CHOLANGIOFIBROSIS INDUCED BY SHORT-TERM FEEDING OF 3'-METHYL-4-(DIMETHYLAMINO)AZOBENZENE: AN ELECTRON MICROSCOPIC OBSERVATION

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The ultrastructure of cholangiofibrosis in a rat liver induced by short-term feeding with 3'-methyl-4-(dimethylamino)azobenzene (3'-Me-DAB) was investigated. The feeding of 3'-Me-DAB for as short a period as 2 weeks was sufficient to induce cholangiofibrosis. After cessation of the feeding of the carcinogen, lesions of cholangiofibrosis continued to grow for up to 25 weeks. Hyalinization of the lesions was seen from 8 weeks after stopping of the dye feeding. However, the newly proliferated lining epithelial cells at the peripheral zone of the hyalinized lesions could always be seen. The ultrastructure of the epithelial lining cells in the lesion of cholangiofibrosis showed a typical intestinal cell metaplasia including goblet cells, enterochromaffin cells, and Paneth cells. A variation in the fine structure of the striated border was often observed.

The first precise description of cholangiofibrosis appeared in the report by Edwards and White3) in 1941. They found areas of cholangiofibrosis in the liver of rats 60 days after the initiation of a diet containing 4-(dimethylamino)azobenzene (DAB). The lesion consisted of epithelial lined, mucus-containing, glandular structures surrounded by connective tissue. Since then numerous investigations have been published on the lesion induced by several hepatocarcinogens.1,12) Until recently, however, there have been no systematic morphological approaches made on the ultrastructural level of this lesion. Furthermore, in many studies on cholangiofibrosis, the hepatocarcinogen-containing diet was given until carcinoma set in. Therefore, the pathological changes were more complicated because of the coexistence of other chronic changes unrelated to cholangiofibrosis.

The present study was undertaken to find the fine structure of cholangiofibrosis induced by 3'-methyl-4-(dimethylamino)azobenzene (3'-Me-DAB) and to study the histogenesis of this lesion. It has been found in the present experiments that feeding of a diet containing 3'-Me-DAB for 2 to 4 weeks followed by a stock diet for 2 to 82 weeks induced cholangiofibrosis in virtually every animal.

MATERIALS AND METHODS

A total of 130 male Wistar rats weighing 90 to 110 g were divided into 5 groups. Group 1: Animals fed with a stock diet (Oriental MF, Oriental Yeast Co., Tokyo) served as a control. Group 2: Thirty-nine rats were fed with a diet containing 0.06% 3'-Me-DAB for up to 21 weeks continuously. They were sacrificed 1, 2, 4, 5, 6, 8, 10, 12, 14, 16, 17, 18, 20, and 21 weeks later. Group 3: Ten rats were fed with a diet containing 0.06% 3'-Me-DAB for 1 week followed by a stock diet for various durations up to 52 weeks. Group 4: Fifteen rats were fed with a diet containing 0.06% 3'-Me-DAB for 2 weeks followed by a stock diet for various durations up to 30 weeks. Group 5: Forty-nine rats were fed with a diet containing 0.06% 3'-Me-DAB for 4 weeks followed by a stock diet for 1, 2, 3, 4, 6, 10, 12, 15, 26, 30, 52, and 82 weeks.

For light microscopy, the tissues were fixed in formaldehyde solution, embedded in paraffin, and stained with Hematoxylin and Eosin, and/or with periodic acid-Schiff. For electron microscopy, samples of the liver were fixed in glutaraldehyde in Eagle's minimum essential medium for 12 hr, postfixed in 1% OsO4 for 2 hr, then dehydrated, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi HU-12 electron microscope. Semi-thin sections were stained with Toluidine Blue at pH 7.4 for light microscopic study.

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RESULTS

Group 1: Rats fed on a stock diet and sacrificed at various intervals showed no abnormalities in their hepatocytes or in their biliary duct cells.

Group 2: Ultrastructural changes in the rat liver after continuous feeding with 3'-Me-DAB were essentially the same as have previously been reported. Vast majority of the rats fed the dye for 12 weeks revealed cirrhosis, nodular hyperplasia, atypical proliferation of hepatocytes, adenoma, and cholangiofibrosis. After 16 weeks of feeding, all the rats revealed a typical hepatoma as well as cholangiofibrosis.

Group 3: As has already been pointed out by many investigators,5,9,10) hepatocytes in the portal area of the rats fed 3'-Me-DAB for 1 week showed an increase in the agranular endoplasmic reticulum, which occupied a greater portion of the cytoplasm. Typical segregation was observed in the nuclei of hepatocytes. However, as opposed to hepatocytes, biliary duct cells in the Glisson’s sheath showed no discernible changes. By 52 weeks after withdrawal of the carcinogen, no cholangiofibrosis could be found in the liver.

Groups 4 and 5: With a light microscope, proliferation of small oval cells was seen in the portal area during the first 2 weeks after beginning of the carcinogen diet. Many ducts of Hering were found in the portal area by electron microscopy. Biliary duct cells in the canal of Hering were attached to hepatocytes and/or to each other by terminal bars (Photo 1). It was often found that the newly proliferated biliary duct cells extended from the canal of Hering into the lobule and replaced the parenchyma. During the second week a few fibroblasts and numerous macrophages, which exhibited considerable phagocytosis, accompanied the proliferated bile ducts, but they were usually outside the basement membrane (Photo 2). Collagen fibers were only rarely found around the proliferating bile ducts.

At the 2-week stage of the feeding of the carcinogen, typical cholangiofibrosis appeared in the liver lobule occasionally. Fig. 1 shows the relationship between the area of the lesion of cholangiofibrosis in the central lobule of the rat liver and the duration after cessation of the carcinogen feeding. The ratio of the area containing the lesion of cholangiofibrosis to the total area of the central liver lobule was determined with a planimeter on figures projected (×10)

![Graph showing percentage of cholangiofibrosis in the central lobule of the rat liver](https://example.com/graph.png)

**Fig. 1.** Percentage of cholangiofibrosis in the central lobule of the rat liver

The percentage means the ratio of the area of cholangiofibrosis to that of the central lobule in each experimental animal. The rats were fed 3′-Me-DAB for 4 weeks followed by a stock diet for the experimental duration.
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on a section paper. After cessation of the feeding of the carcinogen, lesions of cholangiofibrosis continued to grow for up to 25 weeks, while the proliferated bile ducts on the outside of the lesions became atrophied and degenerated. Mitotic figures in the lining epithelium were observed not infrequently throughout the duration of the experiment.

In ultrathin sections, the intestinal epithelium metaplasia was found occasionally in the lining epithelium of the cholangiofibrosis at an early stage. Such metaplasia was seen in most of the tall cylindrical lining epithelial cells of cholangiofibrosis at a late stage (Photo 3). Those of the epithelial lining cells in cholangiofibrosis exhibited a normal intestinal microvillus border at the luminal surface, each with a clearly defined central core of filaments (Photos 3 and 5). A variation in the fine structure of the striated border was also often observed. Abnormal microvilli, such as a border devoid of microvilli, and twin, triplet, and budding microvilli were also present (Photo 4a–d). The border of the whole microvilli showed an enlarged basal portion, in which there were three or more remnants of filamentous cores (Photo 4a). The twin microvillus consisted of two united in a common base (Photo 4b). The triplet microvillus consisted of three branches united in a common base, so that the surface looked cactus-like (Photo 4c). The budding microvillus is of a type that looked like a daughter microvillus attached as a bud to its mother (Photo 4d). Beneath the base of the villi there was a terminal web (Photo 3). Typical junctional complexes were seen. Mitochondria were fairly numerous, whereas ribosomes were not encountered in great abundance. Contrary to the usual intestinal epithelium, prominent interdigitations between the neighboring cells were present. The nuclei of the lining epithelial cells were irregular in contour. Occasionally, goblet cells were also found in the lining cells of the cholangiofibrosis. These cells exhibited various stages of mucus production (Photo 9).

The lining epithelium was always associated with fibroblasts, with a moderate amount of collagen fiber of 0.02 μ in width, with periodicities in the early stage (Photo 6), with a gradual increase in amount with time so that the ducts appeared as scattered islands lying in a matrix of dense collagen. A few macrophages, plasma cells, and lymphocytes were occasionally found in the matrix. Eight weeks after stopping the administration of the dye, however, a hyalinization of the dense collagen was seen around the lining epithelium in the lesions of cholangiofibrosis (Table I). The collagen fiber in the hyalinized lesions consisted of very fine fibrils (0.005 μ in width) (Photo 7). The hyalinization increased in amount up to 30 weeks after withdrawal of the 3'-Me-DAB feeding. However, newly proliferated lining epithelial cells at the peripheral zone of the hyalinized lesions could always be seen.

<table>
<thead>
<tr>
<th>Durations after cessation of 3'-Me-DAB (weeks)</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of hyalinized cholangiofibrosis</td>
<td>0</td>
<td>19.9±2.0</td>
<td>71.5±7.5</td>
<td>85.3±7.0</td>
<td>97.3±2.7</td>
</tr>
</tbody>
</table>

Table I. Percentage of Hyalinized Cholangiofibrosis in the Central Lobule of Rat Liver

The rats were fed with 3'-Me-DAB for 4 weeks and then followed by a stock diet.

a) The number of hyalinized collagen per duct was calculated.

In the hepatocyte there was reconstitution of the granular endoplasmic reticula at that time. In some areas these formed parallel arrangements. Agranular endoplasmic reticula decreased in number. A few fat droplets, varying in size and amount, were scattered throughout the cytoplasm of most hepatocytes. The nucleoli became hypertrophied (Photo 8).
At 52 weeks after 4 weeks of carcinogen feeding, typical cholangiofibrosis was still present. Among the epithelial lining cells in the lesion was found a typical intestinal cell metaplasia, e.g., the Paneth cell and enterochromaffin cell metaplasia (Photo 9).

**Paneth Cell Metaplasia** These cells were located only at the bottom of the glandular structures in the lesion. The nuclei were fairly oval and were located in the basal part of the cells. Also seen were fairly well-developed granular endoplasmic reticulum in the basal part of the cytoplasm. The apical cytoplasm was filled with large secretory granules. These granules showed wide variations in their density. Not like those of goblet cells, they were never fused together in the cytoplasm.

**Enterochromaffin Cell Metaplasia** These cells were found in the lower and intermediate regions of the glandular structure. These areas were well characterized by their highly electron-opaque, polymorphous secretion granules. In most cases, the granules were distributed through the basal area of the cells (Photo 9).

**DISCUSSION**

It has been reported that the ductular proliferation occurs after the administration of almost any hepatocarcinogen to rodents. In the present study, a prominent proliferation of ductular cells or small oval cells in the portal area was observed during the 2 to 4 weeks of 3'-Me-DAB feeding. However, the animals receiving the 3'-Me-DAB-containing diet for 1 week showed no discernible pathological changes in the biliary ductular cells. After withdrawal of the carcinogen, lesions of cholangiofibrosis were induced only in the former case. Within 52 weeks, no such lesion could be observed in the latter. The present data indicated that the feeding of 3'-Me-DAB for as short a period as 2 weeks is sufficient to induce cholangiofibrosis.

Bannasch and Reiss reported feeding the rats with N-nitrosomorpholine and summarized that two distinct stages could be identified before the induction of cholangiofibrosis. In the first stage, as a consequence of toxic necrosis of hepatocytes, the bile duct epithelia and mesenchymal cells develop and then the proliferated bile ductular cells transform into cylindrical cells. The first ductular proliferation in our results may be the response to nonspecific liver cell degeneration as was found by Bannasch and Reiss. Under the effect of the hepatocarcinogen, however, the newly synthesized DNA and/or RNA in ductular cells may act differently from the original ones.

The nodules of cholangiofibrosis increased in number after cessation of the carcinogen. Over 30 weeks after the stopping of a 4-week feeding of 3'-Me-DAB, the lining epithelial cells proliferated always at the peripheral zone of the hyalinized lesions. Furthermore, it has been reported in our previous paper that cholangiocarcinoma was produced from the lining epithelium in the lesion of cholangiofibrosis, which was induced by a 4-week feeding of 3'-Me-DAB. On the basis of these facts, the possibility may not be ruled out that cholangiofibrosis is not merely reactive, but an autonomous response.

Since the classical report by Borghese, it is now widely accepted that epithelia require mesenchyme for normal cytodifferentiation. Mathan et al. described epithelio-mesenchymal contact sites during the differentiation of fetal rat duodenal mucosa and postulated that such an interaction may facilitate the maturation of the duodenal mucosa. In our present experiments, a direct contact between the proliferating ductal cells and the mesenchyme was not manifested. In the histogenesis of these metaplasia, however, the epithelio-mesenchymal interaction may play an important role.

Abnormalities of microvilli in normal small intestine were studied by several investigators. Until recently, however, there have been no descriptions about our kind of observations.
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on th intestinal metaplasia. The variety as well as the frequency of occurrence of pathological microvilli in the intestinal cell metaplasia was greater than those of normal intestinal epithelium. It has been calculated by Wehman et al.\textsuperscript{15}) that abnormalities in the microvilli probably represent a stage of suppression of the microvillous border rather than its regeneration. In the closed lumen of the glandular structure, the microvilli of the lining epithelium may be more easily suppressed by excrescence into the closed lumen than the normal small intestinal epithelium.

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REFERENCES


EXPLANATION OF PLATES

Photo 1. The liver of a rat fed with 3'-Me-DAB for 4 weeks continuously (Group 2). The proliferated bile ductal cells (BD) at the canal of Hering (CH) is shown. There is a marked increase in agranular endoplasmic reticulum in the hepatocytes (Hy). \( \times 4,000 \).

Photo 2. The liver of a rat fed with 3'-Me-DAB for 4 weeks continuously (Group 5). The outside of the basement membrane of the proliferated bile ducts (BD) is surrounded by numerous macrophages (M). The majority of macrophages shows a marked phagocytosis of the remnants of degenerated hepatocytes. \( \times 3,500 \).

Photo 3. A survey micrograph of the intestinal metaplasia in the lesion of cholangiofibrosis. Eight weeks after a 4-week feeding with 3'-Me-DAB (Group 5). At the top left, typical microvilli (MV) of the intestinal type is seen. Beneath the base of the microvilli there is a terminal web (TW). A prominent interdigitation (ID) and desmosomes (arrows) are seen. Outside of the basement membrane there are some fibroblasts (F) with dense collagen fiber. \( \times 7,000 \).

Photo 4. Abnormal microvilli on the lining epithelial cells in the cholangiofibrosis. The microvilli of epithelial lining cells in cholangiofibrosis of rats fed with 3'-Me-DAB for 4 weeks followed by normal diet for 8 weeks (a, b), for 52 weeks (c), and for 2 weeks (d) (Group 5). (a) Fused microvilli. Four remnants of the filamentous cores are seen in an enlarged basal portion. (b) Twin microvilli. Two branches unite in a common base. (c) Triplet microvilli. The micrograph shows cactus-like appearance. (d) Budding microvilli. The daughter microvilli look like a bud attached to the mother (arrows). \( \times 4,000 \).

Photo 5. Tangential section of striated border of the lining epithelium in the cholangiofibrosis. Central core of the lamellae in the microvilli is connected with fine fibrils in the terminal web. (D) desmosome. \( \times 34,000 \).

Photo 6. Stroma of cholangiofibrosis. The liver of a rat fed with 3'-Me-DAB for 4 weeks followed by a stock diet for 6 weeks (Group 5). The stroma is composed of fibroblasts (F) associated with a moderate amount of collagen fiber with periodicities. (Ep) lining epithelium. \( \times 12,000 \).

Photo 7. Hyalinized stroma of cholangiofibrosis. The liver of a rat fed with 3'-Me-DAB for 4 weeks followed by a stock diet for 82 weeks (Group 5). The collagen fiber in the hyalinized lesion consists of very fine fibrils. Fibroblasts are degenerated. \( \times 7,000 \).
Photo 8. The liver of a rat fed with 3'-Me-DAB for 4 weeks followed by a stock diet for 6 weeks (Group 5). Reestablishment of the granular endoplasmic reticulum and a very prominent fatty droplets (L) in the hepatocyte (Hy) are seen. Kupffer cell (K), bile ductal cell (BD). ×10,000.

Photo 9. The liver of a rat fed with 3'-Me-DAB for 4 weeks followed by a stock diet for 52 weeks. Electron micrograph of the epithelial lining cells in the lesion of cholangiofibrosis. At the left of the picture is a Paneth cell (Pa). Its secretory granules show a wide variation in density. There is an enterochromaffin cell (Ch) at the bottom of the picture. The highly electron-opaque, polymorphous secretion granules in it are distributed through the basal area of the cytoplasm. Some goblet cells (Go) are seen at the right side. ×7,000.
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