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Abstract

The three-dimensional arrangement of ductular structures formed by oval cells in rats fed 2-acetylaminofluorene (2-AAF) was studied by scanning electron microscopy (SEM) of biliary tract casts and light microscopy of sections of liver injected with india ink via the biliary tract. Both resin and india ink were well injected up to bile ductules, and the findings of each method correlated with each other. By the second week after 2-AAF administration, a few oval cells appeared in the periportal areas forming ductular structures which connected with the portal bile ducts. At the 4th week, increased ductular structures occupied two thirds of the lobule and formed networks communicating with each other, and with the portal bile ducts. At the 8th week, such ductular structures were compressed around hyperplastic nodules and appeared like a basket in biliary casts examined by SEM. Although a histochemical study of gamma-glutamyl transpeptidase revealed activity both on the luminal side of the ductular structures and hepatocytes in hyperplastic nodules, no transition was observed between these two cell populations. These results suggest that oval cells have characteristics more similar to those of biliary epithelia than of hepatocytes, and have no relation to the development of hyperplastic nodules.

KEYWORDS: oval cells, biliary tract casts, scanning electron microscopy, hyperplastic nodules, hepatocarcinogenesis

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Three-Dimensional Arrangement of Ductular Structures Formed by Oval Cells during Hepatocarcinogenesis

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The three-dimensional arrangement of ductular structures formed by oval cells in rats fed 2-acetylaminofluorene (2-AAF) was studied by scanning electron microscopy (SEM) of biliary tract casts and light microscopy of sections of liver injected with india ink via the biliary tract. Both resin and india ink were well injected up to bile ductules, and the findings of each method correlated with each other. By the second week after 2-AAF administration, a few oval cells appeared in the periportal areas forming ductular structures which connected with the portal bile ducts. At the 4th week, increased ductular structures occupied two thirds of the lobule and formed networks communicating with each other, and with the portal bile ducts. At the 8th week, such ductular structures were compressed around hyperplastic nodules and appeared like a basket in biliary casts examined by SEM. Although a histochemical study of γ-glutamyl transpeptidase revealed activity both on the luminal side of the ductular structures and hepatocytes in hyperplastic nodules, no transition was observed between these two cell populations. These results suggest that oval cells have characteristics more similar to those of biliary epithelia than of hepatocytes, and have no relation to the development of hyperplastic nodules.

Key words : **oval cells, biliary tract casts, scanning electron microscopy, hyperplastic nodules, hepatocarcinogenesis**

Although proliferation of oval cells is a common event in the early phase of chemical hepatocarcinogenesis (1-5), the origin and fate of oval cells are controversial. Two opposing opinions on their origin have been reported. One is that oval cells arise from biliary epithelia (3, 6-8), and the other is that some of them are derived from stem cells which are capable of developing into both hepatocytes and biliary epithelia (9-11). Morphological studies support the former because of the similarity between oval cells and biliary epithelia (2, 6, 12-14). On the other hand, histochemical studies support the latter in that albumin and α-fetoprotein (AFP) are shown in proliferating oval cells (9, 15). The fate of oval cells is also unclarified. Autoradiographic studies showed in a carcinogenesis model employing 3'-methyl-4-dimethylaminoazobenzene as the carcinogen that tritium-thymidine, at first labeled in nuclei of oval cells, was observed in nuclei of hepatocytes after a few mitoses, suggesting that oval cells develop into

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hepatocytes (16). However, neither production of AFP nor transition into hepatocytes was reported in oval cells induced by α-naphthylisothiocyanate (17).

In this paper, the three-dimensional arrangement of ductular structures formed by oval cells is described with special reference to the relation of the structures to preexisting portal bile ducts. Scanning electron microscopy (SEM) of biliary tract casts is a useful method for studying the three-dimensional structure of the biliary tract (18, 19), and it was applied in this study in conjunction with retrograde injection of india ink into the biliary tract. Furthermore, γ-glutamyl transpeptidase histochemistry was utilized to identify oval cells (20, 21) and to study their relation to hyperplastic nodules.

Materials and Methods

Animals. Thirty-two male Fischer-344 rats weighing from 160 to 180 g were divided into two groups. The control group of 8 rats was fed a basal diet (CE-2, Clea Japan Inc., Tokyo) alone, and the other group of 24 rats was fed a basal diet containing 0.02% 2-acetylaminofluorene (2-AAF). One control and three 2-AAF rats were sacrificed at the end of every week from the first to 8th week. Animals were given food and water *ad libitum* with a 12-h light and dark cycle.

Histochemistry for γ-glutamyl transpeptidase (GGT). GGT histochemistry was performed according to the method of Rutenburg *et al.* (22) with slight modification. The liver tissue was cut 5 to 7 mm in thickness, fixed in ice-cold acetone overnight and embedded in paraffin. The sections were cut into 6-micron sections and incubated in freshly prepared medium containing γ-glutamyl-4-methoxy-2-naphthylamide (Sigma, USA) as a substrate and fast blue BB salt (diazotized 4'-amino-2', 5'-diethoxybenzanilide, Sigma, USA) as a coupling agent.

Biliary tract casts and india ink injection. Under anesthesia by intraperitoneal injection of sodium pentobarbital (50 mg per kg body weight), the common bile duct was cannulated with a polyethylene tubing (PE-50, Becton Dickinson, Labware, Lincoln Park, NJ, USA). The liver was perfused with Ringer solution through a catheter inserted retrograde into the abdominal aorta below the renal arteries. Just after starting the perfusion, the aorta was clamped above the celiac artery and the inferior vena cava was cut above the diaphragma. For making biliary tract casts, a resin of relatively low viscosity, prepared by mixing one part of Mercox (Oken Shoji Co., Ltd., Japan) with one or two parts of methylmethacrylate monomer (Wako Pure Chemical Ind., Ltd., Osaka, Japan), was injected into the common bile duct keeping the pressure under 14 mmHg. The liver was immersed in a water bath at 60°C, and the tissue was macerated in a 30% NaOH solution. The biliary tract casts were washed gently in distilled water, freeze-dried and trimmed under a stereomicroscope. Small specimens were mounted on metal stubs, sputter-coated with gold and observed in a scanning electron microscope (JSM-U3, JEOL Inc., Tokyo, Japan) with an accelerating voltage of 5 kV. By tilting the stage, stereopairs of scanning electron micrographs were taken for three-dimensional analysis.

India ink was injected retrograde into the common bile duct, as was the resin, and the liver was perfusion-fixed with 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The liver was cut into small pieces, dehydrated through a graded series of ethanols, embedded in paraffin and sectioned both 6 μm and 1 mm in thickness. Six-micron sections were stained with hematoxylin and eosin and examined by light microscopy. One-millimeter-thick sections were dehydrated through ethanol, immersed in methylsalicylate to make them transparent and observed under a light microscope.

Results

Histological findings. By the second week after 2-AAF treatment, a small number of oval cells with spindle shaped nuclei and scanty cytoplasm were observed in the periportal area. They were situated just next to the portal tract and radiated toward

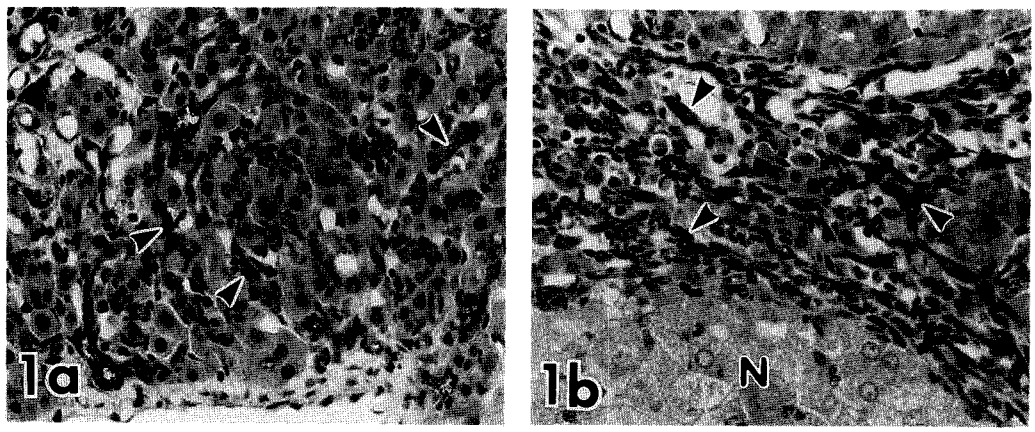

Fig. 1 Light micrographs of rat liver at the 4th (a) and 8th (b) week after the start of 2-acetylaminofluorene feeding. (a) Oval cells form ductular structures in which injected india ink (arrowheads) is observed. H-E stain, ×250. (b) Oval cells are compressed between the hyperplastic nodules (N). India ink is also observed in the lumen (arrowheads). H-E stain, ×280.

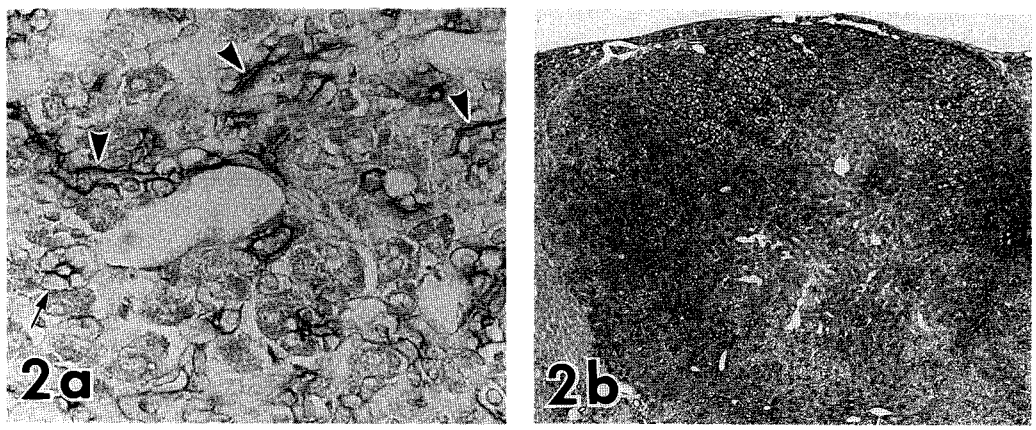

Fig. 2 Histochemical demonstration of γ-glutamyl transpeptidase (GGT) activity in rat liver at the 4th (a) and 8th (b) week after the start of 2-acetylaminofluorene feeding. (a) GGT activity is confined to the luminal sides of the portal bile duct (arrow) and oval cells (arrowheads). ×500. (b) GGT positive hyperplastic nodule. ×40.

the lobule. By the 4th week, they increased in number and occupied about two thirds of the lobule. Some of them formed ductular structures in which india ink was observed (Fig. 1a). At the 6th week, hyperplastic nodules composed of large cells with prominent nuclei and eosinophilic cytoplasm appeared and compressed oval cells and preexisting hepatocytes. By the 8th week, hyperplastic nodules increased in number, and oval cells were compressed between the nodules (Fig. 1b).

GGT histochemistry. In control rats, GGT activity was localized in the luminal side of the portal bile duct epithelia. Hepatocytes showed no activity. In 2-AAF rats, the luminal side of oval cells (Fig. 2a) and cell membranes of hyperplastic nodules showed positive activity (Fig. 2b). Sequential studies showed that oval cells and GGT positive hepatocytes were discrete lesions and no transition was observed between these

two cell populations.

Biliary tract casts and india ink injection. Resin and india ink were injected well into the intrahepatic biliary tract up to bile ductules. Some india ink was observed in the lumina of periportal bile canaliculi. SEM of biliary tract casts allowed three-dimensional observation of the biliary system, while light microscopy of transparent india ink injected samples transparency supplemented understanding of the relationship between the biliary tract and other portal and parenchymal structures.

Control biliary tract casts appeared as described before (19). At the second week of 2-AAF treatment, small side branches were observed radiating from the portal bile duct into the lobule (Fig. 4a). These side branches, about 10 μm in diameter, corresponded to ductular structures formed by oval cells when compared with the specimens of india ink injected liver (Fig. 3a). Ductular structures were observed as tortuous channels which connected with the portal bile ducts when biliary casts were observed by SEM. A ductular plexus (19) in the large portal tract also radiated such side branches. At the 4th week, these side branches increased in number and formed inter-communicating networks around the portal bile duct (Fig. 4b). However, at the 8th week, such anastomosing networks were compressed around hyperplastic nodules and appeared like a basket (Figs. 4c, d). Resin and india ink were not observed in the hyperplastic nodules (Figs. 3b, 4c).

Discussion

Oval cell proliferation is commonly observed during the administration of various hepatocarcinogens (1-5). Sequential stages from early oval cell proliferation to late hyperplastic nodule formation have been followed in a model using 2-AAF as the hepatocarcinogen (4). This study demonstrated that oval cells arose from the periportal area, extended into the lobule, and formed ductular structures which were revealed by SEM of biliary casts to connect with pre-existing portal bile ducts (Fig. 4a). Massive proliferation of oval cells ceased after the 4th week, and oval cells were compressed around hyperplastic nodules after the 6th week, when hepatocytes resistant to 2-AAF proliferated and hyperplastic nodules appeared (Figs. 1b, 4c). A histochemical study showed no transitional stages between oval cells and hepatocytes in hyperplastic nodules. These results suggest that oval cells have characteristics more similar to those of biliary epithelia than of hepatocytes.

The appearance of injected materials in ductular lumina is in agreement with the results of the india ink injection study of ethionine carcinogenesis by Popper *et al.* (23) and those of the barium gelatin injection study of a carcinogenesis model employing a choline deficient diet and 2-AAF treatment by Dunsford *et al.* (24). SEM of biliary casts clearly demonstrates the three-dimensional arrangement of ductular structures formed by oval cells, which had been difficult to demonstrate in two-dimensional studies by light microscopy. These ductular structures formed tortuous networks continuous with the pre-existing portal bile ducts.

The origin of oval cells remains unclarified. Many morphological studies including this study favor the biliary epithelial origin since oval cells have structures similar to those of biliary epithelia (3, 6-8). It appears that portal bile duct epithelia may not be involved in the proliferation of oval cells because early proliferation starts in the periportal area. The facts that oval cells appear in the periportal area and have bile ductular characteristics suggest that they

might arise from the ductular cells forming the canal of Hering, which is made up partly of hepatocytes and partly of ductular cells (25, 26). A recent histochemical study on carbohydrate residues of cell membranes suggests that biliary epithelia may have different characteristics in bile ducts of different sizes (27). Specific lectin binding of oval cells is under study.

The fate of oval cells is another controversial matter. Our histochemical study for GGT showed positive activity in oval cells and hepatocytes in hyperplastic nodules as reported by others (20, 21). This GGT staining affords discrimination of oval cells and hepatocytes in hyperplastic nodules from other hepatocytes. Our sequential histochemical study shows that oval cells and GGT positive hepatocytes are located in discrete lesions, and that no transition was observed between them. Furthermore, oval cells stop proliferation when hepatocytes resistant to 2-AAF start to proliferate and hyperplastic nodules appear. These results favor the concept that oval cell proliferation is a reactive response to treatment with chemical carcinogens and is not a stage of transformation to hepatocytes or hepatocellular carcinomas. However, the mechanism of their proliferation is still unknown. Metabolites of chemical carcinogens or hepatic injury by carcinogens may stimulate proliferation of oval cells.

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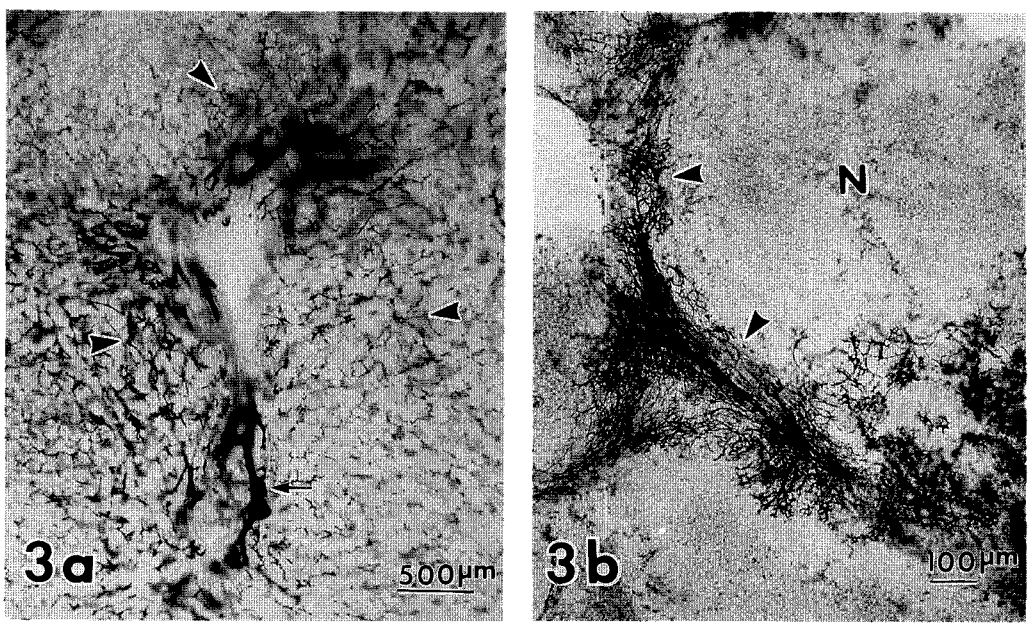
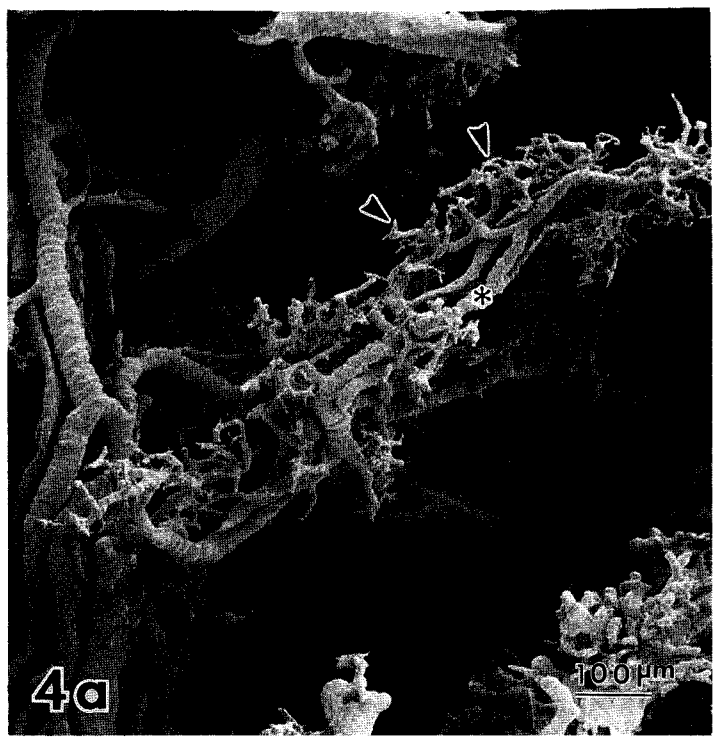


Fig. 3 Light micrographs of transparent thick sections of india ink injected rat liver at the 4th (a) and 8th (b) week after the start of 2-acetylaminofluorene feeding. (a) India ink is observed in both portal bile ducts (arrow) and ductular structures formed by oval cells (arrowheads). ×200. (b) The ductular structures (arrowheads) are compressed around the hyperplastic nodules (N). ×66.



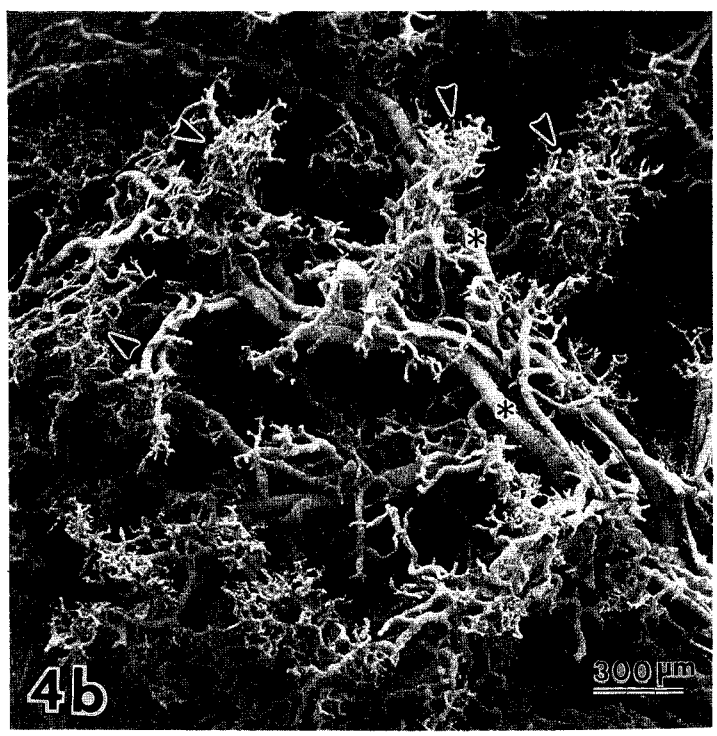


Fig. 4 Scanning electron micrographs of biliary tract casts of 2-acetylaminofluorene-fed rat liver. (a) At the 2nd week, a few side branches (arrowheads) are observed radiating from the portal bile ducts (*). ×100. (b) At the 4th week, numerous side branches anastomose with each other and form networks (arrowheads). Portal bile ducts (*). ×40. (c) At the 8th week, anastomosing networks are compressed around the hyperplastic nodules (arrowheads). ×50 (d) Higher magnification of anastomosing networks (arrowheads). ×150.


4c
200μm


4d
100μm

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