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# Absence of Kupffer cells in carcinogen induced liver hyperplastic nodules: demonstration by intravenous injection of indian ink.\*

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## Abstract

Absence of Kupffer cells in rat liver hyperplastic nodules induced by a chemical carcinogen was demonstrated by intravenous injection of indian ink. Hyperplastic nodules appeared 4 weeks after diethylnitrosamine (DEN) was administered, and the nodules continued growing and became eosinophilic hyperplastic nodules after 5 to 6 weeks. After intravenous injection of indian ink, hyperplastic nodules were observed as carbon-free white nodules, which were macroscopically distinguishable from the black surrounding tissue. As observed by light microscopy, Kupffer cells were absent in hyperplastic nodules in contrast to being present in the surrounding tissue. Scanning electron microscopy confirmed these findings and furthermore revealed that the sinusoidal endothelium of hyperplastic nodules had no fenestrae. Injection of indian ink is a useful method for delineation and enucleation of hyperplastic nodules in the study of morphological and chemical changes of nodules.

**KEYWORDS:** liver hyperplastic nodule, kupffer cell, chemical carcinogenesis, indian ink

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## **ABSENCE OF KUPFFER CELLS IN CARCINOGEN INDUCED LIVER HYPERPLASTIC NODULES: DEMONSTRATION BY INTRAVENOUS INJECTION OF INDIAN INK**

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*Abstract.* Absence of Kupffer cells in rat liver hyperplastic nodules induced by a chemical carcinogen was demonstrated by intravenous injection of indian ink. Hyperplastic nodules appeared 4 weeks after diethylnitrosamine (DEN) was administered, and the nodules continued growing and became eosinophilic hyperplastic nodules after 5 to 6 weeks. After intravenous injection of indian ink, hyperplastic nodules were observed as carbon-free white nodules, which were macroscopically distinguishable from the black surrounding tissue. As observed by light microscopy, Kupffer cells were absent in hyperplastic nodules in contrast to being present in the surrounding tissue. Scanning electron microscopy confirmed these findings and furthermore revealed that the sinusoidal endothelium of hyperplastic nodules had no fenestrae. Injection of indian ink is a useful method for delineation and enucleation of hyperplastic nodules in the study of morphological and chemical changes of nodules.

*Key words:* liver hyperplastic nodule, Kupffer cell, chemical carcinogenesis, indian ink.

Liver hyperplastic nodules (HN) are considered to precede the appearance of hepatocellular carcinoma in experimental carcinogenesis (1-4). Parenchymal cells in HN have been well studied morphologically (2, 3, 5, 6) though the mesenchymal cells in HN have not been described in detail (2, 3).

In this report, the absence of Kupffer cells in HN are revealed by intravenous injection of indian ink and confirmed by scanning electron microscopy (SEM).

### **MATERIALS AND METHODS**

*Hyperplastic nodules.* Liver hyperplastic nodules were induced according to the methods of Solt and Farber (4). Male Fischer-344 rats (Charles River Japan Inc., Atsugi, Japan) weighing 150 to 160 gm were maintained on a basal diet (Oriental Yeast Co. Ltd., Tokyo, Japan). The experimental schedule is shown in Fig. 1. Rats were given diethylnitrosamine

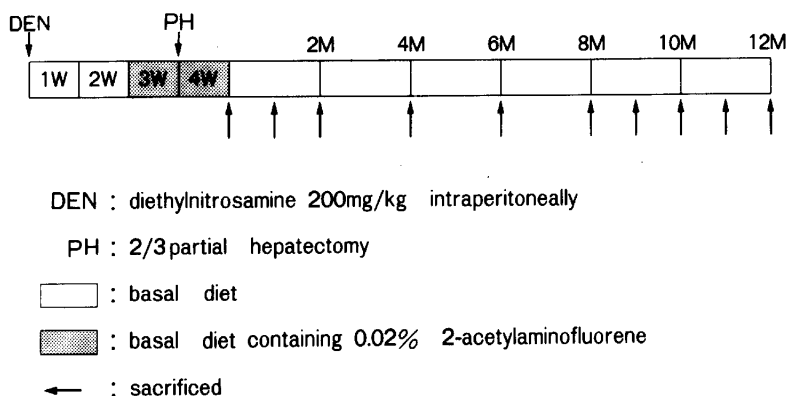


Fig. 1. Experimental schedule.

(DEN) intraperitoneally at 200 mg/kg body weight and were maintained on the basal diet for 2 weeks, after which they were placed on the basal diet containing, in addition, 0.02 % 2-acetylaminofluorene (2-AAF) for the next 2 weeks. They were subjected to 2/3 partial hepatectomy (7) 3 weeks after DEN administration. Three rats were sacrificed at the times shown by arrows in Fig. 1.

*Macroscopic observation, light microscopy and SEM.* One ml of 10 % indian ink (Rotringwerke, Hamburg, West Germany) in physiological saline was injected into the tail vein under ether anesthesia. Ten minutes later, 0.5 ml (500 units) of heparin was injected into the heart to prevent blood coagulation. The abdomen was opened and the liver was observed macroscopically. Then, rats were decapitated. After removal of the anterior chest wall, a polyethylene tube was inserted downwards into the thoracic aorta. The liver was irrigated through the aorta cannula with 500 ml of Ringer solution and perfusion-fixed with 1% glutaraldehyde in 0.1 M phosphate buffer at a pressure of about 200 cm H<sub>2</sub>O. After fixation, the liver was cut into blocks. One of the blocks was used for light microscopy (LM). The other blocks were further fixed in glutaraldehyde overnight for SEM. The SEM blocks were then conductively stained by the revised tannin-osmium method (8), dehydrated in a graded series of ethanol, and dried in an HCP-2 critical point dryer (Hitachi) using liquid carbon dioxide. The dried specimens were fractured with a scalpel under a stereomicroscope (9). The fractured specimens were observed under SEM (Hitachi, HFS-2; JEOL, JSM-U3) at an accelerating voltage of 15 to 20 kV after thin sputter coating with platinum-palladium using an Eiko IB-3 ion coater. For LM, parts of the perfusion-fixed specimens were transferred to 10 % formalin, embedded in paraffin, sectioned 5  $\mu$ m thick and stained with hematoxylin-eosin.

## RESULTS

*Macroscopic observation.* Grayish-white nodules about 1 mm in diameter appeared on the surface of the liver after perfusion-fixation in samples taken 4 weeks after DEN administration. At 6 weeks, HN became larger and were almost uniform in size (2-3 mm in diameter). After 8 weeks, HN were of various sizes (1-5 mm in diameter). HN were observed as carbon-free white lesions which were clearly distinguishable from the surrounding black lesions after injection of indian

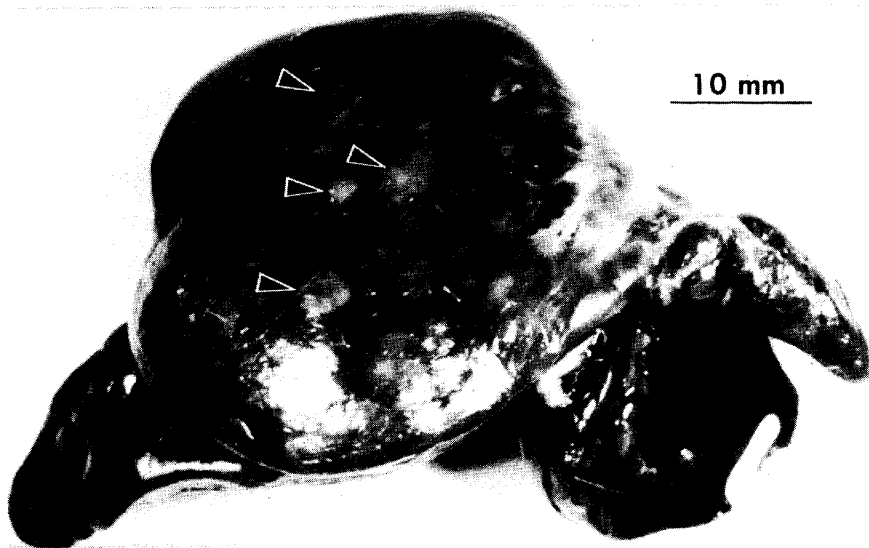


Fig. 2. A rat liver 4 months after DEN administration. White hyperplastic nodules free of indian ink (arrow heads) are clearly distinguishable from the black surrounding tissue.

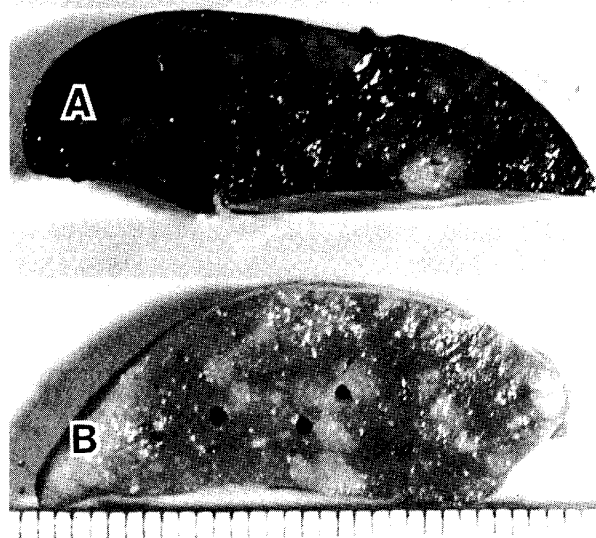


Fig. 3. Cut surface of the liver. Hyperplastic nodules 1 to 5 mm in diameter are devoid of indian ink (A) and more clearly distinguishable from the surrounding tissue than those of rats not injected with indian ink (B).

ink into the tail vein (Figs. 2, 3).

*LM and SEM.* Four weeks after DEN administration, HN were composed

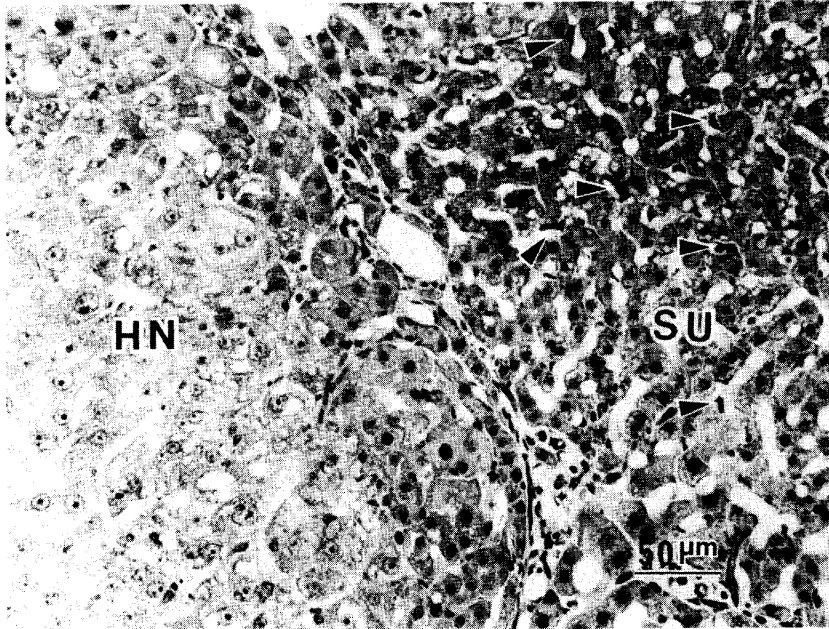


Fig. 4. Light micrograph of a border area of a hyperplastic nodule (HN) 4 months after DEN administration. The hyperplastic nodule compresses normal appearing surrounding tissue (SU). Note the absence of Kupffer cells in the hyperplastic nodule in comparison with numerous Kupffer cells phagocytizing carbon particles in the surrounding tissue (arrow heads). Hematoxylin-eosin stain.

of enlarged hepatocytes 15-35  $\mu\text{m}$  in diameter with basophilic cytoplasm. After 6 weeks, HN were composed of hepatocytes (15-30  $\mu\text{m}$ ) with eosinophilic cytoplasm and often arranged in two-cell-thick plates. Sinusoids in HN were compressed by the enlarged hepatocytes (Fig. 4). Surrounding tissue had atrophic and eosinophilic hepatocytes at 4 weeks, but after 6 weeks, they had neutrophilic hepatocytes (15-20  $\mu\text{m}$ ) and were arranged in one-cell-thick plates (Fig. 4).

Kupffer cells, confirmed by phagocytized carbon particles in their cytoplasm, were observed in the surrounding tissue, but were absent in HN throughout the nodule stage (Fig. 4). By SEM, sinusoidal endothelia were free of their fenestrae (Fig. 5), and Kupffer cells were absent in the HN sinusoids.

#### DISCUSSION

In this study, absence of Kupffer cells in HN was demonstrated by injection of indian ink. Macroscopically, grayish-white HN were clearly distinguishable from the black surrounding tissue (Figs. 2, 3). The procedure makes it easy to enucleate HN for morphological and chemical examinations. Furthermore, it is easy to count the number of HN without any special staining. Microscopically, Kupffer cells could be observed by their phagocytized carbon particles in surrounding tissue,

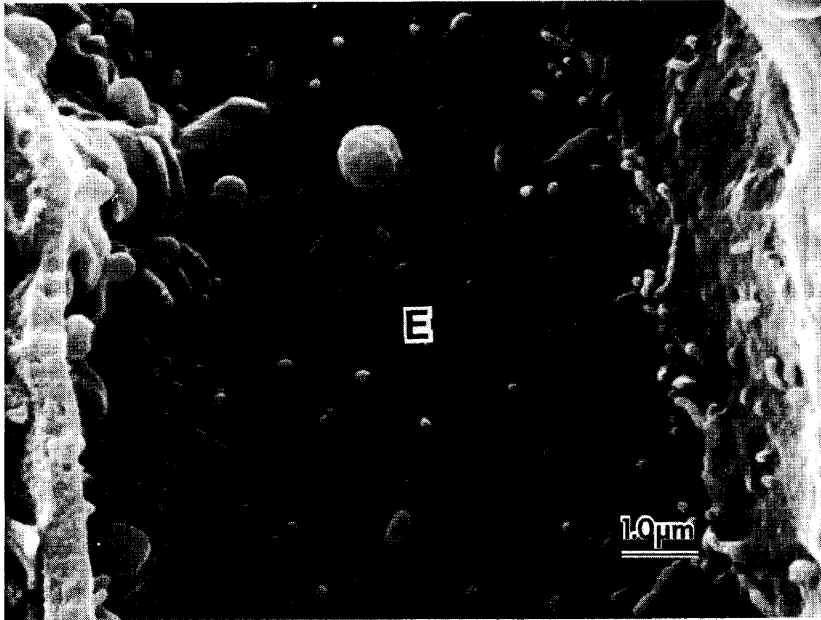


Fig. 5. SEM micrograph of sinusoidal endothelium (E) in a hyperplastic nodule. Sinusoidal endothelium has no fenestrae as indicated by capillarization.

but in contrast, they were not observed in HN (Fig. 4). Kupffer cells were absent in HN throughout the entire HN stage.

SEM confirmed microscopic findings that Kupffer cells were absent in HN in contrast to being present in the normal sinusoids. Sinusoidal endothelia had no fenestration in HN, which is seen as capillarization under SEM (Fig. 5), whereas they had normal fenestrae in the surrounding tissue (10, 11).

In contrast to our study, Reuber has described the presence of Kupffer cells demonstrated by phagocytized carbon particles of indian ink in HN induced by 2-AAF (3), and also Farber has mentioned briefly their presence in HN induced by ethionine by LM (2). Absence of Kupffer cells in HN may depend on the method by which HN is induced and on capillarization of the sinusoid.

It is well known that hepatocellular carcinoma (12) and liver cell adenoma (13) have no Kupffer cells. Absence of Kupffer cells and capillarization of sinusoids may offer beneficial circumstances for selective growth of HN to hepatocellular carcinoma. It is unknown what role Kupffer cells play in the development of HN. The question why Kupffer cells disappear in HN remains to be answered.

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