

## Suppressive Effect of the Angiogenesis Inhibitor TNP-470 on the Development of Carcinogen-induced Hepatic Nodules in Rats

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**Tumor metastasis can be prevented by inhibiting angiogenesis. In the present study, we have demonstrated that the angiogenesis inhibitor TNP-470 also suppresses the development of primary hepatic nodules. Hepatocarcinogenesis was performed by the feeding of 2-acetylaminofluorene to hepatectomized rats during 8–14 weeks of age. Predominantly arterial-to-portal circulation and sinusoidal capillarization were determined by the staining of nodules with arterially infused ink and immunostaining for factor VIII-related antigen, respectively. Intraperitoneal administration of 30 mg/kg b.w. of TNP-470 twice a week significantly reduced the number of hepatic nodules. Among the nodules, hyperplastic nodules stained with ink, atypical hyperplastic nodules and hepatocellular carcinoma, all of which possess structurally altered sinusoidal endothelial cells or capillary-type endothelial cells, were dramatically decreased in number. Suppression was observed equally in nodules of all sizes. TNP-470 was more effective when administered during 8–20 weeks than during 14–26 weeks. In contrast, ink-non-stained hyperplastic nodules, which have normal sinusoidal endothelial cells, were not affected at all. The present results indicate that TNP-470 suppresses the development of primary hepatic nodules whose microvessels are capillaries or transitional forms from sinusoids to capillaries.**

Key words: TNP-470 — Hepatocarcinogenesis — Angiogenesis — Capillarization — Liver

Adenomatous hyperplasia, often associated with human chronic hepatitis or liver cirrhosis, is a pre-neoplastic lesion, which may transform into hepatocellular carcinoma (HCC).<sup>1–5</sup> Human HCC is thus considered to develop in a multi-step fashion.<sup>6</sup> Although most HCCs receive arterial blood as demonstrated by positive staining in arterial angiography,<sup>7</sup> some remain negative.<sup>8</sup> Our recent clinicopathological study has demonstrated that well differentiated HCCs with a diameter of less than 2 cm include a substantial number of tumors which are fed by the portal venous system.<sup>9</sup> This fact suggests that the transition of blood supply from portal venous to arterial may take place at an early stage of the development of well differentiated HCC.

In the carcinogen-induced hepatocarcinogenesis of rats, we have histologically defined three types of hepatic nodules, i.e., hyperplastic nodules (HPN), atypical hyperplastic nodules (AHPN) and HCC, which correspond to human adenomatous hyperplasia, well differentiated HCC and poorly differentiated HCC, respectively.<sup>10</sup> In murine hepatic nodules as well, arterialization proceeds with the advance of stages of hepatocarcinogenesis from HPN to HCC.<sup>10</sup> In parallel with the progress of arterialization and stages, the microvasculature of hepatic nodules undergoes sequential ultrastructural changes from sinusoids to capil-

laries.<sup>8, 11</sup> Non-arterialized HPN show the normal features of hepatic sinusoids, while arterIALIZED HPN and AHPN exhibit transitional figures from sinusoids to capillaries, and HCCs usually have typical capillaries.

Recently, various kinds of angiogenesis inhibitors have been developed for the purpose of preventing hematogenous tumor metastases by interfering with angiogenesis, which is critical for the growth of tumors.<sup>12</sup> The angiogenesis inhibitor TNP-470, a semisynthetic analogue of fumagillin produced by *Aspergillus fumigatus*, has been demonstrated to suppress the growth and metastasis of various tumors including sarcoma, melanoma, lung cancer and colon cancer of mouse, rat or human origin<sup>13–20</sup> by the selective inhibition of vascular endothelial cell proliferation.<sup>21, 22</sup>

In the present study, in order to demonstrate the suppressive effect of TNP-470 on the development of primary hepatic nodules, we performed a morphometric analysis of chemical carcinogen-induced hepatic nodules in rats treated with or without TNP-470, with special reference to the difference in the effectiveness of TNP-470 among non-arterIALIZED HPN, arterIALIZED HPN, AHPN and HCC.

### MATERIALS AND METHODS

**Animals** Thirty specific-pathogen-free male F344 rats (5 weeks old) were purchased from Clea Japan Inc. (Shi-

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zuoka). They were housed at a constant temperature and fed with chow pellets and water *ad libitum*.

**Hepatocarcinogenesis** Experimental hepatocarcinogenesis was performed by using a modification of the procedure of Solt and Farber,<sup>23</sup> as described previously.<sup>10</sup> A single dose of 200 mg/kg b.w. of *N*-nitrosodiethylamine (Wako, Osaka) was injected intraperitoneally into rats at 6 weeks after birth. Rats were then subjected to two-thirds partial hepatectomy at 9 weeks under ether anesthesia. They were fed basal diet (Oriental EA, Tokyo) containing 0.02% 2-acetylaminofluorene (AAF; Tokyo Kasei, Tokyo) during 8–14 weeks of age, and killed at 26 weeks.

**Administration of TNP-470** TNP-470 [*O*-(chloroacetyl-carbamoyl)fumagillol] was obtained from Takeda Pharmaceutical Industries (Osaka). It was suspended in a vehicle composed of 1% ethanol plus 5% arabic gum in saline.

Animals were divided into three groups according to the protocol of TNP-470 administration. Ten rats were used for each group. Control group: animals received no TNP-470 throughout the experiment. Group A: TNP-470 was intraperitoneally injected at a dose of 30 mg/kg b.w. twice a week during 8–20 weeks. This dose is the same as used by Tanaka *et al.*<sup>19</sup> in the experimental hepatic metastasis of colon cancer. Group B: the same dose of TNP-470 as used in group A was administered twice a week during 14–26 weeks.

**Fixation of the liver and arterial infusion of ink** Under ether anesthesia, the liver was perfused with phosphate-buffered saline, pH 7.4, to flush out the intrahepatic blood. The abdominal portion of the aorta was ligated beyond the celiac artery for the efficient guidance of the perfusate into the hepatic artery.<sup>10</sup> Subsequently, the liver was perfused with 10% formalin via the portal vein for 2 min at the flow rate of 10 ml/min. At the same time, 10% formalin containing India ink (Pelikan, Hannover, Germany) was gradually infused via the thoracic aorta for 20 s. Hepatic nodules which mainly received the arterial blood were stained black, as identified macroscopically and light microscopically, while those supplied predominantly with the portal venous blood were not stained because the ink particles entering the nodules from the hepatic artery were quickly flushed out by the ink-free perfusate from the portal vein. After the perfusion-fixation, the liver was sliced at a thickness of 5 mm. The numbers of ink-stained and non-stained nodules with a diameter of larger than 1 mm were macroscopically counted on the surface of liver slices, and their diameters were measured.

**Histology** Formalin-fixed liver slices were dehydrated in ethanol series and embedded in paraffin. Sections were made from all the slices which were subjected to macroscopic examination, and stained with hematoxylin and eosin. The hepatic nodules were histologically classified into HPN, AHPN and HCC, as previously reported<sup>10</sup>: i) HPN showed mild hypercellularity, but exhibited neither

nuclear nor structural atypism; ii) AHPN consisted of pleomorphic tumor cells which showed both nuclear and structural atypism, being arranged in a pseudoacinar pattern; and iii) HCC showed a high degree of nuclear and structural atypism, of which the latter included pseudoacinar, glandular or trabecular arrangement of tumor cells.

De-paraffinized sections were incubated with diluted polyclonal antibody against human FVIII (1:3000, Dako, Kyoto)<sup>24</sup> and the reaction product was visualized by the avidin-biotin complex method using a Dako ABC kit (Dako). Non-immunized rabbit serum was substituted for the primary antibody in the negative controls.

**Statistical analysis** All data were expressed as the mean value $\pm$ SD. The results were analyzed by means of Student's *t* test. A *P*-value less than 0.05 was considered statistically significant.

## RESULTS

### Morphometric analysis of the hepatic nodules which developed in carcinogen-fed rats without TNP-470 treatment

Three types of hepatic nodules, i.e., HPN, AHPN and HCC, developed in the carcinogen-fed rats at 26 weeks. The average diameter of HCC [5.0 $\pm$ 3.0 mm (*n*=44)], was significantly (*P*<0.01) larger than that of AHPN, [3.5 $\pm$ 1.5 mm (*n*=63)], and the latter in turn was significantly (*P*<0.01) larger than that of HPN [2.5 $\pm$ 1.0 mm (*n*=65)]. Some HCCs reached 11 mm in diameter, but such large nodules were not found among HPN or AHPN.

Predominantly arterial-to-portal circulation and sinusoidal capillarization were determined by the positive staining of nodules with arterially infused ink (Fig. 1a) and with antibody against FVIII (Fig. 1b), respectively. With the advance of the stage of hepatocarcinogenesis from HPN to HCC, the percentage of ink-stained (or arterialized) nodules and that of FVIII-positive (or capillarized) ones increased from 63% to 86% and from 30% to 100%, respectively (Table I). Further analysis according to nodular size demonstrated that the percentages of ink- or FVIII-positive HPN and AHPN were low in small nodules and increased with the size, while those of HCC were high even in small nodules (Table I).

### Suppressive effect of TNP-470 on the development of hepatic nodules

The treatment with TNP-470 dramatically decreased the number of hepatic nodules observed macroscopically on the sliced surface of the liver (Fig. 2). Statistical analysis clearly demonstrated that there was significant difference in the number of nodules between TNP-470-treated and non-treated groups, and, furthermore, suppression was more prominent when TNP-470 was administered during the period of 8–20 weeks (group A) than during 14–26 weeks (group B) (Table II). When compared to ink-non-stained nodules, ink-stained ones

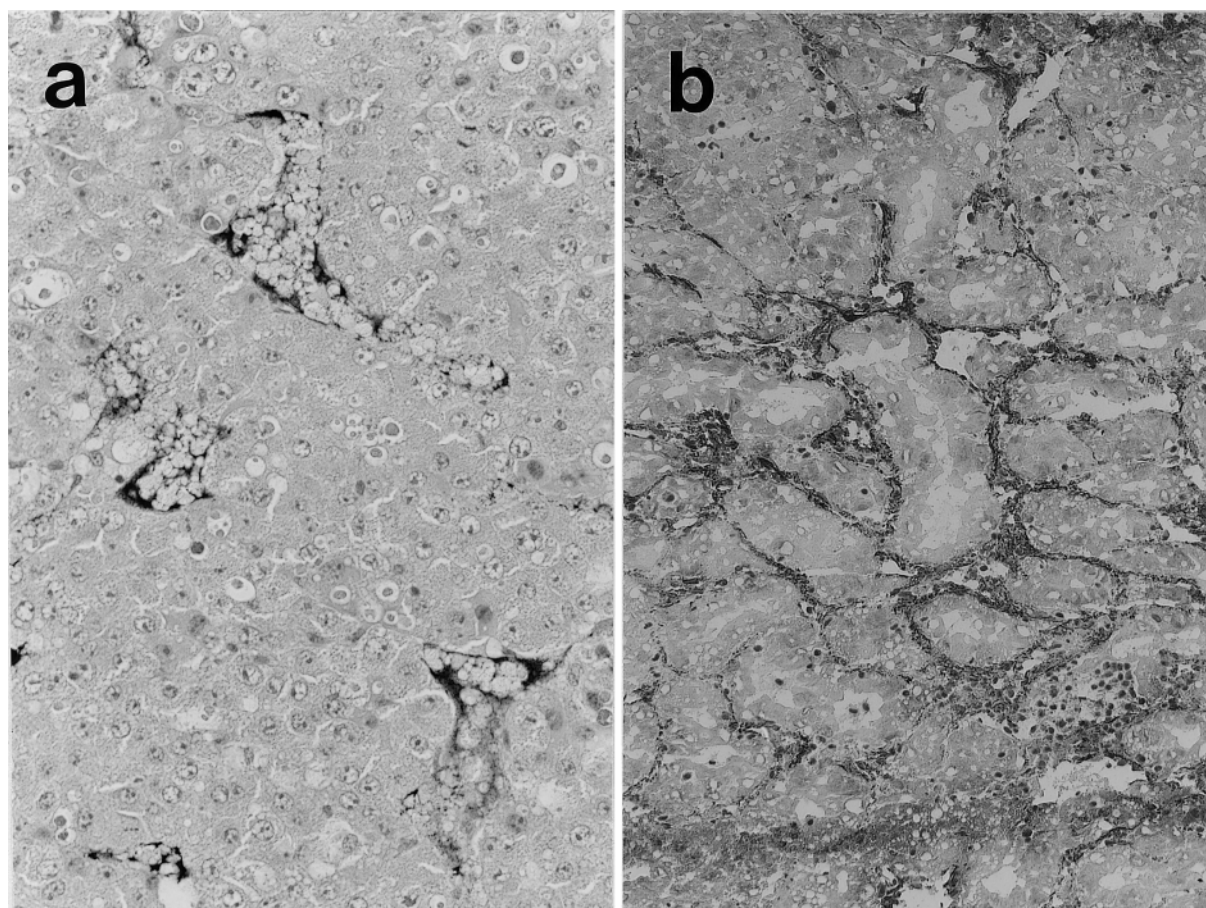


Fig. 1. a, Light micrograph of ink-stained HCC. Carbon particles are present in the sinusoids. a, HE stain.  $\times 60$ . b, Microvascular endothelial cells of HCC are positively stained for FVIII.  $\times 40$ .

Table I. Proportions of Ink-stained and FVIII-positive Nodules in Various-sized HPN, AHPN and HCC from Carcinogen-fed Rats without TNP-470 Treatment

Tumor	Ink-stained nodules <sup>a)</sup>				Total	FVIII-positive nodules <sup>b)</sup>				Total
	diameter (mm)					diameter (mm)				
	1 $\leq$ <2	2 $\leq$ <3	3 $\leq$ <4	4 $\leq$		1 $\leq$ <2	2 $\leq$ <3	3 $\leq$ <4	4 $\leq$	
	Number of positive nodules / number of total nodules (%)									
HPN	2/10 ( 20)	25/35 (71)	10/14 ( 71)	— <sup>c)</sup>	37/59 (63)	1/5 (20)	5/22 ( 23)	5/10 (50)	—	11/37 ( 30)
AHPN	0/4 ( 0)	10/17 (59)	16/19 ( 84)	19/23 (83)	45/63 (71)	—	2/7 ( 29)	3/7 (43)	12/15 ( 80)	17/29 ( 59)
HCC	4/4 (100)	13/16 (81)	5/5 (100)	16/19 (84)	38/44 (86)	—	8/8 (100)	—	11/11 (100)	19/19 (100)

a) Nodules were obtained from 10 livers.

b) Nodules were obtained from 5 livers.

c) —, analysis was not performed because the number of nodules was less than three.

were much more reduced in both groups (Table II). Further analysis of each of HPN, AHPN and HCC demonstrated that all types except ink-non-stained HPN showed

a significant decrease after the treatment with TNP-470 (Table II). [In the case of ink-non-stained HCC, it was difficult to evaluate the suppression because the number was

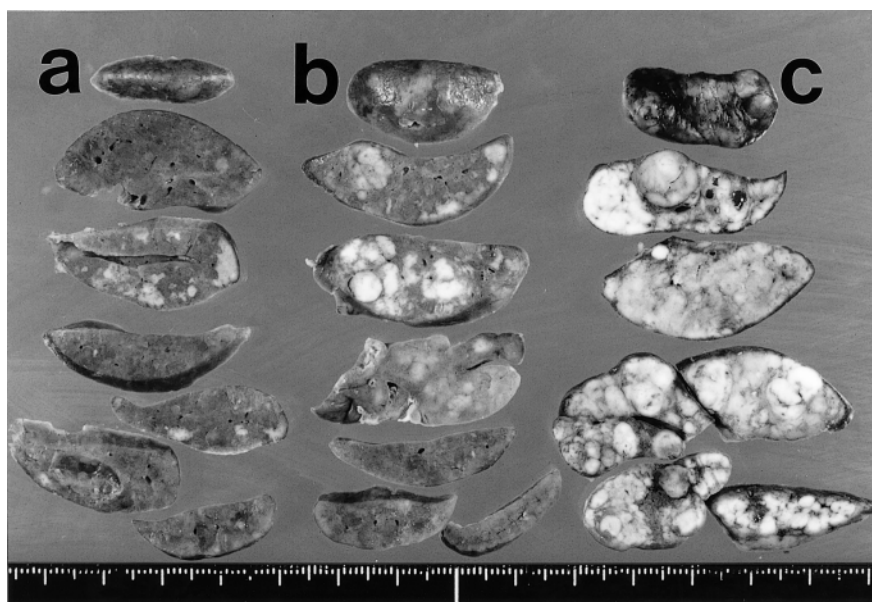


Fig. 2. Macroscopic view of the surface of a liver slice from group A (TNP-470 treatment during 8–20 weeks) (a), group B (TNP-470 treatment during 14–26 weeks) (b) and the control group (without TNP-470 treatment) (c). One minimal division in the scale at the bottom of the figure indicates 1.0 mm.

Table II. Numbers of Ink-stained and Non-stained HPN, AHPN and HCC per Liver in Control, Group B and Group A

Group	Total	Ink-stained nodules				Ink-non-stained nodules			
		HPN	AHPN	HCC	Total	HPN	AHPN	HCC	Total
Control (n=10)	17.0±3.1	4.0±2.1	3.9±1.6	3.8±2.2	11.7±3.6	2.4±2.2	2.3±1.2	0.6±1.0	5.3±2.2
Group B (n=10)	6.9±1.8*	0.8±0.9*	1.1±0.8*	0.2±0.4*	2.1±1.1*	3.4±1.1	1.1±0.8**	0.3±0.5	4.8±1.4
Group A (n=10)	2.9±1.0*	0.2±0.4*	0.3±0.5*	0	0.5±0.7*	2.1±1.3	0.3±0.5*	0	2.4±1.2*

Data represent mean value±SD. \*  $P < 0.01$ , \*\*  $P < 0.05$  vs. control. ( ) number of livers.

Table III. Numbers of Various-sized Nodules of HPN (ink -), HPN (ink +), AHPN and HCC in Control, Group B and Group A

Group	HPN (ink -)	HPN (ink +)	AHPN	HCC
Number of nodules [1≤ <2-2≤ <3-3≤ <4-4≤ mm in diameter]				
Control	8-11-3-2	2-26-11-1	4-17-19-23	4-16-5-19
Group B	5-11-11-7	0-2-0-5	0-2-11-2	0-0-2-3
Group A	4-4-10-3	0-0-2-0	0-2-2-2	0-0-0-0

The data are the sum of values from 10 rats for each group.

very small even without TNP-470 treatment.] The ratio of HPN:AHPN:HCC calculated from the data of Table II was 38:36:26 in the control group, 61:32:7 in group B and 79:21:0 in group A, indicating that the suppressive effect of TNP-470 becomes larger as the stage advances from

HPN to HCC. In the nodules other than ink-non-stained HPN, suppression by TNP-470 was observed equally in every size of nodules (Table III).

Similarly, the number of FVIII-positive nodules per liver was decreased more markedly by TNP-470 treatment

Table IV. Numbers of Ink(+)-FVIII(+), Ink(+)-FVIII(-), Ink(-)-FVIII(+) and Ink(-)-FVIII(-) Nodules in Control, Group B and Group A

Group	Ink(+)-FVIII(+)	Ink(+)-FVIII(-)	Ink(-)-FVIII(+)	Ink(-)-FVIII(-)
	Number of nodules [Total (HPN, AHPN, HCC)]			
Control	49 (11, 15, 23)	19 (11, 8, 0)	5 (3, 2, 0)	22 (16, 6, 0)
Group B	4 (2, 1, 1)	6 (4, 2, 0)	4 (3, 1, 0)	19 (14, 3, 2)
Group A	1 (0, 1, 0)	0 (0, 0, 0)	1 (0, 1, 0)	11 (10, 1, 0)

The data are the sum of values from 5 rats for each group.

than was that of negative ones; the former decreased from  $11.0 \pm 3.0$  ( $n=5$ ; control group) to  $1.7 \pm 1.0$  ( $n=5$ ; group B) or  $0.3 \pm 0.1$  ( $n=5$ ; group A), whereas the latter decreased from  $8.0 \pm 3.0$  ( $n=5$ ; control group) to  $4.0 \pm 2.0$  ( $n=5$ ; group B) or  $2.0 \pm 0.1$  ( $n=5$ ; group A).

Statistical analysis of hepatic nodules base on the combination of ink staining and FVIII expression assay demonstrated that ink(+)-FVIII(+) nodules were significantly ( $P < 0.001$ ) suppressed by TNP-470 treatment relative to ink(-)-FVIII(-) ones; in the former nodules, all types of HPN, AHPN and HCC was almost completely suppressed (Table IV). Between ink(+)-FVIII(+) and ink(-)-FVIII(-) nodules there were two intermediate types, i.e., ink(+)-FVIII(-) and ink(-)-FVIII(+) nodules, of which the former was more frequently observed than the latter (Table IV). Ink(+)-FVIII(-) nodules appeared to be more affected by TNP-470 treatment than ink(-)-FVIII(+) ones.

## DISCUSSION

A potent angiogenesis inhibitor TNP-470 inhibits the proliferation of vascular endothelial cells without affecting other kinds of cells, except fibroblasts, by preventing the cells from entering the G1 phase of the cell cycle.<sup>25</sup> It has been demonstrated in experimental animals that systemic administration of TNP-470 suppresses tumor metastasis and growth by the inhibition of angiogenesis.<sup>13-20</sup>

The present study has for the first time revealed that this agent significantly suppresses the development of primary hepatic nodules that are induced by chemical carcinogen intake and partial hepatectomy. Among the hepatic nodules, ink-stained ones were more susceptible to the suppressive effect of TNP-470 than non-stained ones. To elucidate the reason for the difference between stained and non-stained nodules, further analysis was done for each of HPN, AHPN and HCC. It was found that ink-non-stained HPN was not affected at all by TNP-470 treatment, unlike other types of nodules, including ink-stained HPN. We previously observed by electron microscopy that the microvessels of ink-non-stained HPN were very similar to hepatic sinusoids, being lined by fenestrated endothelial cells with incomplete basement membrane, while those of

ink-stained HPN and AHPN were structurally altered sinusoids consisting of variously defenestrated endothelial cells and continuous basement membranes, and those of HCC were usually typical capillaries with non-fenestrated endothelial cells and one or more layers of thick continuous basement membranes.<sup>10</sup> Thus, the present finding indicates that TNP-470 suppresses the development of hepatic nodules which have either structurally modified sinusoids or capillaries, while it does not affect the development of the nodules with normal sinusoids. This interpretation is supported by the data showing that TNP-470 does not inhibit the multiplication of normal sinusoidal endothelial cells after partial hepatectomy in rats (Ikebe *et al.*, unpublished data).

The mechanism of sinusoidal transformation into capillaries is not fully understood. The present finding of a higher incidence of ink(+)-FVIII(-) nodules compared to ink(-)-FVIII(+) ones indicates that the former is the main transitional form from ink(-)-FVIII(-) nodules into ink(+)-FVIII(+) ones, suggesting that arterialization may precede capillarization. We previously observed that arterialization of liver lobes after portal branch ligation induces the disappearance of fenestrae of sinusoidal endothelial cells (Ikeda *et al.*, unpublished data). The predominantly arterial-to-portal blood supply into hepatic nodules is thus assumed to play a role in inducing the structural alteration of sinusoidal endothelial cells.

The mechanism of the arterialization of nodules is also unresolved. In the present study, both arterialization and capillarization developed as the size of HPN and AHPN increased, in accordance with previous findings that carcinogen-induced hepatic nodules became increasingly dependent on the hepatic artery as they grow.<sup>26, 27</sup> In contrast, HCC seems to be arterialized and capillarized from an early stage of growth, suggesting that there might be different mechanisms for the acquisition of predominantly arterial-to-portal circulation and sinusoidal capillarization between HPN/AHPN and HCC.

In the present study, we have analyzed hepatic nodules larger than 1 mm in diameter to examine the relationship of their histological type and ink-staining to the suppression of development. In all the nodules except ink-non-

stained HPN, not only large-sized nodules, but also small ones were significantly decreased in number by the treatment with TNP-470. This fact suggests that, in addition to the inhibitory effect on angiogenesis, a suppressive effect on the very early process of hepatic transformation might be also involved in the suppression of hepatic primary nodules by TNP-470. Two theories have been proposed to explain the mechanism of hepatocarcinogenesis. One is the clonal expansion of transformed hepatocytes.<sup>28)</sup> Altered cell foci resulting from the clonal growth of glutathione *S*-transferase placental form (GST-P)-positive hepatocytes appear in the post-initiation stage of hepatocarcinogenesis.<sup>29, 30)</sup> To elucidate the action of TNP-470 in the early phase of hepatocarcinogenesis, the numerical changes of GST-P-positive foci after TNP-470 treatment need to be further investigated. It has been demonstrated that the frequency of altered liver cell foci induced by the administration of diethylnitrosamine and phenobarbital is

consistent with that of liver neoplasms, which appear in the late phase.<sup>31)</sup>

Another theory is the aberrant differentiation and proliferation of liver stem cells referred to as oval cells.<sup>32)</sup> Oval cells undergo extensive proliferation after hepatectomy under the conditions of AAF-induced blocking of hepatocyte proliferation.<sup>33)</sup> In the present experiment, suppression was more prominent when TNP-470 was administered during the period of AAF feeding and partial hepatectomy (group A) than it was when the administration was done after the termination of AAF feeding (group B), suggesting that the inhibition of angiogenesis by TNP-470 during oval cell proliferation may be critical for suppressive effects on the development of pre-neoplastic or neoplastic nodules.

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