Lysosomal Changes in Fatty Liver Induced in Rats by High Protein Pyridoxine-Deficient Diet

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Summary The effect of high-protein pyridoxine-deficient diet on the localization of lysosomes and acid phosphatase activity in the rat liver was studied using light and electron microscopy. Numerous lysosomes containing lipid droplets appeared to arise directly from GERL (Golgi apparatus, endoplasmic reticulum and lysosomes) near bile canaliculi and thereafter large crystal clefts were frequently found in these lysosomes. The increased appearance of lysosomes in hepatocytes was compatible with increased lipid droplets and represented an indication of breakdown of stored lipids. Acid phosphatase activity was localized almost exclusively in lysosomes with or without lipid droplets. We postulated that one of the causes of accumulation of lipid in hepatocytes, including that of triglyceride and cholesteryl ester, might be associated with a relative deficiency of intralysosomal digestion in these conditions.

Key Words pyridoxine deficiency, fatty liver, lysosomes, acid phosphatase

The prolonged feeding of high-protein diet lacking vitamin B6 to rats has resulted in the development of fatty liver (1). In a previous study on the morphology of the liver in these conditions (2), increased lysosomes in the pericanalicular areas were demonstrated, which suggested participation in the intracellular digestion of lipid droplets.

The present report deals with the effect of prolonged feeding of the diet on the activity of acid phosphatase and lipase, as "markers" for lysosomes and other organelles, using light and electron microscopy.

MATERIALS AND METHODS

Animals and diets. Male Wister-strain rats weighing about 50 g were placed in

1 岸野泰雄, 阿部ミドリ
2 United States Biochem. Corp., Cleveland, OH.
3 Ratio of cod liver oil to soybean oil, 1:4.
4,5 Harper, A. E. (1958): J. Nutr., 68, 405-418.
individual cages in an air-conditioned room at 20 ± 1°C. The composition of the diet was as follows: vitamin-free casein\(^2\), 70%; sucrose, 10%; corn starch, 7%; cod liver oil-soybean oil mixture\(^3\), 8%; choline chloride, 0.2%; salt mixture\(^4\), 4% and vitamin mixture without pyridoxine\(^5\), 1%. A control group was pair-fed on the same semisynthetic diet supplemented with pyridoxine.

Liver tissues were obtained from animals sacrificed at 2 and 4 weeks after the start of the feeding. After appropriate preparation, detection of fat droplets with Sudan III stain, histochemistry of acid phosphatase and lipase, and quantitative determination of acid phosphatase activity were performed.

**Histochemical localization of acid phosphatase.** Histochemical localization of acid phosphatase was determined by light and electron microscopy.

Light microscopy: Liver tissues frozen at −70°C immediately after sacrifice were subsequently cut into 7 μm sections with a cryostat and placed on microslides. The incubating medium for acid phosphatase was that of Novikoff et al. with cytidine 5′-monophosphate as substrate (3). Following incubation, all sections were rinsed in distilled water. The sections were treated with diluted ammonium sulfide to visualize the site of reaction product. Incubation mixtures lacking the substrate were used as controls.

Electron microscopy: Immediately after sacrifice, small blocks of tissue (2 cu. mm) were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 hr at 4°C. The tissues were then stored overnight in a cold solution of 0.1 M cacodylate buffer and 0.33 M sucrose, pH 7.4. The tissues were fixed for 2 hr in 1% buffered osmium tetroxide, dehydrated in acetone, and embedded in Epon mixture. Ultrathin sections were cut using an LKB Ultratome, and the sections were stained with uranyl acetate and lead nitrate. The electron micrographs were taken with an Hitachi HS-7D electron microscope operating at 50 kV. For the electron microscopic demonstration of acid phosphatase and lipase activities, liver slices were fixed at 4°C in 2.5% glutaraldehyde–0.1 M cacodylate buffer, pH 7.4 for 30 min. After fixation the tissues were stored overnight in cold buffer. The slices were embedded in 7% gelatin and chopped into 40 μm sections on a Sorvall tissue sectioner. The slices were incubated for the detection of acid phosphatase activity in the medium of Novikoff et al. (3) and for lipase activity in the medium of Nagata with Tween 80 as substrate (4). Control sections were incubated in the absence of substrate. After incubation, all sections were fixed for 2 hr in buffered 1% osmium tetroxide and prepared for the observation by electron microscopy as described above.

**Determination of acid phosphatase activity and total lipid.** Acid phosphatase was assayed in liver homogenates according to the method of Barrett and Heath (5). Protein concentration was determined by the method of Lowry et al. (6) with bovine serum albumin used as a standard. Total lipid in liver homogenates was assayed by the procedure of Bligh and Dyer (7).
RESULTS

Total enzyme activity
In comparison with the controls, the liver homogenates obtained from rats fed on experimental diet manifested a slightly greater activity of acid phosphatase with increasing lipid content of the liver after 8 weeks (Table 1).

Light microscopy
In the liver of control rats, there was uniform staining activity for acid phosphatase in the hepatocytes throughout the lobulus (Fig. 1). After 4 weeks on the experimental diet, the materials stained with Sudan III increased mainly in the hepatic centrolobuli (Fig. 2) and particularly in the pericanalicular sites of the liver cell (Fig. 2, Inset). From 4 weeks onward lipid droplets increased in number and size, and were more widely dispersed in the cytoplasm of the liver cell throughout hepatic lobuli. Changes were observed in acid phosphatase activity in liver sections 2 and 4 weeks after feeding on experimental diet. At first, decrease of the enzyme activity occurred in the hepatocytes of centrolobular areas, and to a lesser extent in the peripheral areas. Thereafter, the lysosomes in centrolobular areas recovered some enzyme activity and were irregular in distribution. After 4 weeks, lysosomal enzyme activity gradually returned to levels comparable with those of controls and at 8 weeks some of the lysosomes were larger and more intensely stained. Large aggregates of lysosomes were scattered at random throughout the parenchymal cells of the lobuli (Figs. 3, 4). These changes in distribution of acid phosphatase corresponded so closely with parallel changes in the distribution of lipid droplets as to suggest that the lipids are present in lysosomes.

Electron microscopy
Attention was focused mainly on lysosomes and related structures. In pair-fed rats, the lysosomes were normal in size and distribution and stained positively for

|                      | Control       | Experimental  |
|----------------------|---------------|---------------|
| Total lipid          | 43.8 ± 0.8    | 61.0 ± 3.9*   |
| (mg/g liver wet weight) |               |               |
| Acid phosphatase     | 26.4 ± 4.0    | 36.6 ± 6.0**  |
| (nmol/mg protein/min) |              |               |

Table 1. Total lipid content and acid phosphatase activity of liver homogenates of rats fed on control and experimental diets.

Total lipid content and acid phosphatase activity were determined for liver homogenates of 9 rats after 8 weeks on the diet. Results are means ± SD. Levels of significance are: *p < 0.005, **p < 0.05.
acid phosphatase. Acid phosphatase was markedly seen in Kupffer cells and was occasionally localized in lysosomes containing residual body near a bile canaliculus, but was not detected in the cytoplasm remote from the peribiliary areas. In experimental diet-fed rats, as suggested by light microscopy, most of the lysosomes near bile canaliculi were much larger than those in control hepatocytes and were filled with numerous small dense granules and lipid-like inclusions of various sizes and densities which resembled the triglyceride droplets commonly seen in normal hepatocytes (Fig. 5). The product of acid phosphatase reaction was sharply and irregularly distributed as fine granules in lysosomes or at the periphery of the lipid droplets. Some of the lysosomes containing lipid droplets showed no acid phosphatase reaction (Fig. 5, Insets). Closely associated with the secondary lysosomes was GERL (Golgi apparatus, endoplasmic reticulum and lysosomes) located in the

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Fig. 1. Acid phosphatase activity in the liver of control rat. The activity is seen uniformly in the hepatocytes throughout the lobulus. Inset: Lysosomes in the hepatocytes show pericanalicular arrangement (arrow). CV, central vein. Novikoff's method, ×100, Inset, ×400.

Fig. 2. Four weeks on experimental diet, Sudanophilic materials increase mainly in the pericanalicular regions (arrow of Inset) of the hepatocytes in the centrolobular area. CV, central vein. Sudan III stain, ×100, Inset, ×400.

Fig. 3. Acid phosphatase activity in the liver of rat after 4 weeks of experimental diet-feeding. Reaction product is scattered nonuniformly throughout the hepatic lobulus. Some lysosomes showing the activity in the centrolobular area were much larger and more irregular in shape than those of controls. CV, central vein. Novikoff's method, ×100.

Fig. 4. A higher magnification of Fig. 3 showing the irregular shape and discrete granules of the reaction products in the hepatocytes. Pericanalicular arrangement is less apparent. Novikoff's method, ×400.

Fig. 5. Electron micrograph of hepatocytes from a rat fed on the experimental diet for 8 weeks. Peripheral areas of hepatocytes are representative of the abundance of secondary lysosomes containing lipid-like droplets of various sizes and densities, and dense particles. Insets: Some of the lysosomes containing lipid droplets show no acid phosphatase reaction. Some lysosomes show the activity at the periphery of lipid droplets. BC, bile canaliculus. PM, plasma membrane. ×18,000, Inset, Novikoff's method, ×30,000.

Fig. 6. Electron micrograph of the pericanalicular area of a hepatocyte after 8 weeks on experimental diet. Dilated sac-like structures together with tubules are located near bile canaliculi. They seem likely to consist of a labyrinth-like structure (GERL), and contain electron-dense deposits (arrow). Lysosomes probably separate from GERL contain more lipid-like droplets and electron-dense particles. Inset: Reaction product composed of electron-dense lead deposits is localized to lysosomes. GE, Golgi-endoplasmic reticulum-lysosome region. BC, bile canaliculus. ×23,000, Inset, Novikoff's method, ×33,000.

Fig. 7. Electron micrograph of a hepatocyte from a rat fed on the experimental diet for 16 weeks. Crystal clefts probably composed of cholesteryl ester are seen in lysosomes. ×20,000, Inset: Lipase activity is predominantly seen surrounding crystal clefts within lysosomes. Nagata's method, ×20,000.
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region proximal to the bile canaliculi. This element consisted of sac-like portions together with tubuli suggesting a labyrinth-like structure (Fig. 6). From the evidence of the similarity of their contents and the presence of acid phosphatase activity (Fig. 6, Inset), secondary lysosomes appeared to separate from dilated portions of GERL as suggested by Essner and Oliver (8). In the nucleus, acid phosphatase activity of mitochondria and microbodies was not detectable. Lipase activity also was prominently localized in lysosomes containing crystal clefts probably consisting of cholesteryl ester (Fig. 7, Inset).

Addition of sufficient pyridoxine to the diet of rats fed on experimental diet for 8 weeks resulted in the disappearance of lipid droplets and virtual recovery from these abnormalities except for the presence of crystal clefts in lysosomes.

DISCUSSION

On prolonged administration of high-protein diet lacking pyridoxine, numerous lipid-like materials and crystal clefts appeared in secondary lysosomes near bile canaliculi which we attributed to the impairment of lipid digestion in the lysosomes (2). The investigation into the regulation of hepatic lipid synthesis and secretion has made considerable progress over the past few years. However, very little is known about the degradation of fat in the liver. Recently, some authors have reported that lipolytic activity has been observed in the lysosomal fraction of the liver and that lysosomes are responsible for the breakdown of the hepatic triglyceride stores in acidic media (9, 10). In our present experiment, the increased appearance of lysosomes in pericanalicular areas in the hepatocytes was associated with an increase in the total activity of acid phosphatase assayed in liver homogenates. Histochemically, irregular distribution of acid phosphatase activity in the hepatic lobuli occurred as early as 4 weeks after initiation of the experimental diet in association with fatty deposition in the absence of liver necrosis or inflammation. In electron microscopic findings, most of the lysosomes showed acid phosphatase activity surrounding lipid droplets. However, we occasionally observed lysosomes without acid phosphatase activity containing lipid droplets. After the complete degradation of lipids, the end products may be transported from the lysosomes into the cytoplasm or secreted from the hepatocytes. If the enzyme necessary for the breakdown of lipid or lipoprotein are insufficient in lysosomal particles, the lysosomes will become overloaded with unhydrolyzed lipids in these cells, which become lipid droplets or crystal clefts of cholesteryl ester (11). Pyridoxine deficiency causes a decrease of naphthylesterase activity in the liver cells in contrast with an increase in acid phosphatase activity (12). Further support for the pathogenic model of lipid storage in lysosomes is obtained from studies on acid lipase deficiency (Wolman’s disease) (13, 14), inhibition of hepatic lipolysis in lysosomes by chloroquine (15) and cholesterol-fed Syrian hamster (16). A relative insufficiency of acid cholesteryl esterase activity in lipid-laden lysosomes is suggested as a possible cause of cholesteryl ester accumulation within lysosomes by a
mechanism similar to that postulated in an early stage of development of atherosclerosis (17).

The evidence obtained by use of histochemical and ultracytochemical techniques lead us to suggest that deposition of lipid results from a relative deficiency of hydrolytic enzymes and that this deficiency leads to the accumulation of triglycerides and cholesteryl ester in liver cells.

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