Synopsis

Fluctuation in population of cells and the alteration of cell characteristics in liver of rats during the early stage of carcinogenesis by 3′-methyl-4-(dimethylamino)azobenzene (3′-Me-DAB) were studied histologically, ultrastructurally, and biochemically.

After commencement of 3′-Me-DAB administration at a concentration of 0.06%, the initial change in liver cells was the pronounced degeneration of hepatocytes, and consequently the original hepatocytes gradually disappeared, being replaced by the proliferation of cholangiolar cells (the so-called oval cells) after 2 weeks of ingestion of 3′-Me-DAB. Such quantitative change in cellular population was represented by the decreases in liver weight, glucose-6-phosphatase activity, and content of protein-bound dye. Oval cell proliferation in the periportal area of hepatic lobules reached a maximum in the 4th week occupying about one-half of each lobule and, thereafter, the small hepatocytes transformed from the oval cells appeared in the periportal area. The ultrastructural characteristics of these cells revealed various transitional phases toward hepatocytes. They extended to the central vein of most lobules, increasing in their volume, and by the 11th week, the original hepatocytes were almost completely replaced by these renewed hepatocytes. It seemed that the period of appearance of the renewed small hepatocytes corresponded to that of appearance of α-fetoprotein in the sera.

Introduction

There have been many morphological investigations on the hepatocarcinogenesis in rats induced by 3′-methyl-4-(dimethylamino)azobenzene (3′-Me-DAB). These workers suggested that the hyperplastic nodules of hepatocytes are probably the site in which the carcinogenic process actively takes place. However, it is still uncertain what alterations occur in the liver before the appearance of hyperplastic nodules. Recently, Endo et al. reported that irreversible fixation of an isozyme pattern of aldolase, different from that of adult normal hepatic tissue, appeared in the rat liver in the early stage of hepatocarcinogenesis. Watabe reported that α-fetoprotein, which is produced by fetal hepatocytes or cancer cells of hepatocellular carcinoma, appeared transiently in the sera of rats in the early stage of (dimethylamino)azobenzene carcinogenesis. Previously, Inaoka in this laboratory reported the significance of cholangiolar cell (the so-called oval cell) proliferation in the early stage of 3′-Me-DAB carcinogenesis.
assuming that the proliferated oval cells were transformed into hepatocytes when extensive and prolonged injury of hepatocytes occurred. Therefore, it seemed of interest to know whether the period of appearance of small hepatocytes transformed from oval cells corresponds to that of the alterations in the isozyme pattern of enzymes and that of the transient appearance of α-fetoprotein.

The present study aimed to grasp quantitatively and qualitatively the fluctuation of cell populations in the liver tissue through the whole carcinogenic process, preceding the biochemical investigations. Especially, we focused on the period when the small hepatocytes, derived from oval cells, increased.

**Materials and Methods**

Two hundred male adult rats of the Wistar strain, initially weighing 170~200 g, were individually caged at 20° in an air-conditioned room. Animals were fed on an Oriental solid diet (Oriental Yeast Co., Tokyo; protein content, 26.5%) containing 0.06% 3′-Me-DAB. Ten animals were sacrificed every week during the first 5 weeks, and 5 rats every week from 6 to 33 weeks. Thin slices were taken from each 5 lobes of the liver. Since it was found that there was no remarkable difference in the histological appearance in any of the lobes in an individual during the first 5 weeks, the specimens were always taken from the median and left lateral lobe during 6 to 33 weeks.

**Light Microscopy** Tissues were fixed in cold Carnoy’s and/or 10% cold neutral formal solution for a few hours. Paraffin sections were stained with Hematoxylin-Eosin, periodic acid-Schiff (PAS), or Toluidine Blue.

**Electron Microscopy** Specimens of the liver tissue were fixed for 2 hr in 1 or 2% OsO4 solution buffered with Veronal acetate at pH 7.4, dehydrated in a graded series of ethanol, and embedded in Epon 812. Ultrathin sections were cut on an LKB Ultratome, stained with uranyl acetate and lead citrate, and examined with a Hitachi HS-7D electron microscope.

**Enzyme Assay** After removal of liver slices for the morphological examination, the remainder of each liver was placed in an ice-cold 0.25M sucrose solution, and homogenized with a glass-Teflon homogenizer to make a 20% (w/v) homogenate in 0.25M sucrose solution.

The activity of glucose-6-phosphatase (G-6-Pase) was measured by essentially the same method as that of Swanson,[19] except for the use of 0.02M Tris-maleate buffer (pH 6.75). The reaction mixture contained 0.25M of glucose 6-phosphate (Sigma Chem. Co., St. Louis) and 0.005M of MgCl2, and incubated at 37° for 10 min. Released inorganic phosphorus (Pi) was measured by the method of Fiske and Subbarow.[6]

**Dye Content** The content of protein-bound 3′-Me-DAB in the homogenate (equivalent to 1 g of liver) was estimated by the method of Miller and Miller.[14]

**α-Fetoprotein Test** α-Fetoprotein was tested by double diffusion in agar gel with absorbed anti-fetoprotein antiserum prepared by Watabe.[22] Micro-Ouchterlony plates were made with special Noble agar (Difco), 1% in saline solution.

**Estimation of Fluctuation in Cell Population** The light microscopic specimens were photographed in cabinet size (12 x 16.5 cm) (magnified 100 times). Almost five hepatic lobules were present in each picture, and 10 sheets of photographs were obtained from different parts of a liver. The printed pictures were cut out to separate the 3 areas.
which were occupied mostly by each of different types of cells; the hepatocytes which were hypertrophied and degenerated by dye-feeding, the oval cells, and the regenerated small hepatocytes. The cut-out pictures from each rat were weighed, and the percentage of each type of cells was calculated and expressed by the average value for 10 rats.

**Liver Weight** Liver weight was measured and expressed by the value per 100 g body weight.

**Results**

Under the present experimental conditions, only a few rats died during 3'-Me-DAB feeding. There was only a small difference in histological findings observed among individual liver of rats and also among their liver lobes at each period of feeding.

**Light Microscopic Observations**

In the first week of feeding, hydropic degeneration of hepatocytes appeared in the intermediate area of hepatic lobule and it extended gradually toward the central veins in the second week. Hepatocytes showed atrophic and eosinophilic appearance in the perportal area of the lobules. Some of degenerative hepatocytes, especially those in the central area, were markedly hypertrophied. These alterations reached a peak in the 4th week and a remarkable megalocytosis of hepatocytes, whose cytoplasm was strongly eosinophilic, was observed in the hepatic lobules.

On the other hand, after 2 weeks of ingestion, oval cells began to proliferate around the portal area and extended further toward the central vein in the course of time (Photo 1). This process reached a maximum in the 4th week and proliferated oval cells occupied about one-half of the liver lobules. Although the oval cells frequently revealed mitotic figures, their degenerative appearance was hardly observed. In the PAS-stained specimens the areas of oval cells, which possessed no glycogen particles, were clearly distinguished from those of original hepatocytes.

Small basophilic cells began to appear in the 4th week among the proliferated oval cells at the periportal areas of the lobules, and their increase was followed by decrease in the oval cells and the degenerated original hepatocytes. These small cells showed a cord-like or sheet-like arrangement (Photo 2). Each cell was polygonal or round in shape, and its cytoplasm was more abundant than in the oval cell, and strongly stained with Toluidine Blue, while it was negative or slightly positive to PAS staining. Thus, these cells were becoming to be hardly distinguished from normal hepatocytes in respect to morphological appearance. They rapidly extended near the central veins of most hepatic lobules replacing the original megalocytic hepatocytes (Photo 3).

After 9 to 11 weeks, the hepatic lobules were almost completely occupied by the renewed hepatocytes which showed an appearance similar to normal adult hepatocytes and thus a considerable distortion of the lobular pattern was observed (Photo 4). The hyperplastic foci of hepatocytes began to appear in the periportal areas of the reconstructed hepatic lobules after 10 weeks, and they increased in size and number gradually up to the cancerous stage.

**Electron Microscopic Observations**

The ultrastructural findings of the oval cells and the transitional hepatocytes were essentially the same as those described by Inaoka10) in this laboratory.

Oval Cells: A cluster of a few cells formed a small lumen, and the entire structure was
surrounded by a distinct basement membrane. In the cytoplasm of the most oval cells the development of organelles was rudimentary. Some of these cells possessed small secretory vesicles and small mucinous secretion granules of high density in the cytoplasm near the surface facing the lumen (Photo 5).

Transitional Cells: These cells were identified only ultrastructurally. They possessed the characteristics intermediate of oval cells and hepatocytes, and were frequently observed among oval cells (Photo 6). Most of them had a basement membrane. They frequently formed a common lumen, which seemed to correspond to the canal of Hering, together with the oval cells or hepatocytes. The cytoplasm increased in size and contained more abundant cytoplasmic organelles than that of the oval cells. Mitochondria increased in number and size compared with oval cells, and arrangement of mitochondrial cristae was similar to that of hepatocytes. Rough endoplasmic reticulum (RER), the Golgi apparatus, and free ribosomes were usually developed. No glycogen was present in the cytoplasm. In some instances, secretory vesicles and secretory granules were observed in the cytoplasm.

Renewed Small Hepatocytes: Microbodies and bile canaliculi were observed in the renewed small hepatocytes (Photo 7). RER was well developed showing a tendency of lamellar arrangement, while development of smooth endoplasmic reticulum (SER) was hardly observed. Free polysomes were abundant. Although the number of mitochondria was scanty, their size and arrangement of their cristae were similar to those of the normal hepatocytes. The Golgi apparatus was well developed, and glycogen particles of rosette form increased in the cytoplasm.

Renewed Hepatocytes Showing Maturation: Morphologically there were no essential differences between the matured renewed hepatocytes and normal hepatocytes. Their cytoplasmic organelles showed a good development, and glycogen particles were accumulated in the cytoplasm forming extensive glycogen areas. The aggregation of SER, which was conspicuous in the original megalocytic hepatocytes, was not observed in the majority of them (Photo 8).

Fluctuation of Various Cell Populations

Fig. 1 shows the time course of proportion among the oval cells, original hepatocytes, and newly formed hepatocytes in the liver of rats fed 3'-Me-DAB. These values were estimated roughly by weighing the pieces of photographs cut off in groups of the same type of cells, and it indicates merely the areas of three types of cells in the hepatic lobules and neglected the areas occupied by other types of cells, such as littoral cells and bile duct cells. An alteration in proportion of the cells, which represented the area, means either changes in the number or volume of the cells. As shown in Fig. 2, the original hepatocytes showed hydropic degeneration in the first week and many cells increased their volume, while a few cells shrunk. These original degenerative hepatocytes began to disappear gradually, being replaced by the proliferation of oval cells which reached a maximum in the 4th week and they occupied about one-half of each lobule. The small hepatocytes began to appear in the 4th week and their increase followed the decrease of oval cells and degenerative hepatocytes. In the 6th week, the renewed small hepatocytes occupied about 60% of the area of each lobule. After 11 weeks, most of these small hepatocytes matured and occupied about 90% of the lobule. The original megalocytic hepatocytes appeared to be almost entirely replaced by these renewed hepatocytes.
FLUCTUATION OF CELL POPULATIONS

cytes, while the proliferation of oval cells was not so prominent. After 14 weeks, degenerative changes of the renewed hepatocytes were observed and, concomitantly, slight proliferation of oval cells was observed. At the cancerous stage this tendency continued.
α-Fetoprotein
Up to the third week of ingestion of 3'-Me-DAB, α-fetoprotein was not detected in rat sera. After 4 weeks, this protein began to appear and continued up to the 7th week. Disappearance of α-fetoprotein occurred after 8 weeks most of the renewed hepatocytes assumed morphological appearance similar to that of normal adult hepatocytes. After that, α-fetoprotein was no longer detected until hepatocellular carcinoma began to appear.
Change in G-6-Pase Activity

As indicated in Fig. 2, G-6-Pase activity decreased rapidly after ingestion of 3'-Me-DAB and became about 25% of control liver after 3 weeks. In the 6th week, the activity increased up to 40%. Thereafter, it showed slight fluctuation throughout the experimental period.

Protein-bound Dye Content

As shown in Fig. 3, the content of protein-bound dye showed a first peak in the first week and gradually decreased toward the 5th week. The content began to increase again toward the 8th to 9th week, and thereafter, to decrease, forming a second peak. There were slight fluctuations of the content during the experimental period after this time.

Liver Weight

Fig. 4 shows that liver weight decreased in the 4th week when degeneration of the original hepatocytes was marked. After that, liver weight recovered gradually following the growth of renewed hepatocytes. After 14 weeks, liver weight showed a slight decrease again until the appearance of tumors.

DISCUSSION

There have been some reports3,5,19) on an attempt to determine numerically the alteration of cell populations in rat liver during azo-dye carcinogenesis, but no consideration had been made of the fact that the oval cells are transformed into renewed small hepatocytes in the early stage of 3'-Me-DAB feeding. Although some workers counted cell nuclei of various cell types included in the field, the estimation of numerical variation in cell types seems to be correlated to biochemical changes in the cells. In the present study we divided the cells, which were actively fluctuating in the whole carcinogenic process, into three types; original hepatocytes existing before ingestion of the carcinogen, newly appeared hepatocytes, and oval cells. Simultaneously with fluctuation in the cell population, the G-6-Pase activity and bound dye content were examined biochemically, which are known to be confined exclusively to the hepatocytes.1,2)

According to fluctuation in cell population, it seems pertinent to divide the precancerous period up to 13 weeks of 3'-Me-DAB ingestion into 3 stages; the first stage was up to 4 weeks after commencement of ingestion, the second stage from 5 to 3 weeks, and the third from 9 to 13 weeks.

In the first stage, the sharp increase in dye content of the liver seems to be responsible for the degeneration of hepatocytes. The pronounced degeneration and disappearance of the original hepatocytes, existing before ingestion of 3'-Me-DAB, were observed in this stage and were clearly reflected on the remarkable decrease in G-6-Pase activity and liver weight. With their disappearance, proliferation of oval cells began to appear after 2 weeks and reached a maximum in the 4th week. Thus, this stage was characterized by the disappearance of the original hepatocytes followed by a pronounced proliferation of oval cells.

The second stage was characterized by decrease in the original hepatocytes and oval cells, and marked proliferation of the renewed hepatocytes. It is still a controversy7,9,10,18,23) whether the oval cells are able to transform into hepatocytes or not, since Price et al.16) and Farber19) suggested the transformation of oval cells into hepatocytes. In the
present study a number of transitional cells and small hepatocytes were observed in the periportal area of most lobules in this stage. These findings are essentially the same as those reported by Inaoka\textsuperscript{10} and Price \textit{et al.},\textsuperscript{16} but there is an additional ultrastructural finding to Inaoka's descriptions. Some of the oval cells and transitional cells contained small secretory vesicles and small mucinous secretory granules which have a close resemblance to those of the undifferentiated cells of crypt epithelium of the small intestine.\textsuperscript{21} It is well known that the liver and the small intestine are derived from foregut on the ontogenic process, and the undifferentiated cells of the adult small intestine have been considered to be tripotential blast cells. This finding suggests that the oval cell is also a blast cell with multipotentiality. At the end of this period, most of each lobule was occupied by these renewed hepatocytes.

Biochemically, the activity of G-6-Pase and the content of bound dye in hepatic tissue increased and showed a similar peak around the 6th week in this stage. Since only the hepatocytes in liver tissue possess the G-6-Pase activity and the ability to metabolize azo dye, the higher levels of G-6-Pase activity and bound dye content than those in the first stage might correspond to an increase in renewed hepatocytes.

Recently, Endo \textit{et al.}\textsuperscript{4} reported that the muscle-type aldolase activity in rat liver increased in the early stage of carcinogenesis by 3'-Me-DAB ingestion and this pattern of increased muscle-type aldolase was irreversibly fixed for a long period after cessation of the carcinogen. According to this report, it is evident that the irreversible fixation of isozyme pattern of aldolase occurred during this stage. The change in isozyme pattern of acid phosphatase was also recognized in this stage.\textsuperscript{12} Moreover, it was suggested that the renewed hepatocytes possess, though transiently, properties similar to fetal liver and hepatoma as regards the isozyme pattern. Another noticeable event in this stage is a transient appearance of $\alpha$-fetoprotein in the sera of rats, as reported by Watabe.\textsuperscript{22} In the present experiment, $\alpha$-fetoprotein was transiently detected in the sera during the period when the transitional cells and small hepatocytes proliferated prominently, but this protein was no longer detected at the end of this stage when the small hepatocytes gained the same appearances as normal adult hepatocytes. This fact probably means that on the way of differentiation from the oval cells to the hepatocytes during this stage the transitional cells may synthesize $\alpha$-fetoprotein as fetal hepatocytes do in a certain period of the ontogenic process. Further examinations are being made on these properties of these renewed hepatocytes in the second stage.\textsuperscript{11,12}

In the third stage most of the renewed hepatocytes gained in size, and it became impossible to distinguish them from the normal adult hepatocytes. No remarkable change in population of hepatocytes was observed, but the hyperplastic foci of small hepatocytes began to appear in the periportal area of the hepatic lobules. After this stage of the carcinogenic process by 3'-Me-DAB, the hyperplastic foci enlarged and gained in number in hepatic tissue. Reuber\textsuperscript{17} reported that the hyperplastic foci gradually grew to hyperplastic nodules in which atypical cells similar to cancer cells began to appear in the later stage of carcinogenesis by 0.025% 2-N-fluorenlylacetamide. Karasaki\textsuperscript{13} also observed undifferentiated atypical cells in the hyperplastic nodules by 3'-Me-DAB. According to them, the hyperplastic foci are considered to be the site in which dedifferentiation of hepatocytes will take place and some of these cells will convert into cancer cells in the course of the carcinogenic process.
FLUCTUATION OF CELL POPULATIONS

Fluctuation of cell populations in rat liver may not always occur in carcinogenesis by other chemical carcinogens, but it is reasonable to classify the precancerous stage into 3 stages at least in the case of carcinogenesis by 3'-Me-DAB in a concentration of 0.06%.

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EXPLANATION OF PLATES I~III

Photo 1. The liver of a rat in the 3rd week of dye feeding. Moderate oval cell proliferation is seen around the portal area. Degenerated hypertrophied hepatocytes are observed in the intermediate area of the hepatic lobule. C: Central vein. P: Portal vein. H-E. ×60.

Photo 2. In the 4th week. Small basophilic cells (S) are seen in the periportal area among the proliferated oval cells. These small cells show a cord-like or sheet-like arrangement. Degenerated original hepatocytes, H. H-E. ×150.

Photo 3. In the 6th week. This shows remarkable proliferation of the small hepatocytes, which extend to the central vein replacing the original megalocytic hepatocytes, in the periportal area. H-E. ×60.

Photo 4. In the 8th week. The hepatic lobules are almost completely occupied by the renewed hepatocytes. A considerable distortion of the lobular pattern is observed. H-E. ×60.

Photo 5. Oval cells (O). In the 4th week. They show poor development of the cytoplasmic organelles. Note the small secretory vesicles (V) and small mucinous secretion granules (G) of high density in the cytoplasm near the surface facing the lumen (L). A transitional cell (T) also possesses secretory vesicles and granules in the cytoplasm. ×8,000.
Photo 6. A transitional cell in the 4th week. The cytoplasmic organelles are more abundant than those in the oval cells. Arrangement of mitochondrial cristae is similar to that of hepatocytes. Basement membrane (B) is seen. N: Nucleus. ×10,000.

Photo 7. A renewed small hepatocyte in the 4th week. Microbodies (Mb) and a bile canaliculi (BC) are observed. Rough endoplasmic reticulum is well developed showing a tendency of lamellar arrangement, while development of smooth endoplasmic reticulum is hardly observed. The cell is surrounded partly by a basement membrane (indicated by arrow). ×10,000.

Photo 8. A matured renewed hepatocyte in the 8th week. The cytoplasmic organelles show good development. Glycogen particles (Gl) are accumulated in the cytoplasm, while the aggregation of smooth endoplasmic reticulum is not observed. ×10,000.