FREEZE-REPLICA STUDY OF THE LIPID DROPLETS OF ITO'S FAT-STORING CELLS IN RAT LIVER

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ABSTRACT

Ito's fat-storing cells in the rat liver were examined by the freeze-replica and thin section techniques after an increase in lipid droplets induced by the administration of vitamin A. Many lipid droplets were fractured and had either a concave depression or a convex bulge on which one or more thin, fractured layers were observed. Occasionally, a number of concentric, thin lamellae were observed on the fractured faces of these lipid droplets, which revealed their multilayered structure. The surfaces of both sides of these fractured lamellar layers were smooth and particle-free. On the surface of fused lipid droplets, one or more peripheral multilayered sheets extending over two or more droplets were observed. Besides these droplets, there also appeared lipid droplets exhibiting a flat fractured surface with a coarse and rugged appearance. In thin sections, concentric lamellar structures were found in some lipid droplets intermingled with homogeneous droplets. The results of this study suggest that these lipid droplets with lamellar structure may contain phospholipids, and the probable distribution of vitamin A (retinol) in these lipid droplets is discussed.

KEY WORDS Ito's fat-storing cell / rat liver / vitamin A / freeze-replica / lamellar structure

The fat-storing cells (Ito cells) have been recognized as perisinusoidal cells located in the space of Disse of the liver (6). The cells, which usually contain lipid droplets in their cytoplasm, are clearly distinguishable from endothelial cells and Kupffer cells situated within the sinusoidal lumen attached either by their cytoplasmic processes or by the cell body to the surface of endothelial cells (7-9, 18, 22).

There is as yet little information as to freeze fracture studies of Ito cells. Ito cells are so small and have so few lipid droplets in their cytoplasm that it is very difficult to identify them in replica figures in normal rat liver. Therefore I made freeze replicas after the administration of vitamin A, because it has been previously shown that the lipid droplets in these cells increase in

number and size after the administration of vitamin A (5, 12, 13, 17, 20-22). The present observations revealed lamellar structures in some lipid droplets, and the possibility of the existence of phospholipids in these lipid droplets was discussed in the light of the findings from synthesized phospholipids (1, 2, 19) and from type-II alveolar cells in the lung (3, 4, 16, 24, 25).

MATERIALS AND METHODS

Ten male Wistar strain rats weighing 100-120 g were intramuscularly injected with 15,000 I.U. of vitamin A (Chocola A, Eisai Co., Japan) every other day for two weeks. All animals were sacrificed 48 hr after the last injection. A control group of 5 animals received no medication. Small liver tissue samples were fixed at 0°C in 3% glutaraldehyde in 0.1 M Na cacodylate buffer or in Karnovsky's fixative (10) for 5 hr at 0°C.

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After washing in cacodylate buffer, some specimens were post-fixed at 0°C with 1% OsO₄ solution by Millonig's method (14). After dehydration, the specimens were embedded in araldite for conventional electron microscopy.

The materials for freeze fracture replica were prepared by immersing small pieces of the sample tissues in cacodylate-buffered (pH 7.4) 30% glycerol solution overnight. Glycerinated specimens were placed in liquid Freon 22 for 10 sec and transferred immediately to liquid nitrogen, fractured and shadowed with platinum and carbon in a Hitachi EF-1 at 10⁻⁶ torr (15). The specimens with replica were immersed and removed in a commercially available solution of sodium hypochlorite (Baby Heiter). They were rinsed in distilled water and mounted on a flamed 200 mesh copper grid. Photographs were taken with a specimen JEM 100C electron microscope at 100 kV.

RESULTS

Ito cells were located in the perisinusoidal space (space of Disse) separated from the sinusoid by endothelial cells and contained a small number of lipid droplets which appeared on electron micrographs as empty vacuoles in control animals. They contained also small, rod-shaped mitochondria, dilated cisterns of the granular endoplasmic reticulum, relatively well-developed Golgi apparatus, glycogen particles, free ribosomes, and occasionally multivesicular bodies in the cytoplasm. As shown in Fig. 1, Ito cells became larger after vitamin A administration as a result of the increase of lipid droplets, and some are very close to each other. Numerous depressions on the nuclear envelope were caused by the numerous lipid droplets.

Generally, fractured Ito cells are easily distinguished from other cell types by their unique appearances after vitamin A administration. Thus, numerous lipid droplets with sizes similar to those seen in a thin section (Fig. 1), were observed also in a freeze-fracture image of an Ito cell with several hepatocytes surrounding it (Fig. 2). A large number of lipid droplets are accumulated in the cytoplasm of the Ito cell, creating obvious depressions on the nuclear envelope (Fig. 2). The flat, irregularly shaped area in the center of Fig. 2 is a cross-fractured plane of an Ito cell nucleus, identified by the presence of nuclear pores on a small portion of the fractured face along the nuclear envelope (arrow). In Fig. 3, an Ito cell is shown with numerous round lipid droplets in the cytoplasm

around the nucleus, which shows two different planes of the fractured nuclear envelope. The P-face (P) of the inner membrane of the nucleus has a large number of intramembranous particles. These particles are less numerous on the E-face (E) of the outer membrane, and occasionally, small, irregular portions of free particles can be seen. The nuclear pores are distributed over the entire nuclear envelope, perforating the inner and outer membranes of the nucleus. These nuclear pores are somewhat fewer than in hepatocytes. Each lipid droplet in the Ito cells is fractured, producing either a convex bulge or concave depression. However, a small number of lipid droplets are fractured in a perpendicular plane across the droplet matrix. The fractured surfaces of these lipid droplets are generally quite smooth in appearance when fractured along their thin layer. Fracture planes appear in various patterns on the lipid droplets that show one, two, or more thin concave and convex layers, revealing the multilayered structure in the lipid droplet. In Fig. 4, the concentric lamellar structure is clearly observable on two lipid droplets fractured across the lamellae (arrows in Fig. 4). Also, a number of lipid droplets with partially separated surface layers can be seen (s). Both sides of the thin concentric layer of the lipid droplet in this figure have smooth surfaces without membrane particles. Other cytoplasmic membranes, such as the nuclear envelope, mitochondria, Golgi apparatus and endoplasmic reticulum contain varying numbers of intramembranous particles. Also, some lipid droplets with neither smooth concave or convex surfaces and without multilayered structures can be seen. Fine, fibrous structures with coarse, rugged faces (r) were observed on flat fractured droplets. In thin sections, lipid droplets composed of numerous lamellae were frequently found among homogeneous lipid droplets in the cytoplasm of Ito cell (Fig. 5). The number of these lamellar lipid droplets varied from cell to cell.

Lipid droplets having such a lamellar structure only at the marginal part of the homogeneous lipid droplet were sometimes encountered in the present study (LP-M, Fig. 5). Fig. 6, an enlarged view of a part (in the quadrangle) of Fig. 4, shows a lipid droplet with approximately 10 thin layers of concentric lamellae radiating out from its surface. These layers, fractured diagonally, are seen as long, round steps. Though direct measurement of the thickness of the individual layers is difficult, it can be estimated from the height of some cross-fractured lamellae. In the

Fig. 1 Ito's fat-storing cell (IT) in rat liver following administration of vitamin A. Numerous lipid droplets (LP) of various sizes, expanded cisternae of the granular endoplasmic reticulum, and mitochondria can be seen in the cytoplasm. HC: hepatocyte, N: nucleus. Bar, 1 μ m. $\times$ 6,800

Fig. 2 Freeze fracture image of an Ito cell (IT) and hepatocytes (HC) in rat liver following administration of vitamin A. The nucleus (N) of the cell has obvious depressions caused by lipid droplets (LP). A few pores can be seen on the fractured surface of the nuclear membrane (arrows). Bar, 1 μ m. $\times$ 6,800

upper part (between two small arrows) where the 10 layers are very close, giving the appearance of cross-fractured lamellae, the thickness of each layer is estimated to be 70 Å. All these layer faces were smooth.

Lipid droplets in Ito cells increased after

administration of vitamin A and came into contact with each other. The curved arrows in Fig. 6 indicate such fused droplets. The external layer extends over the adjoining two or more droplets, that is, one side of the layer at the upper left extends over one droplet and the other side

Fig. 3 An enlarged Ito cell with numerous lipid droplets (LP) after administration of vitamin A. The nuclear membrane is exposed with nuclear pores and on the P-face (P) of the inner membrane numerous intramembranous particles can be seen. These are less numerous on the E-face (E) of the outer membrane where small particle-free areas can be seen (arrows). EN: endothelial cell, HC: hepatocyte, S: sinusoid. Bar, 1 μ m. $\times$ 15,000

of this layer at the lower left also extends over yet another droplet (curved arrows, Fig. 6). Thus at least one external sheet covers two or more lipid droplets within the same superficial layer.

DISCUSSION

Electron microscopy revealed unique charac-

teristics of the lipid droplets in Ito cells different from those of other cells. We have frequently found that the inside of the lipid droplets in Ito cells was filled with an electron dense and homogeneous matrix even when the lipid droplets in hepatocytes or adipose cells in connective tissue appeared as electron lucent, empty vacuoles. Watari (23) reported the existence of phospholipid in Ito cells using light microscopy and a

Fig. 4 The two lipid droplets showing clearly defined lamellar structures (arrows). Some lipid droplets show surfaces with a few smooth, thin layers (s). Other droplets have fractured surfaces (flat area) with a somewhat rugged structure (r). Bar, 1 μ m. $\times 22,000$

modified staining of Lillie and Laskey. In synthesized phospholipids, lamellar structures have been observed by both the negative staining method (1, 2) and the freeze-fracture method (18). Bangham *et al.* (2) also reported that the most common phase structure appeared to be the lattice layer, which gave rise to spherulites and myelins, both composite structures consisting of many concentric bimolecular lipid layers separated by an aqueous compartment. It was believed that this lamellar structure would be revealed by the freeze fracture method if the lipid droplets in Ito cells contained phospholipids. This assumption was shown to be correct in the present study. Many droplets in the Ito

cells of the rat showed smoothly fractured surfaces as concave or convex globule with a few fragmented membranous layers. Some lipid droplets filled with lamellar structures were also found in Ito cells. On the other hand there were some lipid droplets with a coarse, rugged, fibrous appearance rather than a smooth or lamellar appearance when they were fractured perpendicularly to the surface. If the droplet containing lamellae is fractured at an inadequate angle, the lamellar structure may not be visible within the droplet. In thin sections, some droplets were found with lamellar structures which had concentric layers or somewhat irregularly shaped myelinous figures among the homogeneous lipid

Fig. 5 Electron micrograph of thin section showing a part of an Ito cell from rat liver after the administration of vitamin A. Four lipid droplets filled with lamellar structures or myelin figures are found besides light, homogeneous lipid droplets (LP). One lipid droplet has a lamellar structure at one side (LP-M). Granular endoplasmic reticulum (ER) with dilated cisterns characteristic of Ito cells is seen in the cytoplasm. HC: hepatocyte. Bar, 1 μ m. $\times 27,000$

droplets of the Ito cells. In some lipid droplets such lamellar structure was encountered only in a portion of the lipid droplets with homogeneous content. Besides these structures Kobayashi and Shibasaki (11) reported some heterogeneous figures in the droplets in thin sections and many similar figures also in freeze replicas. Therefore, they thought that these figures corresponded to each other. If all these lamellar structures found in the lipid droplets by the

freeze-fracture technique correspond to the myelin-like figures found in thin sections, the lipid droplets fractured with coarse, rugged faces as mentioned above may correspond to the lipid droplets without any lamellar structure in thin sections. In some lipid droplets such lamellar structure was encountered only in a portion of the lipid droplets with homogeneous content. So, it may be concluded that there are three different types of lipid droplets in Ito cells: the

Fig. 6 Enlarged micrograph from Fig. 4. Lamellar structures consisting of about ten sheets can be seen at the edge of the droplet. At the upper part of the droplet, the straight arrows (small arrows) indicate ten cross-fractured lamellae. A thin sheet of the outer lamellae expands over the surfaces of three lipid droplets (curved arrows). Bar, $0.1 \mu\text{m}$. $\times 85,000$

first contains lamellar structures, and the second do not and has homogeneous content, and the third contains lamellar structures only in one portion.

The lipid droplets increased in number after the administration of vitamin A, and the cells were consequently enlarged, with the droplets in close contact. In the present study two or more fused droplets were also observed, some with slightly concave connected areas. On the surface of the fused droplets, superficial, thin layers covered one or more droplets, enveloping them with more than one thin common layer. It is very probable that the superficial outer layers fuse with each other when the lipid droplets make close contact, but the possibility of additional layer synthesis around the fused droplets can not be excluded.

In type II alveolar cells of the lung, the structure of lamellar bodies revealed by Williams (25)

has a strong resemblance to that of the lipid droplets in Ito cells. These lamellar bodies of type II alveolar cells are thought to be stores of phospholipid-rich pulmonary surfactant (3, 4, 16, 24). It is therefore very likely that phospholipids are incorporated in the lamellar lipid droplets in Ito cells. Concerning the presence of retinol in the lipid droplets in Ito cells, it is improbable that retinol molecules disperse in neutral fat droplets, because they have a hydrophilic moiety at one end of each molecule, unless they form spheroidal micelles, turning their hydrophilic head toward the inside, and the hydrophobic tail toward the outside. On the other hand, it is quite probable that retinol molecules are incorporated among the phospholipid molecules when the latter form lamellae in the lipid droplets in Ito cells.

Phospholipid molecules can form layers of bimolecular arrangement and retinol molecules

also can be thought to form layers because they have both a hydrophilic head and a hydrophobic tail like phospholipid molecules. Whether phospholipids and retinol can exhibit the same multilayered structure when they are mixed together is a subject for future research.

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