectomized Cirrhotic Patients with Hepatocellular Carcinoma

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To examine whether the marked increase in DNA synthesis of hepatocytes in cirrhotic liver might elicit multicentric hepatocarcinogenesis, we examined the relationship between new development of hepatocellular carcinoma (HCC) and the bromodeoxyuridine (BrdU) labeling index (LI) of hepatocytes in the residual liver of hepatectomized patients with liver cirrhosis (LC) and HCC. Eighteen hepatectomized patients with LC and HCC, whose resected liver revealed neither portal nor hepatic vein invasion by histologic examination, were studied (to exclude cases with intrahepatic metastasis). DNA synthesis activity of hepatocytes from the residual cirrhotic liver was measured by a BrdU/anti-BrdU *in vitro* method. The incidence of HCC recurrence was studied during a 3-year follow-up period. Among 18 patients, 9 patients had recurrence and 9 did not. The average BrdU LI in the recurrent patients was 2.6±1.3% and was significantly higher than that in patients without recurrence (1.4±0.5%, $P<0.05$). All five patients who had a BrdU LI of 2.4% or above showed recurrence within 3 years, as compared with 4 of 13 (30.8%) patients with BrdU LI of less than 2.4% ($P<0.05$). Our data indicate that abnormally high DNA synthesis in hepatocytes in the background cirrhosis might lead to the development of multicentric carcinogenesis in human cirrhotic liver, and in the residual cirrhotic liver of hepatectomized patients with HCC and LC, it may be a predictor of new development of HCC.

Key words: Multicentric carcinogenesis — Residual liver — Recurrence of HCC — DNA synthesis activity — Hepatocellular carcinoma

It is generally accepted that multicentric carcinogenesis occurs very often in the development of hepatocellular carcinoma (HCC). We demonstrated previously by a bromodeoxyuridine (BrdU)/anti BrdU *in vitro* method[1] that HCC tended to develop or become detectable when DNA synthesis in the background cirrhosis was increasing.[2,3] Thus, increased DNA synthesis in hepatocytes may lead to multicentric carcinogenesis in the cirrhotic liver. To examine this possibility, we followed the new development of HCC in the residual liver after hepatectomy in patients with both liver cirrhosis (LC) and HCC, whose HCC showed neither portal nor hepatic vein invasion (no tendency to intrahepatic metastasis).

MATERIALS AND METHODS

Patients Forty-two hepatectomized patients with Child A[4] posthepatitic cirrhosis and HCC (34 men, 8 women) were studied initially. The reason for this selection was that HCC develops more frequently in such patients in the Far East, including Japan.[5–9] All patients had undergone hepatectomy at the Kanagawa Cancer Center Hospital between June 15, 1986, and August 13, 1990. No drugs that could influence the results had been administered before surgery. Transcatheter arterial embolization or arterial infusion of anticancer drugs was not performed. Those who died within 1 month of hepatectomy or whose resected liver showed portal or hepatic vein invasion by HCC cells on histopathological examination were excluded from the 3-year follow-up study of new development of HCC, to avoid cases of intrahepatic metastasis at the time of hepatectomy.

The patients were examined preoperatively by ultrasonography (US), computed tomography, (CT), dynamic CT and angiography, and during operation by US to detect remaining HCC lesions in the residual liver.

Biopsies of the residual liver were regularly performed from the lobe of the liver opposite to the resected lobe. If the resection was segmental, specimens were obtained from both right and left lobes. Liver biopsy specimens (1.0–1.5 cm in length) were obtained with a Tru-cut needle (Travenol Laboratories Inc., IL) during surgery.

The diagnosis of posthepatitic LC and HCC was made histologically.

After discharge from hospital, US was performed every 3 months, and serum α-fetoprotein level (radioimmunoassay, <15.0 ng/ml) was measured every month

on an outpatient basis. If recurrence of HCC was suspected, further imaging examinations including CT, dynamic CT, Lipiodol CT[10] (Ultra-Fluide, Laboratoire Guerbet, France) and angiography were performed on readmission. Patients were followed up for 3 years. Finally, this follow-up study of recurrence of HCC included 18 patients (12 men and 6 women). Their ages ranged from 42 to 66 years, with a mean age of 57.6±6.1 years. The clinicopathological features of these patients are given in Table I.

***In vitro* BrdU assay** BrdU was purchased from Takeda Chemical Industries Ltd. (Osaka), RPMI 1640 medium was a product of Nissui Pharmaceutical Co. (Tokyo), the monoclonal antibody against BrdU[1] was from Becton Dickinson Immunocytometry Systems (Mountain View, CA), and the antisera and other necessary agents for the avidin-biotin-peroxidase complex (ABC) method were obtained from Vector Laboratories (Burlingame, NJ).

The liver biopsy specimens were immediately incubated for 45 min in 0.1% BrdU solution in RPMI 1640 medium at 37°C in a shaking water bath under a pressure of 3 atmospheres (95% O_2/5% CO_2). The specimens were fixed in 10% phosphate-buffered formaldehyde solution for approximately 1 day, embedded in paraffin, and cut into 4-μm sections. After deparaffinization, they were treated with 4 *N* HCl for 30 min at 37°C to denature DNA. Immunohistochemical detection of BrdU was performed by the routine ABC method, using an anti-BrdU monoclonal antibody[1] as the primary antibody. BrdU-positive nuclei stained brown and could be clearly distinguished under the light microscope (Fig. 1). Adjacent sections were stained with hematoxylin and eosin and Azan-Mallory for histologic examination. The sections were examined under a light microscope. Histologic diagnosis was based mainly on the sections stained with hematoxylin and eosin. The degree of tumor cell differentiation was assessed according to Edmondson and Steiner[11]; in this classification, Grade 1 indicates extremely well-differentiated tumor and Grade 4 indicates poorly differentiated tumor. The histologic grade listed on Table I represents that observed in the predominant part of the liver biopsy specimen that was obtained from the center of the nodules seen by ultrasonography. To determine the BrdU LI, more than 1000 hepatocytes were counted in randomly selected high-power fields and the number of BrdU-positive nuclei were recorded in each field. The LI was defined as the ratio of BrdU-labeled hepatocytes to the total number of hepatocytes counted, expressed as a percentage.

Regarding the reliability of the BrdU LI in the biopsied specimens, we estimated the BrdU LI twice in the same six control subjects (liver biopsied specimens from operated early gastric cancer patients without liver involvement, etc.), and the result was 0.25±0.09% in one series, and 0.25±0.08% in the other series.[12] Furthermore, we examined the BrdU LIs of cirrhotic portions of both tumor-bearing lobes and non-tumor-bearing lobes, and the result was 2.2±0.8% in the cirrhotic portion of the tumor-bearing lobe, and 2.0±0.9% in the non-tumor-bearing cirrhotic lobe.[12] Thus, the reliability of the BrdU LI in the biopsied specimens seems to be sufficient.

For statistical analysis, Student's *t* test (Welch's method) and the chi-square test were used.

RESULTS

Of the 18 hepatectomized cirrhotic patients with HCC and without portal or hepatic vein invasion, 9 had recurrence within three years after hepatectomy, and 9 had no recurrence. The post-operative interval before recurrence was within one year in 4, within one to two years in 3, and within two to three years in 2. The mean postoperative recurrent interval was 16.4±9.7 months.

The mean BrdU LI in the recurrent group was 2.6±1.3, and was significantly higher than that in the non-recurrent group (1.4±0.5%, $P<0.05$, Table I).

In our recent investigation of HCC development from HCV-positive LC, the high DNA synthesis group whose BrdU LIs were 2.4% or above frequently developed HCC (7 out of 9, 78%) in the three year follow-up period.[13] In the present study, all five patients who had a BrdU LI of 2.4% or above showed recurrence within 3 years, as compared with 4 out of 13 (30.8%) patients who had a BrdU LI of less than 2.4% ($P<0.05$, Table II).

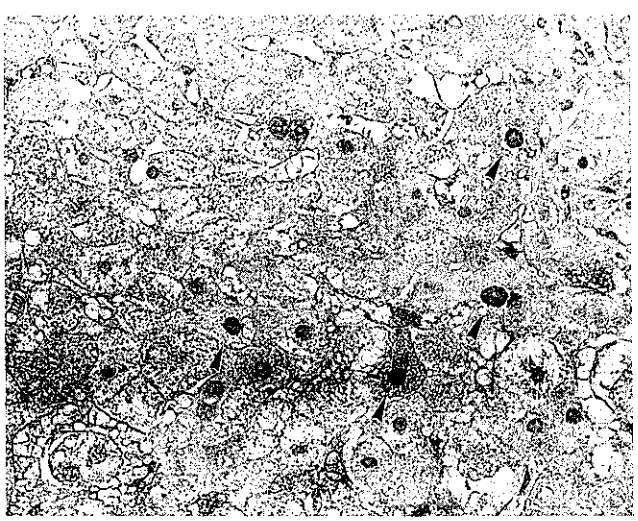

Fig. 1. Representative photomicrograph of BrdU stained hepatocytes. Many nuclei of hepatocytes are positively stained by BrdU (arrows). BrdU immunostain, ×320.

Table I. Clinicopathological Features and Bromodeoxyuridine Labeling Index (%) of Hepatocytes from Residual Cirrhotic Liver in 18 Hepatectomized Patients with Liver Cirrhosis[a] and Hepatocellular Carcinoma

Patient No.	Age/Sex	Recurrence (month)	HBsAg	HCV-Ab	Size of tumor (cm^3)	Capsule		Peri-nodular tumor	Portal vein invasion	Hepatic vein invasion	Edmondson and Steiner's classification	Labeling index (% labeled) of residual cirrhosis
						Formation	Invasion					
1	57/M	(−)	(−)	(−)	3.2×3.0×3.5	(+)	(+)	(−)	(−)	(−)	I-II	1.1
2	60/M	(−)	(−)	(+)	5.5×4.0×3.0	(+)	(−)	(−)	(−)	(−)	II	1.9
3	63/M	(−)	(−)	(−)	4.5×3.0×4.8	(+)	(−)	(−)	(−)	(−)	II>I	0.8
4	42/F	(−)	(−)	(+)	7.0×8.0×8.1	(−)	/	(−)	(−)	(−)	II	2.1
5	49/F	(−)	(+)	(−)	2.8×2.5×2.5	(+)	/	(−)	(−)	(−)	I-II	0.6
6	64/M	(−)	(−)	(+)	4.5×4.5×4.0	(+)	(+)	(−)	(−)	(−)	II	2.2
7	59/F	(−)	(+)	(+)	2.0×1.5×1.7	(+)	(−)	(−)	(−)	(−)	III	1.3
8	63/M	(−)	(−)	(+)	3.2×2.9×3.0	(+)	(−)	(−)	(−)	(−)	II	1.3
9	52/M	(−)	(+)	(−)	1.2×0.8×0.7	(+)	(−)	(−)	(−)	(−)	II<III	1.5
10	57/M	(+) (7)	(−)	(+)	3.5×3.5×3.0	(+)	(+)	(−)	(−)	(−)	I-II	2.4
11	56/M	(+) (7)	(+)	(−)	4.1×3.5×5.0	(−)	/	(−)	(−)	(−)	II	0.7
12	54/F	(+) (8)	(−)	/	6.2×4.5×4.5	(−)	/	(−)	(−)	(−)	II	4.0
13	62/F	(+) (11)	(−)	(+)	6.0×6.0×5.5	(+)	(+)	(−)	(−)	(−)	II	1.7
14	66/F	(+) (15)	(−)	(+)	3.5×3.5×3.5	(+)	(+)	(−)	(−)	(−)	II	1.4
15	62/M	(+) (17)	(−)	(+)	5.0×4.8×4.5	(+)	(−)	(−)	(−)	(−)	II	3.9
16	62/M	(+) (21)	(−)	(+)	7.2×6.0×6.2	(+)	(−)	(−)	(−)	(−)	III	4.5
17	52/M	(+) (27)	(−)	(+)	3.5×3.2×3.0	(−)	/	(−)	(−)	(−)	III	2.1
18	57/M	(+) (35)	(+)	(−)	2.8×2.2×2.0	(+)	(+)	(−)	(−)	(−)	II	2.4

a) Posthepatitic liver cirrhosis in all 18 patients.

Table II. Bromodeoxyuridine Labeling Index (BrdU LI) of Hepatocytes from Residual Cirrhotic Liver and Recurrence Rate of Hepatocellular Carcinoma in 3 Years

	Recurrence (+)	Recurrence (−)	
BrdU LI < 2.4%	4/13 (30.8%)	9/13 (69.2%)	$P<0.05$
BrdU LI ≧ 2.4%	5/5 (100.0%)	0/5 (0%)	

DISCUSSION

Among the many hypotheses proposed to explain the pathogenesis of carcinoma, one is that increased mitotic activity of cells in tissue correlates with the development of carcinoma, presumably owing to an increased rate of random mutation[14, 15] and promotion.[16, 17] The mitotic index (MI) has been used as a practical means for determining the mitotic activity of cells. It was recently shown that the MI correlates well with the tritiated thymidine LI[18] and that BrdU LI is also comparable with the tritiated thymidine LI.[19]

In accordance with the above mentioned hypothesis, we demonstrated previously by a BrdU/anti BrdU *in vitro* method[1] that HCC tended to develop or become detectable when DNA synthesis in the background cirrhosis was increasing[2, 3]; we prospectively followed the changes in DNA synthesis activity in hepatocytes from cirrhotic patients (Child A[4] stage) and examined its relation to the development of HCC. During the follow-up period of 2 years, HCC developed in 11 of 33 LC patients; 8 of 15 patients who showed high DNA synthesis activities initially and 3 of 18 patients who had low activities initially ($P<0.05$).[3] It is generally considered that histological changes might occur in the whole liver in cirrhotic patients, so it is likely that the abnormal increase in DNA synthetic activity of hepatocytes might occur throughout the cirrhotic liver. We demonstrated that the DNA synthetic activities (BrdU LIs) of hepatocytes in biopsied specimens from right and left hepatic lobes were nearly the same in hepatectomized cirrhotic liver of patients with LC and HCC.[12] We speculated that the abnormal increase in the DNA synthesis of hepatocytes in the whole cirrhotic liver might lead to or accelerate multicentric carcinogenesis in the cirrhotic liver.

To examine the validity of this idea clinically in cirrhotic patients, it seemed best to investigate whether new development of HCC might occur in residual cirrhotic liver showing an abnormal increase in DNA synthesis of hepatocytes. Therefore, in this study, the relationship between DNA synthesis activity of hepatocytes from the residual cirrhotic liver and the new development of HCC was studied in hepatectomized cirrhotic patients with HCC whose resected liver showed neither portal nor hepatic vein invasion by histological examination. We found that the DNA synthesis activity (BrdU LI) of hepatocytes from the cirrhotic liver was significantly

higher in the hepatectomized patients who had new development of HCC within three years after hepatectomy than in the patients who had no such development within this period. No inverse correlation was found between LI and period before new development, but we think that the number of cases examined here might be too small to detect such a correlation.

Two possibilities can be considered to explain the recurrence of HCC in hepatectomized patients; the development of intrahepatic metastasis after hepatectomy, and the growth of new HCC independent of the original one. Okuda *et al.*[20] studied histopathologically 31 cases of recurrent tumors with diameter of less than 20 mm, which developed after hepatectomy, and found 16 (51.6%) to be newly developed HCC, while in the other 14 cases (48.4%) the recurrence was thought to be metastatic. However, they did not exclude cases that showed portal vein invasion microscopically, and this would be the main route of intrahepatic metastasis.

As we strictly excluded hepatectomized cases with portal or hepatic vein invasion apparent on histopathological examination, the recurrent cases in our study should be predominantly newly developed HCC (non-synchronous multicentric carcinogenesis in the cirrhotic liver). Thus, our study indicates that multicentric carcinogenesis in human cirrhotic liver might require abnormally high DNA synthesis activity in the hepatocytes of the background cirrhosis.

So, HCC might develop in the cirrhotic liver with high DNA synthetic activity, while cirrhotic patients with low DNA synthetic activity might be at low risk of HCC development.

In conclusion, our present finding that new development of HCC is favored by a high DNA synthetic state in the residual cirrhotic liver suggests the importance of abnormally high DNA synthesis in the background cirrhosis for the development of multicentric carcinogenesis in human cirrhotic liver.

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