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Relationship of the Microvascular Type to the Tumor Size, Arterialization and Dedifferentiation of Human Hepatocellular Carcinoma

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Unlike normal liver with the sinusoids, hepatocellular carcinomas (HCCs) possess capillaries. Whether these capillaries derive from the sinusoids remains unclear in human HCCs. This study aimed to examine sinusoidal capillarization in human HCCs and its relationship to the tumor size, arterialization and dedifferentiation. Thirty-eight HCCs with a diameter of 10–140 mm were pathologically and angiographically examined. By electron microscopy, the microvasculature of tumors was classified into sinusoidal, intermediate and capillary types, which were all negative, partially positive and all positive, respectively, for four parameters, i.e., endothelial defenestration, continuous basement membrane, lack of Kupffer cells, and lack of lipid-containing hepatic stellate cells. Well-, moderately and poorly differentiated HCCs displayed sinusoidal/intermediate/capillary types, intermediate/capillary types and only capillary type, respectively, suggesting the transition from the sinusoids to capillaries in well-differentiated (and probably moderately differentiated) HCCs. Furthermore, well-differentiated HCCs with a diameter of less than 30 mm often received preferential portal venous blood, while moderately and poorly differentiated ones were all supplied with arterial blood, indicating a relationship between dedifferentiation and arterialization. In contrast, the microvascular type displayed no significant relationship with tumor size or arterialization in well-differentiated HCCs. The present study has demonstrated that sinusoidal capillarization occurs in human well-differentiated HCCs and seems to be related to dedifferentiation of parenchymal tumor cells, but not to tumor size or arterialization.

Key words: Hepatocellular carcinoma — Sinusoidal capillarization — Blood supply — Electron microscopy

The normal liver parenchyma has hepatic sinusoids which are ultrastructurally characterized by fenestrated endothelial cells with an incomplete basement membrane, intravascular macrophages (i.e., Kupffer cells), and hepatic stellate cells (HSCs) with vitamin A-associated lipid droplets,[1)] and is supplied with both portal venous and arterial blood in the proportion of 70% and 30%, respectively.[2)] In contrast, advanced hepatocellular carcinomas (HCCs) usually have capillaries[3, 4)] and are preferentially supplied with arterial blood.[5)] They are recognized as hypervascular tumors by arterial angiography and hypovascular ones by portal angiography.[6, 7)] Of clinical importance is that this single-source blood supply permits therapeutic efficacy in tumor embolization and direct infusion of antineoplastic agents into the tumor.

Different from advanced HCCs, early-stage, small HCCs are not always positively stained by arterial angiography, indicating preferential supply with portal venous blood.[8)] Pathological studies revealed that such unstained tumors were usually well-differentiated HCCs which involved the portal tracts within the tumor and lacked a fibrous capsule.[8, 9)] Furthermore, they had sinusoid-like microvessels which retained some morphological characteristics of the sinusoids,[10, 11)] raising the possibility that "sinusoidal capillarization"[12)] may occur in HCCs. We previously observed in rat hepatocarcinogenesis that arterialization and sinusoidal capillarization proceeded in parallel as the stage advanced from hyperplastic nodules to HCCs.[13)] In human subjects, early-stage HCCs are mostly well-differentiated ones and, after the initial growth, the tissues are gradually replaced by moderately to poorly differentiated HCC tissues, often displaying the feature of "nodule-in-nodule."[14)] It is, however, still unclear in human HCCs whether tumorous capillaries derive from the sinusoids through a process of capillarization.

In this study, in order to examine sinusoidal capillarization in human HCCs and its relationship to the tumor size, arterialization and dedifferentiation, we conducted morphological and statistical analyses on 38 HCCs.

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MATERIALS AND METHODS

Patients Thirty-eight patients with HCCs (35 men and 3 women; 42–76 years old), admitted to the Osaka City University Hospital between 1991 and 1999, were examined. None of them had been treated with transarterial embolization prior to operation or biopsy. Informed consent was obtained from each patient.

Transarterial angiography Transarterial angiography was performed prior to operation or biopsy by injecting 15–30 ml of iopamidol (Iopamiron, Nihon Schering, Osaka) at the rate of 4–8 ml/s through the hepatic artery. Images were obtained by either conventional angiography or computer-tomographic (CT) angiography.

Histology Liver specimens were obtained by surgical resection or needle biopsy. The tumor size was measured macroscopically or microscopically. Specimens were immediately fixed in 1.5% glutaraldehyde in 0.067 *M* cacodylate buffer, pH 7.4, plus 1% sucrose. A part of each specimen was further fixed in 10% formalin. Paraffin sections were stained with hematoxylin and eosin (H-E). The stage of dedifferentiation and the cell pattern were histologically diagnosed by two experienced pathologists. Another part of each specimen was post-fixed in 1% OsO_4 in 0.1 *M* phosphate buffer, pH 7.4, for 2 h, dehydrated in an ethanol series, and embedded in Epon 812 resin (TAAB, Reading, England). Semi-thin sections were stained with toluidine blue. Thin sections were stained with saturated uranyl acetate and lead citrate, and observed under a JEM-1200EXII electron microscope (JEOL, Tokyo) at 100 kV. One of the serial thin sections cut for electron microscopy was stained with toluidine blue and observed by light microscopy to confirm the histologic diagnosis.

Statistics A χ^2 test, a Mann-Whitney *U* test, a Fisher's exact test, ANOVA, and a Kruskal-Wallis test were used. A *P* value of less than 0.05 was considered significant.

RESULTS

In early-phase angiography, HCCs showed either negative (Fig. 1a) or positive staining (Fig. 1b), representing preferential supply with portal venous or arterial blood, respectively. Tumors were divided into 3 groups by size; small (≤2 cm in diameter), medium (>2 cm, ≤3 cm) and large (>3 cm). The reasons for setting the 2-cm diameter as the boundary between small and medium are that HCCs with a diameter of ≤2 cm without intrahepatic metastasis have been defined as "small liver cancer," [15] and the 2-cm diameter cut-off is an important yardstick for monitoring the capsule formation and prognosis in imaging techniques. The completeness of the fibrous capsule was determined macroscopically or microscopically. The histologic grades were diagnosed as follows in H-E- or toluidine blue-stained sections; well-differentiated HCCs had proliferative parenchymal cells of thin trabeculations with a low incidence of atypical cells and less peripheral invasion, while moderately and poorly differentiated ones had more pleomorphic and proliferative parenchymal cells of thick trabeculations or solid masses with a high incidence of atypical cells and more peripheral invasion. Tumor cell patterns were classified as follows[15]: i) thin-trabecular pat-

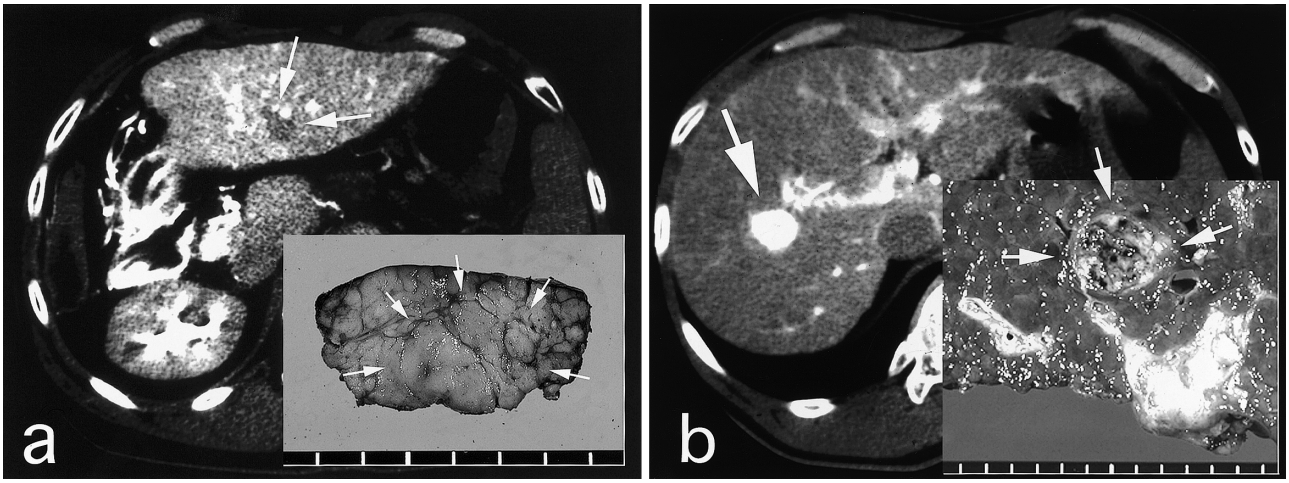

Fig. 1. CT-angiographic and macroscopic views of two cases of HCCs. a. A negatively stained HCC (large arrows) in CT-angiography. Macroscopically, the tumor (small arrows) was not encapsulated. Histology revealed that it was a well-differentiated HCC. One scale unit=5 mm. b. A positively stained HCC (large arrow) in CT-angiography. The tumor was encapsulated and included necrotic lesions within the tumor (small arrows). Histology revealed that it was a moderately differentiated HCC. One scale unit=5 mm.

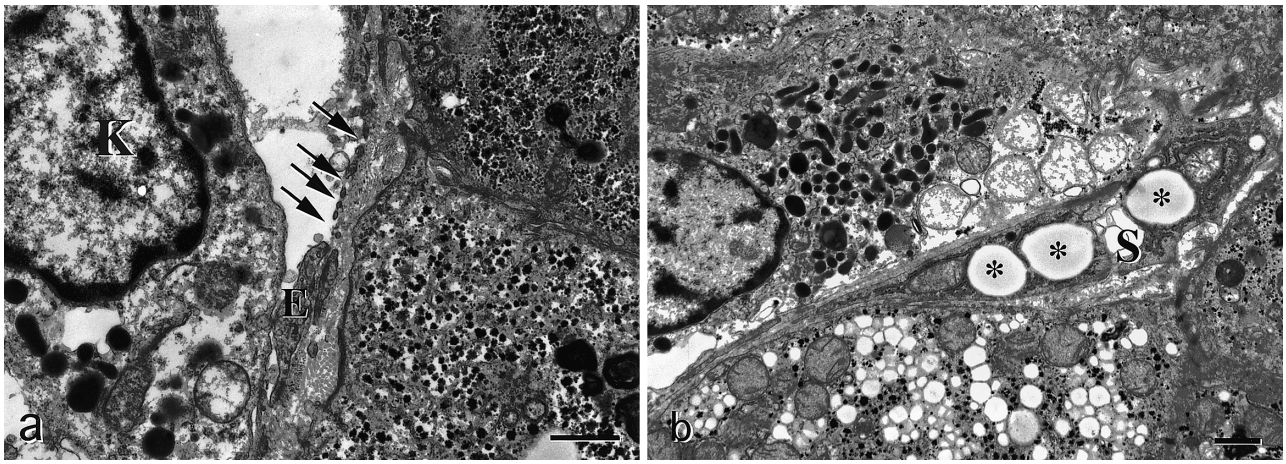

Fig. 2. Electron microscopy of the sinusoidal structure in well-differentiated HCCs. a. Endothelial cells (E) have fenestrae (arrows). Kupffer cells (K) are present in the lumen of sinusoids. Bar=1 μm. b. Hepatic stellate cells (S) in the space of Disse contain several lipid droplets (asterisks). Bar=1 μm.

tern; tumor cells were arranged in a single hepatic cell cord with one or two lines, surrounding the sinusoids; ii) thick-trabecular pattern; they were arranged in three or more lines, surrounding the sinusoids; iii) solid pattern; they were gathered like islets; iv) pseudoglandular pattern; they were arranged in a round or glandular manner.

By electron microscopy, the parenchymal cells of well-differentiated HCCs had microvilli and abundant glycogen particles in the cytoplasm, and enclosed the bile canaliculi (Fig. 2a and b), while those of moderately and poorly differentiated HCCs had few microvilli and a negligible amount of glycogen particles with rare formation of bile ductules (data not shown). The microvascular types were classified into three by four electron microscopic parameters for capillarization, i.e., endothelial defenestration, formation of complete basement membrane, lack of Kupffer cells and lack of lipid droplet-containing HSCs. Endothelial defenestration was defined as the situation when no fenestrations were found in 10 microscopic fields at $\times$5000, and the lack of Kupffer cells and HSCs was defined as the situation when these cells were not observed in 10 microscopic fields at $\times$2000. Sinusoidal type of microvessels was negative for all four parameters, namely, they had endothelial fenestration (Fig. 2a), incomplete basement membrane, lipid droplet-containing HSCs (Fig. 2b) and intravascular Kupffer cells (Fig. 2a). Capillary type was positive for all these parameters, and intermediate type was positive for one to three parameters.

Table I summarizes the data on the tumor size, arterialization, histology and microvascular type of HCCs examined here. Fig. 3 depicts the size distribution of sinusoidal, intermediate and capillary types of tumors in each well, moderately and poorly differentiated HCCs. It was noted that well-differentiated HCCs alone included all of the sinusoidal, intermediate and capillary types, while moderately differentiated ones included intermediate and capillary types, and poorly differentiated ones were composed of only the capillary type. We therefore focused our attention on well-differentiated HCCs for analysis of the correlation between the microvascular type and the arterialization and tumor size (Table II). The frequency of tumors with preferentially portal venous blood supply was similar among the sinusoidal, intermediate and capillary types, thus giving no significant correlation between the microvascular type and arterialization. There was also no correlation between the microvascular type and tumor size. On the other hand, a significant correlation was found between the tumor size and arterialization (Table III). This may be due to the fact that preferential portal venous blood supply was only seen in HCCs of less than 30 mm in diameter (Fig. 3). Different from well-differentiated HCCs, moderately and poorly differentiated ones were preferentially supplied with arterial blood (Fig. 3).

Statistical analysis was further conducted on the average size and number of endothelial fenestrae in the sinusoidal and intermediate types of HCCs (Table IV). No significant difference in average size was found between them. Average number was decreased in the intermediate type, but with no significant difference.

DISCUSSION

Previous studies reported that early-stage, well-differentiated human HCCs often displayed the sinusoidal structure,[11, 16] suggesting that the normal sinusoids might acquire the characteristics of capillaries in the development

Table I. Size, Arterialization, Histology and Microvascular Type of HCCs Examined Here

Size (mm in diameter)	Arterialization[a]	Histology			Microvascular type[d] (defenestration, complete basement membrane, lack of HSCs, lack of KCs)[e]				
		Grade[b]	Pattern[c]	Capsule					
Small (≤20 mm in diameter)									
15	–	W	thin	–	S	(–	–	–	–)
15	–	W	thin	–	S	(–	–	–	–)
20	–	W	thin	?	S	(–	–	–	–)
15	+	W	thin	–	S	(–	–	–	–)
12	–	W	thin	–	I	(–	+	–	–)
13	–	W	thin	–	I	(–	+	–	–)
15	+	M	thick	+	I	(–	+	–	+)
10	–	W	thin	?	C	(+	+	+	+)
20	+	W	thin	+	C	(+	+	+	+)
20	+	M	thin	+	C	(+	+	+	+)
Medium (>20 mm, ≤30 mm)									
25	+	W	thin	+	S	(–	–	–	–)
25	+	W	thin	+	I	(–	–	+	–)
30	–	W	thin	?	I	(–	–	+	+)
30	+	W	thin	–	I	(–	–	+	+)
25	+	M	thick	+	I	(–	+	–	–)
24	+	M	thick	?	I	(–	+	–	+)
26	–	W	thin	+	C	(+	+	+	+)
25	+	W	thin	+	C	(+	+	+	+)
25	+	M	thick	+	C	(+	+	+	+)
30	+	M	thick	+	C	(+	+	+	+)
25	+	M	solid	–	C	(+	+	+	+)
25	+	P	solid	+	C	(+	+	+	+)
Large (>30 mm)									
40	+	W	thin	–	S	(–	–	–	–)
35	+	W	thin	+	I	(–	–	–	+)
35	+	W	thin	–	I	(+	–	–	–)
110	+	W	thin	–	I	(+	+	+	–)
32	+	M	thick	+	I	(–	+	+	+)
35	+	M	solid	+	I	(+	–	–	–)
40	+	M	solid	+	I	(+	+	–	–)
40	+	M	solid	+	I	(+	–	+	+)
45	+	M	pseudo	+	C	(+	+	+	+)
34	+	M	solid	–	C	(+	+	+	+)
44	+	M	solid	–	C	(+	+	+	+)
40	+	M	solid	+	C	(+	+	+	+)
50	+	P	thick	+	C	(+	+	+	+)
100	+	P	thick	+	C	(+	+	+	+)
140	+	P	thick	+	C	(+	+	+	+)
40	+	P	solid	+	C	(+	+	+	+)

a) Arterialization of tumors was determined by early-phase arterial angiography.
b) W, well-differentiated HCCs; M, moderately differentiated HCCs; P, poorly differentiated HCCs.
c) thin, thin-trabecular pattern; thick, thick trabecular pattern; pseudo, pseudoglandular pattern; solid, solid pattern.
d) S, sinusoidal type; I, intermediate type; C, capillary type.
e) HSC, hepatic stellate cells; KC, Kupffer cells.

Table II. Correlation of the Microvascular Type to Arterialization and Tumor Size in Well-differentiated HCCs

	Microvascular type		
	S	I	C
Arterialization			
–	3	3	2
+	3	5	2
Size			
S	4	2	2
M	1	3	3
L	1	3	0

No significant correlation was found between the microvascular type and arterialization or between the microvascular type and size.

Table III. Correlation of the Tumor Size to Arterialization and Defenestration in Well-differentiated HCCs

	Size		
	S	M	L
Arterialization*			
–	6	2	0
+	2	4	4
Defenestration			
–	6	4	2
+	2	2	2

* $P<0.01$.

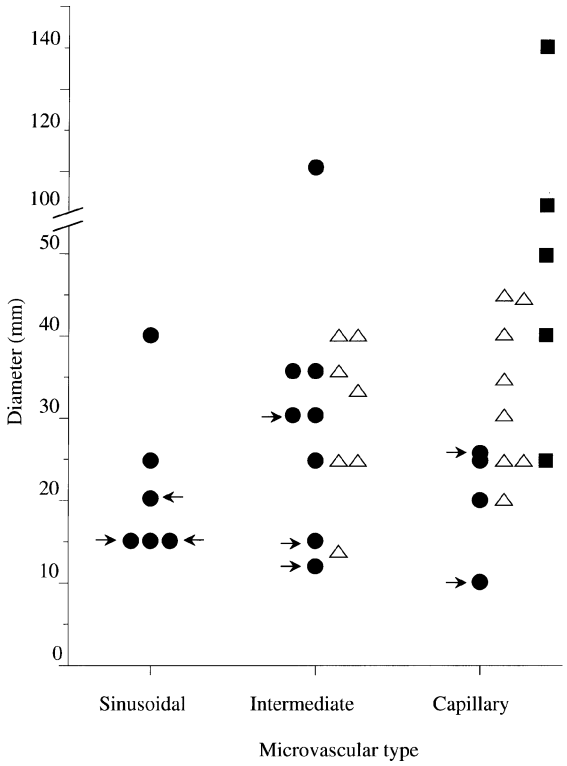

Fig. 3. Distribution of size of sinusoidal, intermediate and capillary types of well (●), moderately (△) and poorly (■) differentiated HCCs. Arrows indicate the tumor with preferentially portal venous blood supply.

of HCCs. This view was supported by immunohistochemical findings that expression of factor VIII-related antigen[17] and CD34,[18, 19] both of which are markers for capillary endothelial cells, was intense and diffuse in advanced HCCs, while it varied from negative to focal in early-stage HCCs. In this study, we used four ultrastructural parameters including endothelial defenestration, and defined three microvascular types, i.e., sinusoidal, intermediate and capillary types. Since electron microscopic observation is limited to small areas, we examined here 10 microscopic fields per sample. Well-differentiated HCCs displayed all three microvascular types, while moderately differentiated ones consisted of intermediate and capillary types, and poorly differentiated ones had only a capillary type, suggesting that the transition from the sinusoids to capillaries may proceed in well-differentiated HCCs (and probably in moderately differentiated ones as well).

The hypothesis that human HCCs undergo a replacing growth in the early stage[20] raises the possibility that fenestrated sinusoids in the tumor might derive from pre-existing normal sinusoids. It is reported that, in the fetal period, the capillaries of the septum transversum undergo differentiation into hepatic sinusoids once they are surrounded by growing plates of hepatocytes,[21] suggesting that interaction of capillaries with normal hepatocytes may play an important role in inducing the characteristic endothelial fenestration of the sinusoid. The predominant occurrence of sinusoidal-type microvessels in well-differentiated HCCs may be related to the matured parenchymal tumor cells present in them. Matured cells possessing cell organelles comparable to those of normal hepatocytes must be engaged in some of the metabolic functions of the

Table IV. Average Numbers and Diameters of Endothelial Fenestrae in Sinusoidal and Intermediate Types of HCCs

Types	Average number	Average diameter
S (n=6)	30±14	0.16±0.03
I (n=10)	17±11	0.17±0.12

The average number and diameter of fenestrae per 20-μm length of sinusoidal walls in 10 microscopic fields were calculated. No significant differences in average number and average diameter were found between sinusoidal and intermediate types.

normal hepatocytes and therefore require the sinusoidal structure for efficient substance-exchange between parenchymal cells and the portal venous blood.

The tumor-nontumor boundary of the HCCs with a replacing growth has a hematogenous continuity that allows the influx of sinusoidal blood from the surrounding nontumor parenchyma into the tumor,[20] thus giving rise to the preferential portal venous blood supply seen in some well-differentiated HCCs. An inducing role of arterialization in sinusoidal capillarization has been suggested both in human subjects[17] and experimental animals.[13] However, we found in this study no appreciable correlation between the microvascular type and arterialization in well-differentiated HCCs. Our previous study using rat liver after portal branch ligation demonstrated that arterialization reduced the endothelial fenestrae to some extent, but not completely, indicating that arterialization is not the sole determinant for inducing sinusoidal capillarization.[22] Furthermore, the tumor size had no significant correlation with sinusoidal capillarization in well-differentiated HCCs, while it did with arterialization. The reason for the latter fact is that tumor growth requires arterial blood supply.

It was also noted that there was a difference in the mode of blood supply among well, moderately and poorly differentiated HCCs, i.e., some well-differentiated HCCs were preferentially supplied with portal venous blood, while moderately and poorly differentiated ones were all supplied with arterial blood. This finding may indicate that, as HCCs become increasingly dedifferentiated, their dependence on arterial blood supply becomes larger.

In conclusion, sinusoidal capillarization is likely to occur in human well-differentiated (and probably moderately differentiated) HCCs. This event is closely related to the histological grade, but not to the tumor size or arterialization, suggesting that dedifferentiation of parenchymal tumor cells rather than tumor growth may play a major role in inducing sinusoidal capillarization in human HCCs. The present finding provides a pathological basis for understanding the development of HCCs and is also important for angiographic differential diagnosis and the choice of treatment (transarterial therapy, microwave coagulation, or surgical resection) of HCCs.

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