MORPHOLOGICAL STUDIES ON ADIPOCYTES FROM OBESE RATS—SCANNING AND FREEZE-FRACTURING ELECTRON MICROSCOPY

TOSHIMI MIZUNUMA,* YUKARI TAKAHASHI,* YASUO KISHINO*
AND MICHIMASA FUJIMOTO**

Department of Nutrition and Central Laboratory of Electron Microscopy,**
School of Medicine, Tokushima University,
Kuramoto-cho, Tokushima 770*

Received for publication September 22, 1980

Comparative morphological studies on epididymal fat cells from young, old and obese rats were made by scanning and freeze-fracture electron microscopies. Scanning microscopy showed that adipocytes of young rats were spherical with a smooth surface, but with a few crater-like pits. As the rats became older, their fat cells increased in size and the surface of the cells became rough with an increase in the number of craters. In obese rats, there were more numerous craters in various sizes than in old rats. In ultra-thin sections, the accumulated lipid of adipocytes appeared uniform and amorphous. But in freeze-fractured adipocytes of young and old rats the central lipid was seen to have a multi-lamellar structure, like an onion. The fracture-face of lipid droplets in adipocytes from the obese rat was disfigured and complicated by the crossing of membranous lamellae. These findings suggest that the modes of lipid accumulation in the fat cells of old rats and obese rats differ. This difference may be related to the difference in the distributions and numbers of craters on the surface of adipocytes in these animals.

The fine structure of adipocytes in young rats has been examined by conventional thin-section electron microscopy by many investigators (2, 3, 10, 11, 21, 23, 27, 30, 31, 32, 33), but there are few reports on the morphology of adipocytes of obese rats. This may be because of the technical difficulty of examining these cells by electron microscopy owing to their very high lipid content. Recently, Carpentier *et al.* (8, 9), and Jarett *et al.* (26) examined the morphology of adipocytes of young rats during lipolysis by the freeze-fracture method and scanning electron microscopy. Our previous papers described the morphology of isolated adipocytes examined by transmission and scanning electron microscopies (18, 19). With these techniques we observed that the cells developed a wrinkled plasma membrane with an increase in the number of vesicles in the cytoplasm during starvation, and that they developed numerous projections composed of lipid droplets on their surface on treatment with insulin as a lipogenic agent. However, these experiments did not provide any information on the accumulation of fat in the central lipid inclusion of adipocytes. The application of the freeze-fracturing technique to adipocytes makes it possible to study the mechanism of fat storage in the cells (12) and the structural features

of the plasma membrane and cytoplasmic particles under various conditions, such as obesity.

In the present experiment, we used these techniques to study the relationship between surface structure of fat cells and internal molecular architecture of the lipid droplets of adipocytes from old rats and obese rats.

MATERIALS AND METHODS

Male Wistar strain rats of 27 weeks old and young adult rats of 7 weeks old were used as controls. For studies on adipocytes in obesity, hypothalamic obesity was induced by bilateral electrolytic lesions of the ventromedial hypothalamus of young adult rats (16). Rats with ventromedial hypothalamic lesions were kindly supplied by Dr. Chikamori (2nd Department of Internal Medicine of this University). The rats were kept in cages and given laboratory chow *ad libitum*. Before use, the animals were starved for 15 hr and then killed by decapitation between 9:30 and 10:30 AM. The tip of the epididymal fat pads was rapidly removed and was treated with crude collagenase (Worthington Biochemical Corp., Freehold, N. J.) for isolation of adipocytes as described by Rodbell (25). For this, the fat tissue was minced and incubated in polyethylene tubes containing 0.5 mg glucose per ml and 5% bovine serum albumin, Fraction V (Armour Pharmaceutical Co., Kankakee, Ill.) in Krebs-Ringer bicarbonate buffer, in the presence of collagenase, pH 7.4, at 37°C for 30–40 min. Then, the preparations were filtered through nylon to remove undigested fibrous tissue. The cell suspension was washed once with 5 ml of phosphate buffer at room temperature and then part was fixed with an equal volume of 2.5% glutaraldehyde in Krebs-Ringer phosphate buffer (pH 7.4) for 2 hr. For thin sectioning electron microscopy, the cells were then washed once more with 10 ml of Krebs-Ringer phosphate buffer. One drop of suspension of glutaraldehyde-fixed cells was postfixated in 4% osmium tetroxide in phosphate buffer for 2 hr, dehydrated by passage through a graded series of concentrations of acetone, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate, and examined in a Hitachi HS-7D transmission electron microscope operated at 50 KV. For scanning electron microscopy, the cell suspension fixed with glutaraldehyde was allowed to adhere to a poly-L-lysine coated coverglass (28). The adherent adipocytes were postfixated overnight at room temperature in 4% osmium tetroxide. Doubly fixed adipocytes were dehydrated by passage through a graded series of concentrations of acetone and dried in a critical point drying apparatus with liquid CO₂ (1). Specimens were shadowed with gold (1-B2 Eiko Engineering Co.). The cells were examined in a Hitachi SSM-2 scann-

FIG. 1. Scanning electron micrograph of isolated fat cells from a 7 week-old rat. The cells are spherical with a smooth surface and with a few craters. Bar, 10 μ m.

FIG. 2. Higher magnification of Fig. 1. Bar, 5 μ m.

FIG. 3. Scanning electron micrograph of isolated fat cells from a 27 week-old rat. The cell surface shows numerous craters with some fragments of fibrous tissue. Bar, 10 μ m.

FIG. 4. Higher magnification of Fig. 3. Bar, 5 μ m.

FIG. 5. Scanning electron micrograph of isolated fat cells from an obese rat. The surface of the cells has numerous craters of various sizes covered with fragments of incompletely digested fibrous tissue. Bar, 10 μ m.

FIG. 6. Higher magnification of Fig. 5. Bar, 10 μ m.

ing electron microscope operated at 15 KV. In each experiment, more than 200 cells were examined to determine the diameter of adipocytes. For freeze-fracturing electron microscopy, small pieces of adipose tissue were fixed for 2 hr in 2.5% glutaraldehyde solution in phosphate buffer, pH 7.4, and then treated for at least 30 min with 20% glycerol in Krebs-Ringer phosphate buffer. They were then rapidly frozen in liquid nitrogen, fractured and shadowed in an FD-2A apparatus (Eiko Engineering Co.) as described by Carpentier *et al.* (9). Adipocytes were difficult to freeze-fracture. Large adipocytes from old rats and obese rats were damaged more easily than small ones from young rats during the procedure for cleaning freeze-fracture replicas.

RESULTS

Adipocytes have a signet-ring appearance with a large central amorphous fat deposit, an eccentric nucleus and a thin layer of cytoplasm containing mitochondria, the endoplasmic reticulum and the Golgi apparatus (2, 10, 11, 21, 22, 30, 31, 32, 33). The adipocyte membrane shows numerous microvesicular invaginations and is covered by a basal membrane (Fig. 7). The accumulated lipid is bounded by a thin membrane. We compared the surfaces of adipocytes from young and old rats and obese rats by scanning electron microscopy (Figs. 1–6). Adipocytes, in general, appeared smooth and spherical, without microvillous projections (Figs. 1 and 2). In several cases, fine fragments of fibrous tissue were seen attached to, or close to, adipocytes prepared from old or obese rats (Figs. 3–6). These may be formed during digestion of the basement membrane with collagenase. The mean diameters of adipocytes, determined from scanning electron micrographs, were $35 \pm 5 \mu\text{m}$, $103 \pm 10 \mu\text{m}$ and $230 \pm 14 \mu\text{m}$, in young, old and obese rats, respectively. Craters on the cell surface tended to be more numerous and more varied in size in old and obese rats. These craters located on the cell surface may be related to the release and/or uptake of free fatty acids.

In freeze-fractured preparations from young rats, investigations of the cytoplasmic membrane of adipocytes appeared as small circular depressions on the P-face (6), corresponding to the microvesicular invaginations in ultra-thin sections (Fig. 8). The cleared surface of central lipid droplets showed many wave-like or globular structures with multiple layers, like an onion, in young, old and obese rats (Figs. 8, 9 and 10). This characteristic lamellar structure was remarkable in oblique fractures in which the concentric outer layers had been partially removed. Direct measurement of the thickness of each layer was difficult, because these layers were

FIG. 7. Thin section micrograph of a fat cell from a 7 week-old rat. The cytoplasm surrounding the central lipid droplet contains the Golgi apparatus (G) and mitochondria (M). The arrows show microvesicular invaginations (MI). A well-defined basal lamina (BL) and fragmented collagen fibers (CF) are seen above the cell membrane. Bar, $1 \mu\text{m}$. N; nucleus, L; stored lipid. The inset shows the signet-ring appearance of an isolated fat cell containing a single large lipid droplet. Bar, $10 \mu\text{m}$.

FIG. 8. A freeze-fractured micrograph of epididymal adipose tissue from a 7 week-old rat. The cross-fractured cytoplasm (C) shows microvesicles and microvesicular invagination (arrows). The lipid accumulation is limited by a thin smooth membrane. The central lipid has a lamellar, oyster shell-like appearance. Bar, $1 \mu\text{m}$. The inset shows the higher magnification of the cytoplasm, cytoplasmic membrane and microvesicular invaginations with a regular arrangement.

only visible in oblique cross-fractures. However, the thickness of one layer could be measured from a partially splintered fragment at the outer side of a cross-fractured shell, and appeared to be similar in young, old and obese rats. The fracture faces of these layers were all smooth. The central lipid droplets of young and old rats had a concentric periodic lamellar structure (Figs. 8 and 9). This lamellar structure could not be seen in conventional electron micrographs of ultra-thin sections.

On the other hand, the arrangement of the lamellar structure of obese rats (Fig. 10) appeared to be different from that of young and old rats. The cleared surface of lipid from obese rats appeared as a multi-centric lamellar structure in which the membranous lamellae crossed each other. This was a characteristic feature of the fat cells of obese rats.

DISCUSSION

In previous biochemical studies on isolated adipocytes (17), we found that lipolysis in the absence of a fat mobilizing hormone was much higher in obese rats than in young rats, and that the response to insulin was less in obese rats than in young ones. We also found a close relationship between the surface structure and biochemical findings in isolated adipocytes treated with insulin (19). In the present experiments, the surface of adipocytes isolated from obese rats had craters which were more variable in size and more numerous than those of control adipocytes. In a state of either active lipolysis or lipogenesis in obesity, these structures might be related to increased fat metabolism in adipocytes. Although there is little evidence for the existence of areas of active lipolysis and lipogenesis, these craters on the surface of adipocytes suggest the existence of sites of operation of transport or metabolic systems (19).

In general, it is difficult to preserve lipid substances in adipocytes during routine procedures for preparation of ultra-thin sections for electron microscopy. During dehydration, the lipid is extracted with organic solvents resulting in a collapse of adipocytes. Moreover, chemical fixatives are believed to alter the fine structure of lipid (15). In the freeze-fracture technique it is not necessary to use chemical fixatives, and so the fine structure of lipid droplets can be seen. Thus in this work by the freeze-fracture technique we could see a characteristic lamellar structure, like an onion, rather than the amorphous structure seen in ultra-thin sections. Branton (5) demonstrated that fractures preferentially pass along the midline of the membrane through hydrophobic phospholipid. In this technique the substance in lipid droplets may develop a lamellar structure during freezing. Similar lamellar figures have been observed in phospholipid granules in yeast cells (20) and lung (4), and a lipid-water system *in vitro* (13, 14). Daikoku *et al.* (12) observed that the cleared surface of rat adipose tissue had a granular appearance showing lamellar structures and that the lamellae were less distinct in the center of

FIG. 9. Fat cell from a 27 week-old rat. The fractured surface of lipid accumulation shows layers like an oyster-shell or onion. Stored lipid has a multilayered structure, indicating concentric deposition of unit membranes of lipid. Bar, 0.5 μ m.

FIG. 10. Fat cell and its lipid accumulation from epididymal adipose tissue of an obese rat. The lamellar structure is disorderly, with crossing of membranous lamellae, suggesting multicentric deposition of lipid. Bar, 0.5 μ m.

lipid accumulations. In starving rats, these structures were not observed, but canalicular or cylindrical structures were seen arranged at right angles to the limiting membrane. In the present study we found that the lipid droplets of obese rats, unlike those of the control, contained numerous crossing lamellae. The multicentric layers in these droplets, together with the fact that there were numerous craters on the surface of obese adipocytes, suggests the presence of multiple centers of lipid synthesis in the cytoplasmic layer. There may also be morphological alterations, since there are biochemical and biophysical changes in the accumulated lipid, such as differences in the ratio of triglyceride, phospholipid and cholesterol (7, 24, 29).

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