Immunohistochemical and Ultrastructural Studies on the Sinusoidal Lining Cells of Canine Hepatocellular Carcinoma

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ABSTRACT. Immunohistochemical and ultrastructural studies were performed on the sinusoidal lining cells of eight canine hepatocellular carcinomas. The sinusoidal endothelial cells of the tumors had a positive reaction for both Factor VIII-related antigen and peanut agglutinin, but did not bind with *Ulex europaeus* agglutinin-I. Desmin- and lysozyme-positive cells were present along the sinusoids and perisinusoidal spaces of the tumor tissues, respectively, but were fewer in number compared with those of normal canine liver. α-Smooth muscle actin-positive cells outlining the sinusoids were frequently observed. Electron microscopy revealed that basement membranes were often formed beneath the sinusoidal endothelial cells, with rare fenestration. Macrophages were present around or within the sinusoids and tended to increase in number relative to the degree of tumor differentiation. Myofibroblast-like cells with various morphological features, consistent with α-smooth muscle actin-positive cells, were frequently found in the perisinusoidal space. The present study indicates that the sinusoidal lining cells of canine hepatocellular carcinoma have some phenotypic characteristics. — KEY WORDS: canine, hepatocellular carcinoma, immunohistochemistry, sinusoidal lining cell, ultrastructure.

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Hepatocellular carcinoma is uncommon in dogs, but the incidence in dogs is relatively high compared with other domestic animals [18]. However, there may be difficulties to differentiate hepatocellular carcinoma from nodular hyperplasia in aged dogs or small biopsy specimens. It is known that the sinusoid in normal liver is composed of endothelial cells, Kupffer cells, Ito cells, pit cells, and extracellular matrix components [21]. Several investigations have shown the characteristic structures of the sinusoids in the tumor tissues of human hepatocellular carcinoma [3, 4, 9, 11–13, 16, 19, 22, 23], while the characterization of canine hepatocellular carcinomas has been limited to the tumor cells [15, 25]. In small animal practice, liver biopsy have been frequently performed for diagnosis of massive lesions in addition to biochemical analysis. There have been no reports on the characteristics of sinusoids in canine heptocellular carcinoma. The purpose of this study is to characterize the features of the sinusoidal lining cells in canine hepatocellular carcinoma.

MATERIALS AND METHODS

Eight cases of canine hepatocellular carcinomas were obtained at necropsy (2 cases) or surgery (6 cases). The degree of differentiation of each tumor determined based on the Edmondson-Steiner's classifications [5]: grades I and I-II were defined as well-differentiated, grades II and II-III as moderately-differentiated, and grades III and IV as poorly-differentiated. In addition, these hepatocellular carcinomas were classified into three histologic types (solid, pseudoglandular, and trabecular) according to the World Health Organization classification [8]. The growth of hepatocellular carcinoma was expressed in three patterns according to the description by Nakashima *et al.* [14]. A normal liver tissue was obtained from a beagle dog and served as control for comparison. The liver tissues were fixed in 10% phosphate buffered formalin and embedded in paraffin according to standard operating procedures. Paraffin sections were cut at 3 μm and stained with hematoxylin and eosin (HE) and also with special stainings including Masson's trichrome, Watanabe's silver impregnation, Perls' methods for iron pigment, periodic acid-Schiff (PAS) reaction (with or without diastase digestion), and Hall's methods for bile pigment. Immunohistochemical studies were performed using a histofine kit (Nichirei Corp., Tokyo, Japan). After blocking endogenous peroxidase activity with 3% hydrogen peroxide, normal rabbit or goat serum was applied to the deparaffinized sections to inhibit non-specific binding. Primary antibodies used in this study were as follows: anti-human desmin antibody (Dako A/S, Denmark), anti-human lysozyme antibody (Dakopatts A/S, Denmark), anti-human factor VIII-related antigen (FVIII-RAg) antibody (Dakopatts A/S, Denmark), and anti-α-smooth muscle actin (α-SMA) antibody (Bio-science, U.S.A.). In addition, lectin-histochemistry was performed with peanut agglutinin (PNA; E-Y Laboratories, U.S.A.) and *Ulex europaeus* agglutinin-I (UEA-I; E-Y Laboratories, U.S.A.). All the immunoreactions with primary antibodies were performed under the same conditions for 1 hr at 37°C. Thereafter, biotinylated anti-rabbit or mouse IgG and peroxidase-labeled streptavidin were sequentially applied to the sections for 15 min at room temperature. The reaction was visualized with 3,3'-diaminobenzidine in 0.1 M Tris buffer (pH 7.6) and hydrogen peroxide. Counterstaining with Mayer's hematoxylin was carried out. To enhance the immunoreaction, some deparaffinized sections were pretreated with pepsin digestion or microwave oven heating. Sections were washed in phosphate buffered saline after each step. Lysozyme-positive cells were counted in 10 high-

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power fields (HPF) for each case.

For electron microscopy, 1 mm^3 blocks of normal and neoplastic livers were fixed by immersion in 2.5% glutaraldehyde buffered in phosphate (pH 7.4) for 2 hr at 4°C, post-fixed in 1% osmium tetroxide (OsO_4) buffered in phosphate (pH 7.4) for 2 hr at 4°C, then dehydrated and embedded in Epoxy resin. Formalin-fixed materials were also post-fixed by OsO_4, followed by dehydration and embedding in the same manner described above. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate. Electron microscopic observation was performed using a Hitachi H-300 transmission electron microscope at 75 kV.

RESULTS

The profiles of the dogs with hepatocellular carcinomas are shown in Table 1. Of 8 cases examined one case (Case No. 4) showed distant metastasis and two cases (Case Nos. 2 and 7) exhibited intrahepatic metastasis. The size of the neoplasms ranged from 3 to 10 cm in diameter. No capsule formation was observed around the tumor tissues. Macroscopically, three tumors were massive in shape and five tumors were nodular.

Histopathology: Histopathologic features of each tumor are summarized in Table 2. According to Edmondson and Steiner's classification [5], four were well-differentiated, one was moderately-differentiated, and three were poorly-differentiated. The histologic types of the tumors were trabecular in four cases (Figs. 1a, b), pseudoglandular in one case (Fig. 1c), and solid in three cases (Fig. 1d). There were no tumors producing bile or complicating with cirrhosis. Extravasation of erythrocytes was observed frequently in the tumor tissues of all cases. Vascular lakes were found in 6 of the 8 cases. Hemosiderin-containing cells were occasionally observed in the sinusoids of the tumor tissues. Although reticulin fibers were scant in the hepatocellular carcinomas compared with the normal dog liver, they were present along the sinusoids in the tumors. In the most well-differentiated tumor (Case No. 8), reticulin fibers were more abundant.

Lectin-and Immunohistochemistry: The results are summarized in Table 3. Sinusoidal endothelial cells in the tumor tissues had a positive reaction for both FVIII-RAg (Fig. 2a) and PNA (Fig. 2b), but did not bind with UEA-I. PNA and UEA-I did not bind with the normal canine sinusoidal endothelium. α- SMA-positive cells outlined the sinusoids in the tumors (Fig. 3) as well as those in the normal liver. However, the immunoreactivity in the tumors and the surrounding tissues including borderline regions was more intense than that in the normal liver tissue. The number of α- SMA-positive cells in the tumor tissues tended to increase in proportion with the volume of stromal collagen. In the normal liver, lysozyme-positive cells were present along the sinusoids and were more abundant in the periportal regions of hepatic lobules (Fig. 4a). These cells were considered to be Kupffer cells because of their characteristic morphology and anatomical location. The number of lysozyme-positive cells in the various types of the canine hepatocellular carcinomas, the surrounding tissues, and the normal control liver is shown in Table 4. The number of these cells in the tumor tissues was less than that in the normal liver (Table 4). Furthermore, in the surrounding tissues, lysozyme-positive cells exhibited hypertrophy and increased in number (Fig. 4b). Lysozyme-positive cells were observed within and around the sinusoids. Well-differentiated tumors (grade I, I-II) contained more lysozyme-positive cells than did poorly-differentiated ones (grade III, IV). In the surrounding tissues, lysozyme-positive cells increased in number,

Table 1. Profile of dogs with hepatocellular carcinoma

Case No.	Sex[a]	Age (Years)	Breed
1	M	11	Mongrel
2[b]	F	13	Shiba
3	M	15	Mongrel
4[c]	F	11	Maltese
5	M	13	Beagle
6	M	15	Yorkshire terrior
7[b]	F	12	Beagle
8	F	10	Mongrel

a) M: male, F: female. b) Cases with intrahepatic metastasis. c) Case with distant metastasis.

Table 2. Histopathological features of canine hepatocellular carcinoma

	Case No.							
	1	2	3	4	5	6	7	8
Cell grading[a]	III	II	I-II	III-IV	I-II	I-II	III	I
Histologic type[b]	S	P	T	S	T	T	S	T
Growth pattern[c]	E	E	E	R/S	E	E	E	E
Bile production	–	–	–	–	–	–	–	–
Glycogen	+	–	+	+	+	+	+	+
Fatty change	+	–	+	+	+	–	–	+
Vascular lake	+	–	+	–	+	+	+	+

a) According to Edmondson and Steiner's classification [5]. b) According to WHO's classification (S: Solid, P: Pseudoglandular, T: Trabecular) [8]. c) According to the description by Nakashima *et al.* (E: Encapsulated, R: Replacing, S: Sinusoidal) [14].

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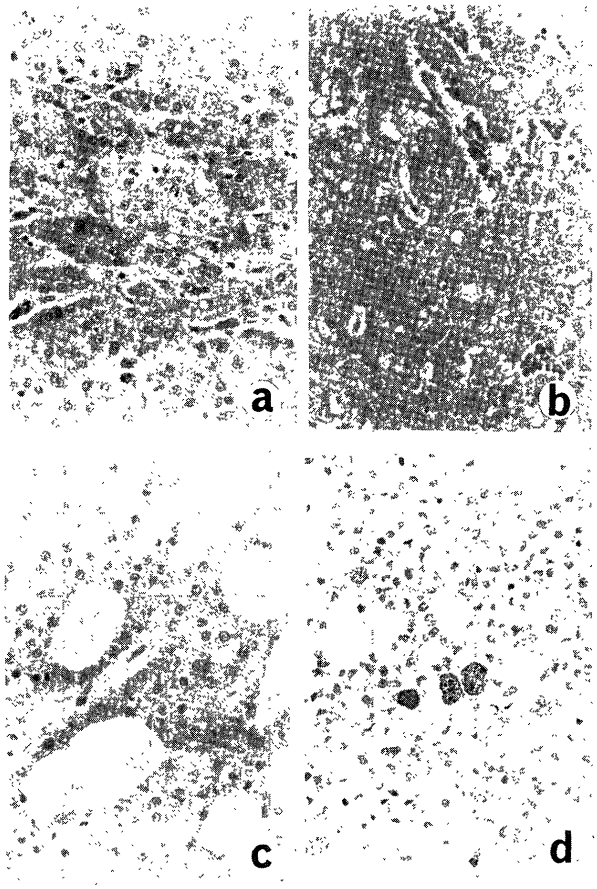


Fig. 1. The histologic types of canine hepatocellular carcinomas. a) Trabecular type (thin). Case No. 8. HE stain. × 460. b) Trabecular type (thick). Case No. 1. HE stain. × 460. c) Pseudoglandular type. Case No. 2. HE stain. × 460. d) Solid type. Case No. 4. HE stain. × 460.

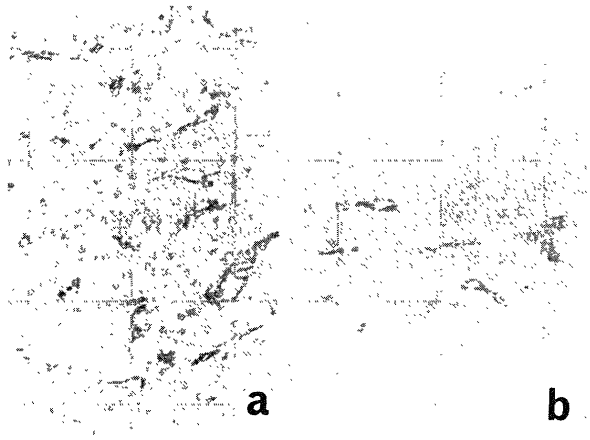


Fig. 2. a) FVIII-RAg expression along the sinusoids. Immunostaining with anti-human FVIII-RAg antibody. Mayer's hematoxylin counterstain. Case No. 1. × 460. b) PNA binding with sinusoidal endothelial cells. Lectin-histochemistry with PNA. Mayer's hematoxylin counterstain. Case No. 1. × 460.

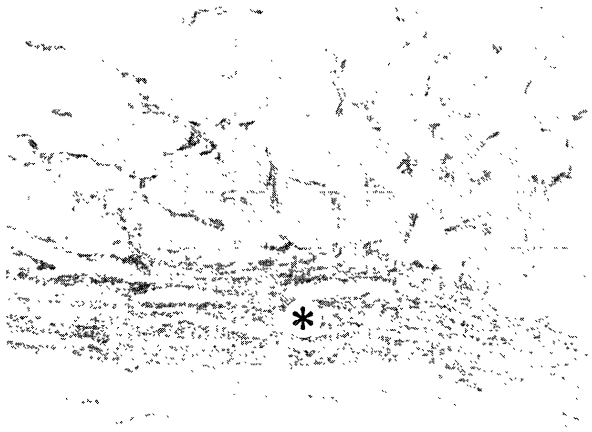


Fig. 3. α- SMA positive cells in the perisinusoidal space and the fibrous septa (asterisk) of tumor tissue. Immunostaining with anti-α-SMA antibody, and counterstained with Mayer's hematoxylin. Case No. 6. × 460.

Table 3. Lectin - and immunohistochemical findings of sinusoidal linings in the normal liver and hepatocellular carcinoma of dogs

	UEA-I	PNA	FVIII-RAg	Desmin	α -SMA
Normal liver					
endothelial cell	–	–	–	–	–
Ito cell	–	–	–	+	+
Hepatocellular carcinoma					
endothelial cell	–	+	+	–	–
myofibroblast-like cell	–	–	–	±	+

+: positive, –: negative, ±: partially positive.

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Table 4. Number of lysozyme-positive macrophages in the tumor and control tissues

	Case No.	Mean No. of Macrophages/ HPF
Hepatocellular carcinoma		
Solid type (n=3)	1	7 (22)[b]
	4	3 (30)
	7	4 (25)
Pseudoglandular type (n=1)	2	4 (111)
Trabecular type (n=4)	3	5 (7)
	5	5 (20)
	6	10 (25)
	8	18 (13)
Normal control liver[a] (n=1)		22

a) from a normal beagle dog. b) data within parentheses indicate the mean number of macrophages in the surrounding tissue.

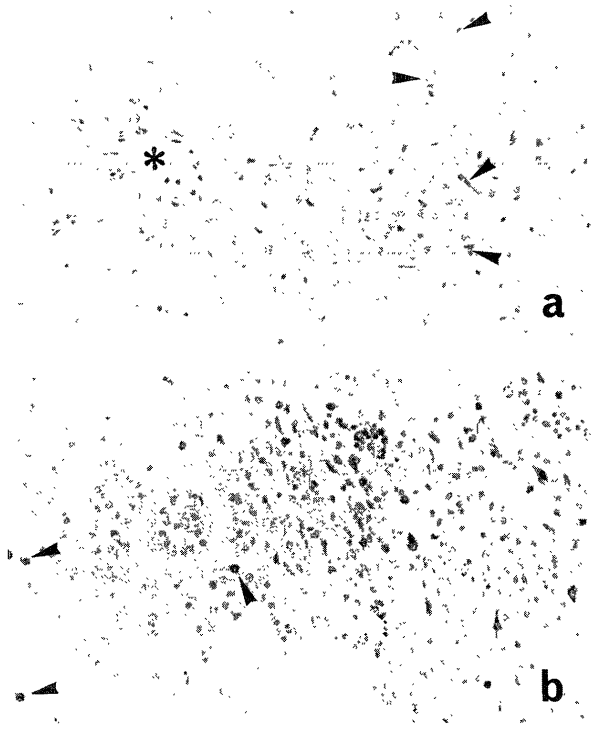


Fig. 4. Immunostaining with anti-lysozyme antibody. a) Irregular distribution of lysozyme-positive cells (arrowheads) in the lobules of normal canine liver. (asterisk): central vein. × 460. b) No lysozyme-positive cells except for a few leukocytes (arrowheads) within the tumor tissues (left half), but abundant in the surrounding tissue (right half). Case No. 2. × 460.

especially in the two malignant cases (Case Nos. 2 and 4, see Tables 1 and 4). Desmin-imunoreactivity of Ito cells was also enhanced in the surrounding tissues (Fig. 5).

Electron microscopy: Sinusoidal endothelia of all the hepatocellular carcinomas had no fenestrae and were accompanied by basement membranes (Fig. 6). Sinusoidal

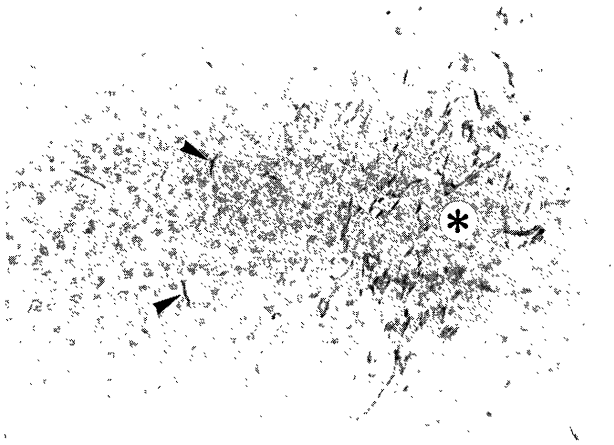


Fig. 5. A few desmin-positive cells within the tumor tissue (left half, arrowheads) and enhanced expression of desmin in the surrounding tissue (asterisk). Immunostaining with anti-human desmin antibody, and counterstained with Mayer's hematoxylin. case No. 4. × 460.

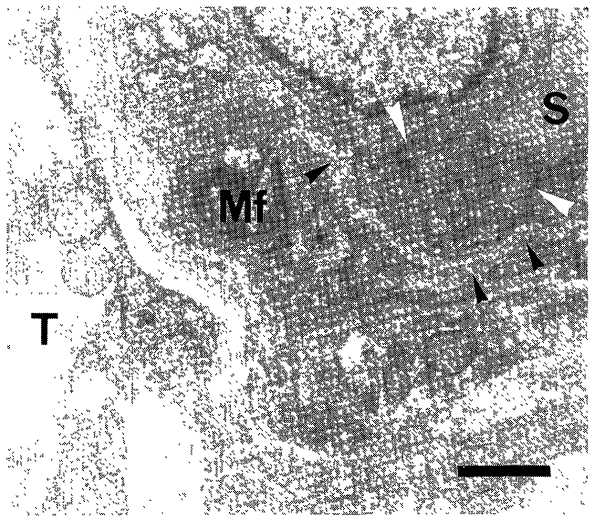


Fig. 6. Sinusoidal endothelial cells accompanying duplicated basement membrane (arrowheads) and intermediate junctions between neighboring cells (white arrowheads). Abundant collagen fibrils and myofibroblast-like cells (Mf) between sinusoidal endothelial cells and tumor cells (T). S: sinusoid. Case No. 7. Bar=2 μm.

endothelial cells were often multi-layered, and intermediate junctions were formed between the neighboring cells (Fig. 6). In the subendothelial space, mesenchymal cells with various cytological features such as pinocytotic vesicles, subplasmalemmal dense patches, and basement membranes were frequently observed (Fig. 6). Macrophages were also occasionally present around (Fig. 7a) or within (Fig. 7b) the sinusoids of the tumors.

DISCUSSION

Histopathologic and immunohistochemical characteristics

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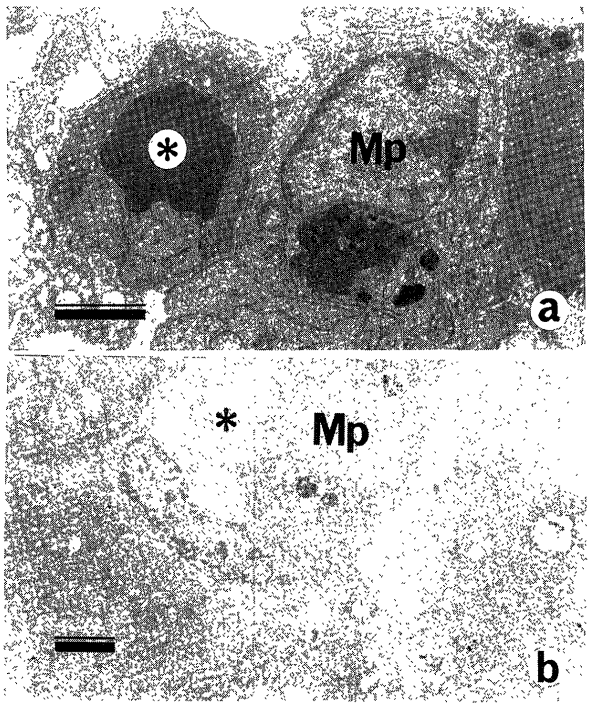


Fig. 7. a) Macrophage (Mp) in the vicinity of the sinusoid (asterisk). Case No. 7. b) Kupffer cell-like Mp in the sinusoid. Case No. 8. Bar=3 μm.

of the sinusoidal lining cells of the present canine hepatocellular carcinomas were almost the same in all cases examined, irrespective of the histologic type of tumor and the degree of differentiation. The characteristic features of these canine hepatocellular carcinomas are similar to those of human hepatocellular carcinoma [3, 4, 11–13, 19, 20, 23].

Sinusoidal endothelial cells in the canine hepatocellular carcinoma had a positive reaction to FVIII-RAg as is observed in human hepatocellular carcinoma [4, 13]. Lectin UEA-I is a useful marker for human vascular endothelial cells [26], but does not bind with sinusoidal endothelial cells of the normal human [17] or canine [1] liver. In human hepatocellular carcinoma, UEA-I binds with sinusoidal endothelium [4, 11, 19, 23]. However, UEA-I did not bind with the sinusoidal endothelial cells of the present canine hepatocellular carcinomas. On the other hand, PNA, which is a marker for canine vascular endothelial cells [10], did not bind with sinusoidal endothelial cells of the normal canine liver, but did bind with sinusoidal endothelial cells of the canine hepatocellular carcinomas. Electron microscopy revealed that the sinusoidal endothelial cells in the canine hepatocellular carcinomas had no distinct fenestrae and were accompanied with basement membranes. These immunohistochemical and ultrastructural findings suggest capillarization of the sinusoids in the canine hepatocellular carcinoma. A recent study on 2-acetylaminofluorene-induced hepatocellular carcinoma of rats, however, revealed that the vascular structures of hepatocellular carcinoma did not represent capillarization of sinusoids, but rather capillaries branched from the hepatic artery [20].

Lysozyme-positive cells and desmin-positive cells which are indicative of Kupffer cells and Ito cells, respectively in the normal liver tissue were also detected along the sinusoids and perisinusoidal areas of the tumor tissues. However, these cells were fewer in number in the tumor tissues than in the normal liver. The number of hemosiderin-containing cells which was consistent with that of lysozyme-positive cells seemed to increase relative to the grade of differentiation of the tumor cells. Haratake and Scheuer [11] have speculated that the degree of deviation of sinusoidal structure in hepatocellular carcinoma may be proportional to the degree of immaturity of the cancer cells.

In human hepatocellular carcinoma, the capsule formation may be a part of the host defense mechanism against tumor growth [24]. In the present study, there were no cases with macroscopic capsule formation. However, lysozyme-positive cells had hypertrophic changes and increased in number in the surrounding tissues, especially in the malignant cases. This finding may represent a response of the host defense mechanism in dogs with hepatocellular carcinoma.

A recent study on adult human liver has suggested that liver cell atrophy and degeneration due to chronic congestion and tumor compression may induce the myofibroblastic transformation of Ito cells [7]. Our findings of enhanced immunoreactivity to desmin and α-SMA, which are expressed in Ito cells, in the surrounding tissues may support this possibility. Electron microscopy revealed that α-SMA-positive mesenchymal cells frequently observed in the perisinusoidal areas of the tumor tissues are consistent with myofibroblast-like cells. On the other hand, proliferation and hypertrophy of Ito cells within the nodular hyperplastic lesions have been reported in the dog [2]. According to the immunohistochemical and ultrastructural findings in this study, these mesenchymal cells may be collagen producers in the tumor tissue similar to the perisinusoidal stromal cells described by Enzan *et al.* [6].

In conclusion, the sinusoids in hepatocellular carcinoma have the distinct characteristic features as compared with normal sinusoid in dogs. Some of them are similar to those of human hepatocellular carcinoma. Nodular hyperplasia (NH) frequently develops in the liver of aged dogs and is sometimes difficult to distinguish from hepatocellular carcinoma, especially in small biopsy specimens. Shoji *et al.* [20] have speculated that neoplastic changes of the hepatocytes may be followed by changes in sinusoidal cells in hepatocarcinogenesis. Therefore, in the diagnosis of hepatic nodular lesions in aged dogs, it is important to focus on the characteristics of the sinusoidal linings as well as of hepatocytes. The characteristic features of sinusoidal lining cells of non-neoplastic nodular lesions in canine liver are to be clarified.

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