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Light and Electron Microscopic Analysis of Liver Sinusoids during Hepatocarcinogenesis with 2-Acetylaminofluorene in Rats

Yutaka Shoji,[1] Kenji Kaneda,[2,3] Kenjiro Wake[2] and Yoshio Mishima[1]

[1]*Second Department of Surgery and* [2]*First Department of Anatomy, Faculty of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113*

To clarify the sequential changes and morphological differences of the sinusoidal structures between hepatocellular carcinoma (HCC) and hepatocellular adenoma (HA), we examined morphological changes of sinusoidal cells and related structures such as basement membrane during hepatocarcinogenesis in the rat. During continuous feeding of carcinogenic diets containing 2-acetylaminofluorene to rats, HA appeared at the 8th week in the periportal area and then extended toward the centrolobular area. The appearance of HCC was recognized at the 27th week. In the HA lesion, the morphology of sinusoidal cells and related structures was basically the same as that of normal liver except for a slight thickening of the basement membrane and a decreased amount of vitamin A-lipid droplets of stellate cells. In HCC, the fenestrations of endothelial cells disappeared and the basement membrane became continuous, thick and often multilayered. Stellate cells contained almost no vitamin A-lipid droplets and were associated with abundant collagen fibers. Kupffer cells and pit cells were not seen inside the sinusoid. All these features of the sinusoids in HCC resembled the morphological characteristics of the capillary. The present study has revealed that HCC possesses sinusoid structures distinct from those of HA. This suggests that HCC may not derive directly from HA but may develop newly within the HA.

Key words: Hepatocarcinogenesis — Liver sinusoid — Ultrastructure — Rat — 2-AAF

Many studies have been reported on the ultrastructural analysis of human hepatocellular carcinoma (HCC).[1,2] Most reports dealt with the carcinoma cells, but recently interest has been focused on the sinusoidal cells in the HCC, including pit cells.[3] It is considered that there is a close relationship between the changes of sinusoidal structures and hepatocarcinogenesis. Haratake and Scheuer[4] also reported the ultrastructural changes of sinusoidal cells in human primary hepatomas. Unfortunately, these reports were limited to the ultrastructural analysis of the parenchymal and the non-parenchymal cells in only hepatocellular carcinoma. In other words, there was no mention of the sequential changes of sinusoidal structures.

Recently it has been suggested that hepatocellular adenoma (HA) might be a precancerous lesion.[5-7] It is, however, difficult to detect HA in surgical specimens of human liver under an electron microscope because of its small size, and there has been no report about their ultrastructural characteristics.

An experimental model of HCC chemically induced in the rat is available.[8] This experimental HCC is developed from HA and has been used widely for the analysis of hepatocarcinogenesis.[9,10] Attention, however, has been paid mostly to the change of parenchymal cells[11] and there has been no report on the morphological changes of sinusoidal cells in this model.

To clarify the sequential changes of the sinusoidal cells and the morphological differences between HCC and HA, we have examined the morphological changes of the sinusoidal cells,[12] i.e., endothelial cells,[13] Kupffer cells, stellate cells, fat-storing cells, pit cells,[14,15] and related structures such as basement membrane and collagen fibers during hepatocarcinogenesis, and compared them in HCC and HA.

MATERIALS AND METHODS

Animals Fifty male Donryu rats (Sankyo Lab. Service, Tokyo), weighing 150–200 g, were used. The animals were housed in cages in an air-conditioned room.

Experimental carcinogenesis Synthetic diets containing 0.03% 2-acetylaminofluorene (2-AAF) (CE2, CLEA Japan Inc., Tokyo) were used. The diet and water were given *ad libitum*. According to Kitagawa,[8] all rats were continuously fed the carcinogenic diet except for a 5-day pause in the third week after initial administration of the diet to prevent early death.

Light and electron microscopy At the 4th, 8th, 12th, 18th, 22nd, 24th, 27th and 32nd weeks, five or six rats were examined. Under ether anesthesia, livers were perfused via the portal vein with 0.1 *M* phosphate buffer, pH 7.4, for 20 s to remove blood. We examined four kinds of

[1] To whom requests for reprints should be addressed.

[3] Present address: Second Department of Anatomy, Osaka City University Medical School, 1-4-54 Asahimachi, Abeno-ku, Osaka 545.

stainings (hematoxylin-eosin (H-E) staining, Ishii's silver staining, gold staining and uranyl acetate and lead citrate staining).

After this perfusion, one lobe of the liver was resected, fixed in 10% formalin solution and embedded in paraffin for light microscopy. Paraffin sections were stained with H-E or by Ishii's silver staining.[16] HA or HCC was diagnosed according to histologic typing of liver tumors of the rat using paraffin sections of H-E specimens.[17] Sections for Ishii's silver staining were treated in 0.25% potassium permanganate for 8 min and 1% oxalic acid for 6 min, then stained with silver ammonium solution for 30 min and finally transferred to 0.05% gold chloride for 1–2 h under shielding from light. After staining, sections were rapidly dehydrated and mounted in Canada balsam. At normal, HA (12 weeks) and HCC (32 weeks) stages, frozen sections were stained by the gold chloride method.[18] Frozen sections were cut on a freezing microtome. The sections were treated in 0.05% chromic acid solution for 10 min and then exposed to the gold staining solution (1 ml of 1% gold chloride, 1 ml of 1% HCl and 98 ml of distilled water). The sections were put into an incubator for 12–16 h and, after staining, washed in distilled water, dehydrated through graded alcohol, and mounted in Canada balsam.

The remaining lobes of the liver, perfused via portal vein with 0.1 *M* phosphate buffer, were further perfused with fixative solution containing 1.5% glutaraldehyde in 0.062 *M* cacodylate buffer, pH 7.4, and 1% sucrose.[13] The whole liver was taken out and selected areas with HA or HCC were cut into small pieces (30 or 40 pieces). After post-fixation in 1% OsO_4, they were dehydrated in ethanol and embedded in Polybed. Thin sections were stained with Toluidine blue and diagnosed as HA or HCC by comparing them with H-E stained specimens of the same liver. Second, embedded blocks including HA or HCC were trimmed for ultrathin sections. Finally, ultrathin sections were stained with uranyl acetate and

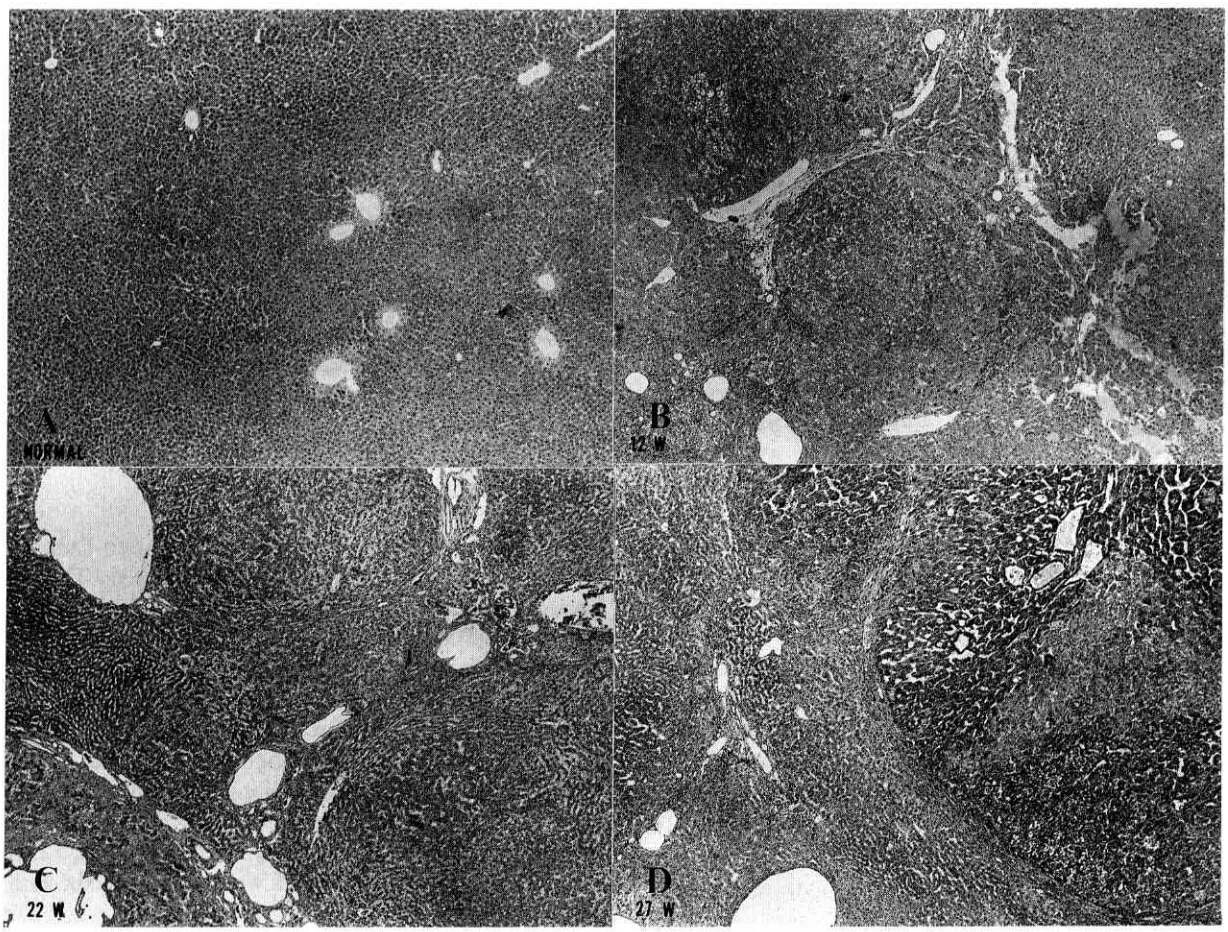

Fig. 1. Histology of the liver at various stages of hepatocarcinogenesis in the rat (H-E staining). A: normal liver. ×10. B: an early stage of HA at the 12th week. ×10. C: a late stage of HA at the 22nd week. ×10. D: HCC formed at the 27th week. ×10.

lead citrate, and observed under a JEOL 100CX electron microscope operated at 100 kV.

RESULTS

Light microscopy Among normal tissue, small areas of HA that consisted of immature hepatocytes started to appear at the 8th week after the start of administration of 2-AAF. They occurred preferentially in the periportal area of the liver lobule. Then, at the 12th week, HA extended toward the central area (Fig. 1B). Proliferation of spindle cells, probably fibroblasts, was recognized around such hyperplastic nodules. At the 18th week, the nodules became larger, showing apparent compression against surrounding normal tissue. At the 22nd and 24th week, heterogeneity of the hepatocytes in size became apparent in the nodules. Sinusoidal lumens in the HA at this period tended to show dilatatoin (Fig. 1C). At the 27th week, HCC was recognized (Fig. 1D). In the HCC, atypism was obvious in the arrangement of the hepatocytes: it was of trabecular or compact type. Nuclear atypism was also seen in the hepatocytes. The HCC nodules were surrounded by many spindle cells. We divided the hepatocarcinogenesis seen in this study into four stages, i.e., normal stage (–7th week after administration of 2-AAF), early HA stage (8–21st week), late HA stage (22–26th week) and HCC stage (27th week–).

In normal liver, the stellate cells were stained black by the gold chloride method, which indicated the presence of vitamin A. They were distributed evenly in the liver

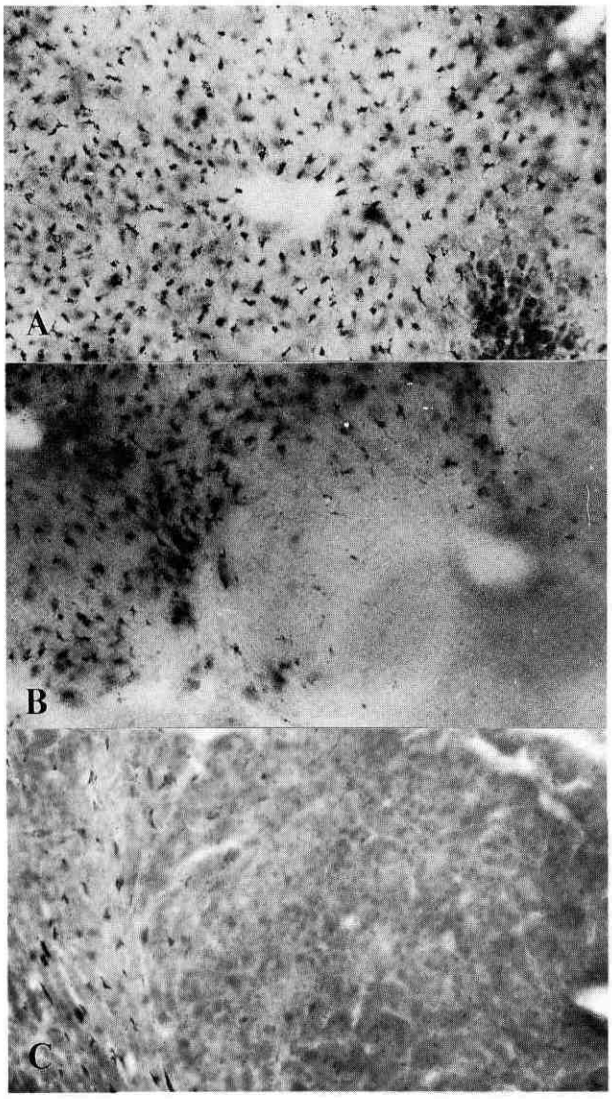

Fig. 2. Distribution of vitamin A-containing stellate cells in the liver. Gold chloride staining. A: normal liver. ×20. B: an early stage of HA at the 12th week. ×20. C: HCC at the 32nd week. ×20.

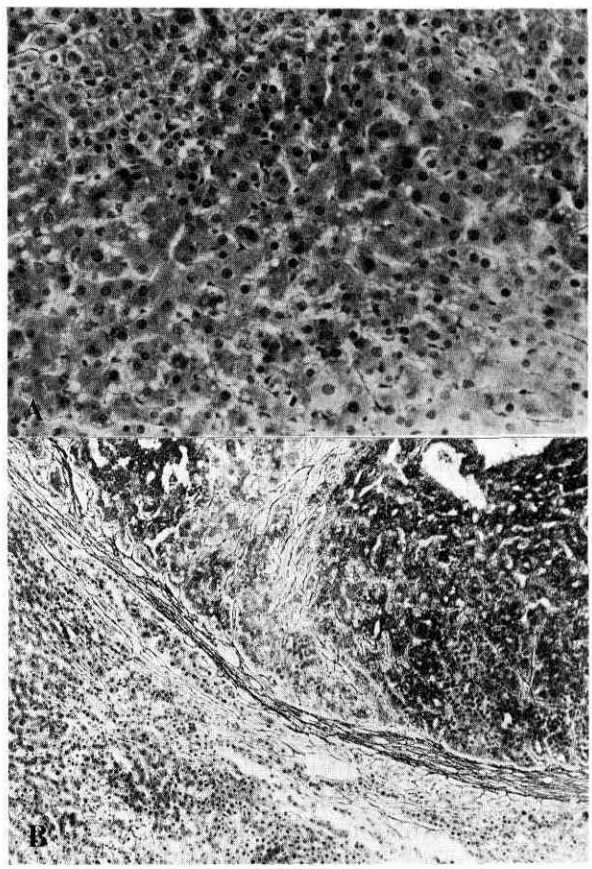

Fig. 3. Formation of collagen fibers in the liver. Silver impregnation method. A: a late stage at the 22nd week. ×40. B: HCC at the 32nd week. ×20.

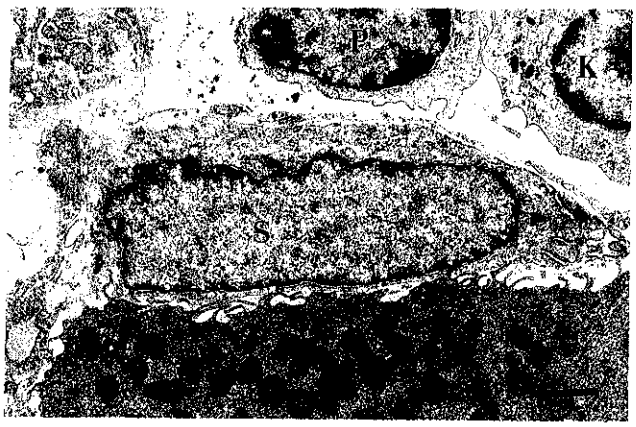

Fig. 4. Ultrastructure of liver sinusoid at an early stage of HA at the 12th week. Pit cells (P) and Kupffer cells (K) are seen inside the sinusoid. Uranyl acetate and lead citrate staining. ×2700. Bar=2 μm.

lobule (Fig. 2A). HA at both early and late stages contained only a few positive cells, while positive cells were abundant in surrounding normal tissue (Fig. 2B). Within the HCC, there were almost no positively stained stellate cells (Fig. 2C).

When liver tissues of HA were examined with silver impregnation staining, a small amount of collagen fibers or reticular fibers was seen along the sinusoid (Fig. 3A). As shown in Fig. 3B, the bundles became thicker at the HCC stage. Abundant collagen fibers were observed around the nodule, in which spindle cells were present in H-E stained specimens.

Electron microscopy The sinusoid of the normal liver consisted of four types of cells, as follows. i) Endothelial cells had a characteristic sieve plate feature, being associated with thin and interrupted basement membranes beneath the cell. ii) Stellate cells usually contained vitamin A-lipid droplets. They existed in the space of Disse, being associated with a small amount of collagen fibers. iii) Kupffer cells. iv) Pit cells. The latter two types of cells extended their cytoplasmic projection into the pores of endothelial cells.

At an early stage of HA, the architecture of the sinusoid is almost the same as that of normal liver. Endothelial cells had obvious sieve plates and the basement membrane was not continuous, particularly beneath the sieve plates (Figs. 4 and 5A). Vitamin A-lipid droplets in the stellate cells were, however, much fewer than those of normal liver.

At a late stage of HA, the basement membrane became thicker and continuous (Fig. 5B). The sieve plates of endothelial cells were, however, well preserved. The stellate cells had only a few lipid droplets and the amount of

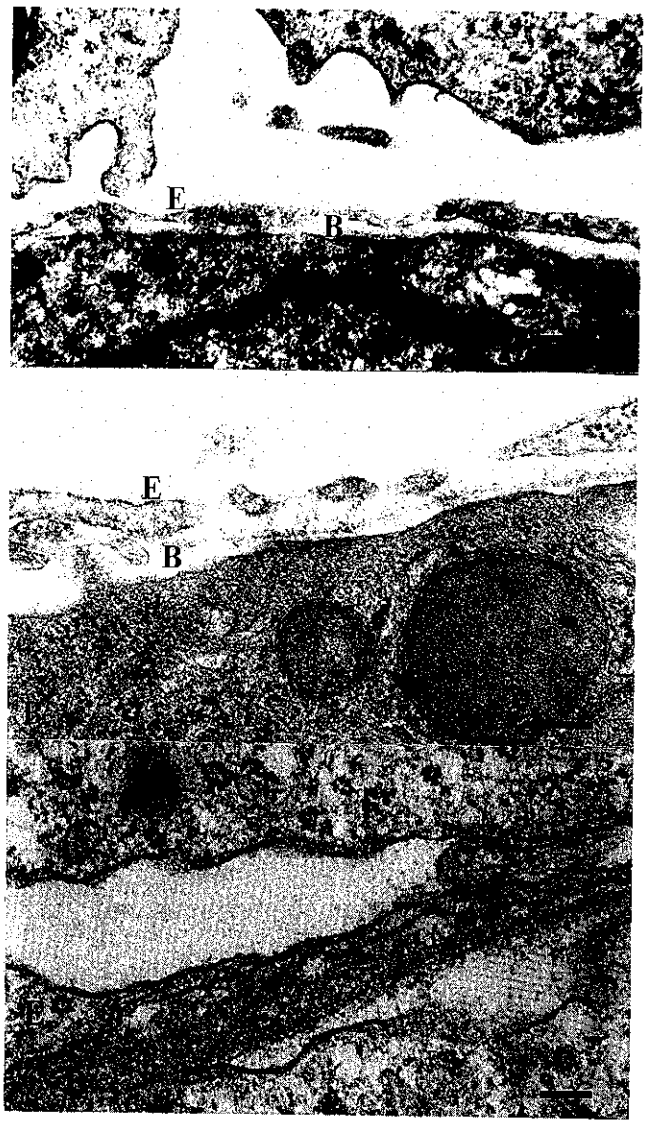

Fig. 5. Sinusoidal endothelial cells (E) and basement membrane (B). Uranyl acetate and lead citrate staining. A: at the 12th week. ×2700, Bar=2 μm. B: at the 22nd week thin and continuous basement membrane exists beneath the sieve plates. ×33000. Bar=0.2 μm. C: at the 27th week thickened and continuous basement membrane of endothelial cells is recognized. ×26000. Bar=0.2 μm.

associated collagen fibers increased in the space of Disse. Kupffer cells and pit cells were observed within the sinusoid.

In HCC, endothelial cells had no sieve plates. The basement membrane became continuous and much thicker than that seen in HA (Fig. 5C). It often formed a multilayered configuration of basement membrane (Fig. 6). Stellate cells contained few lipid droplets and

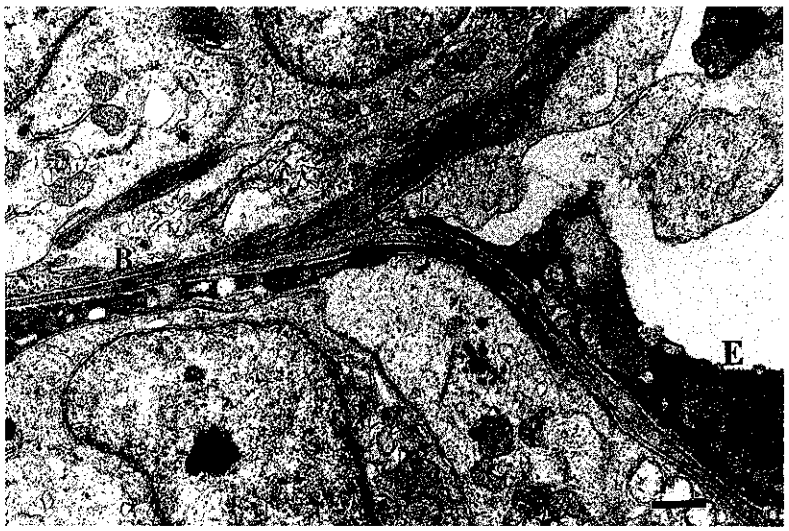

Fig. 6. Mutilayered basement membrane (B) beneath the endothelial cells (E) at 27th week. Uranyl acetate and lead citrate staining. ×1700. Bar=4 μm.

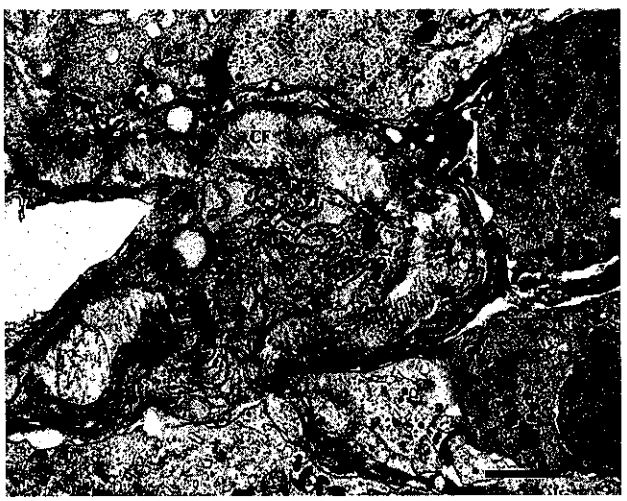

Fig. 7. A stellate cell (S) surrounded by a large amount of collagen fibers (CF) in the space of Disse at the 27th week. Uranyl acetate and lead citrate staining. ×3400. Bar=2 μm.

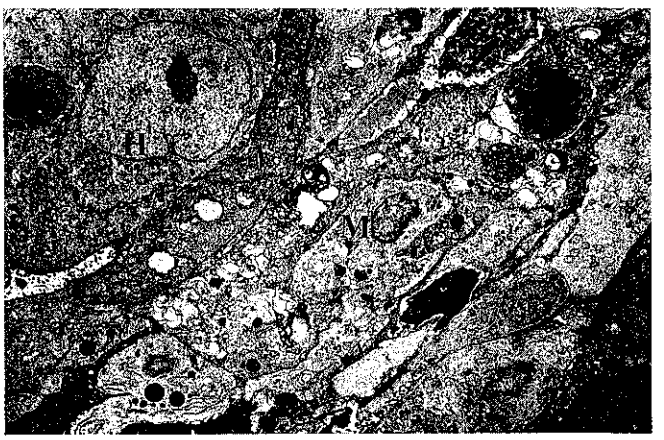

Fig. 8. Macrophages (M) infiltrating HCC (H) at the 27th week. Uranyl acetate and lead citrate staining. ×1700. Bar=2 μm.

were surrounded by a large amount of collagen fibers (Fig. 7). Within the sinusoid, Kupffer cells and pit cells were not observed. They did, however, exist among the carcinoma cells, as well as other leukocytes such as neutrophiles and lymphocytes, directly contacting the carcinoma cells (Figs. 8 and 9).

DISCUSSION

We have described the light and electron microscopic features of the sinusoidal cells and surroundings of the hepatic sinusoid during the course of experimental carcinogenesis of the liver, i.e., normal, early and late stages of HA and the HCC stage, induced by the chemical carcinogen 2-AAF. The results are summarized in Table I. The morphological characteristics of the sinusoid in the experimentally induced HCC of the rat were consistent with the morphological changes in human primary HCC reported by Haratake and Scheuer.[4] Therefore this experimental model may be valuable to clarify the natural history of hepatocarcinogenesis.

Kitagawa[8] reported that there was no marked difference in histological features of hyperplastic nodules between rats continuously fed carcinogen and rats returned to basal diet after being fed on carcinogenic diet, though development of HCC in the former was more frequent

than in the latter. So toxic effects of the chemical on the development of HCC should be minimal. In our study, only 8 of 50 rats died up to the 10th week. Therefore, we continuously fed carcinogenic diet to the rats until they were killed.

We recognized a slight morphological difference of sinusoidal structure between normal tissue and HA, i.e., the amount of lipid droplets of stellate cells was less and the basement membrane became slightly more continuous or thicker in HA (Table I). It has been reported that stellate cells can take up much vitamin A when they are localized in the vicinity of the sinusoidal lumen but this function decreases as the cells depart from their juxta-sinusoidal position owing to thickening of the basement membrane between endothelial cells and stellate cells.[19-21] As a result, they gain fibroblastic nature and produce more collagen fibers. The defenestration of the sinusoidal endothelium, by acting as a barrier to chylomicron catabolism and hence retinol metabolism, is important in the initiation of perisinusoidal fibrosis.[22] It has also been reported that in human hepatocellular carcinoma the amount of vitamin A is decreased compared with non-cancerous regions.[23, 24] Our study supported those reports. In other words, depression of the amount of vitamin A was recognized even in HA.

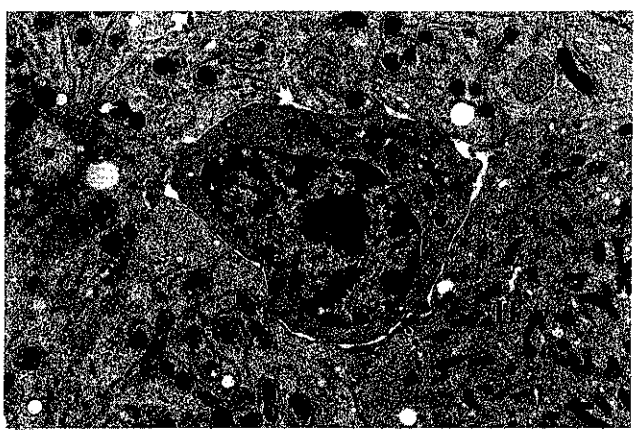

Fig. 9. Pit cell (P) in HCC (H) at the 32nd week. Uranyl acetate and lead citrate staining. ×6000. Bar=1 μm.

The difference of sinusoidal structures between HA and HCC was distinct. Sieve plates, which are characteristic of liver sinusoidal endothelial cells, disappeared in HCC. The basement membrane became more obvious and abundant collagen fibers were recognized around the endothlial cells and stellate cells. These features were common to capillaries. Kupffer cells and pit cells were not found in the sinusoids of HCC. This fact also indicates a difference of properties between endothelial cells of sinusoids in HCC and the sinusoidal endothelial cells in normal liver. Kupffer cells do not exist in the capillaries of the Glisson's sheath but only in the sinusoid. According to these results, it might be reasonable to designate the sinusoids in HCC as capillaries.[25-27] There is an obvious discontinuity between the capillaries in the HCC and the sinusoid in HA in the rat, which suggests that HCC would not be directly formed from HA, but rather generated from HA and then develop rapidly, producing new capillaries within the HCC. It is also known that the blood flows in the capillaries of the HCC

Table I. Morphological Characteristics of the Sinusoidal Cells during Hepatocarcinogenesis Induced by 2-AAF

Sinusoidal cells and related structures	Normal	Hepatocellular adenoma		HCC (27 wk)
		early (12 wk)	late (22 wk)	
Endothelial cells				
sieve plate	+	+	+	−
basement membrane				
continuity	−	−	+	+
thickening	−	−	−	+
multilayering	−	−	−	+
Stellate cells				
vitamin A droplets[a]	+	−	−	−
collagen fibers				
around the cell	−	−	+	+
Kupffer cells				
existence in the sinusoid	+	+	+	−
Pit cells				
existence in the sinusoid	+	+	+	−

a) The results were obtained by using the gold impregnation method.

come directly from the hepatic artery,[28,29] while the blood flows in the sinusoids of normal liver or HA come from both portal vein and hepatic artery. Such differences of blood supply between HA and HCC easily explain the morphological disparity of the capillaries in HCC; in other words, the capillaries seen in HCC would be formed independently of the sinusoids in HA. Tsuda *et al.*[30] reported that neovascularization from the arterial rather than the portal vein might play an important role for further development of preneoplastic lesions in the liver, and sinusoids of HCC were rich in anastomosing branches that connected to the hepatic artery. The structures that we could observe in the HCC did not represent capillarization of sinusoids but capillaries of the hepatic artery.

The sudden appearance of sinusoidal changes implies a gap between HA and HCC in terms of sinusoidal cells. In other words, there is no morphological continuity of sinusoidal cells during hepatocarcinogenesis. Therefore, we may speculate that changes of hepatocytes occur first followed by the changes of sinusoidal cells. More detailed analyses on the sequential changes in terms of the relationship between sinusoidal cells and hepatocytes will be necessary to solve this problem.

In the present study, we followed the morphological changes in experimental hepatocarcinogenesis, i.e., HA and HCC, and observed distinct differences of the morphological characteristics of the sinusoidal walls between HA and HCC.

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