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Takuzo Oda* Koyo Yoshizawa† Takashi Nakamoto‡

Yutaka Kubo** Hiroaki Okazaki††

*Okayama University,
†Okayama University,
‡Okayama University,
**Okayama University,
††Okayama University,

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Takuzo Oda, Koyo Yoshizawa, Takashi Nakamoto, Yutaka Kubo, and Hiroaki Okazaki

Abstract

With the purpose to elucidate morphologically the site where fat synthesis takes place in the cell, electron-microscopic observation has been conducted on the interscapular brown fat tissue of mice at various periods of carbohydrate introduction after starvation. By starving mice, the depot lipids in the brown fat have been discharged almost completely, and the carbohydrate introduction has caused the biosynthesis of lipids from carbohydrtates in the same tissue. Observations on the tissues proved that the lipogenesis in the brown fat tissue cells takes place in the ground substance keeping the intimate correlation with the endoplasmic reticulum but not in the mitochondria.

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AN ELECTRON-MICROSCOPIC STUDY ON LIPOGENESIS

Takuzo ODA, Kōyō YOSHIZAWA, Takashi NAKAMOTO,
Yutaka KUBO and Hiroaki OKAZAKI

Department of Pathology, Okayama University Medical School
Okayama, Japan (Director : Prof. S. Seno)

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Studies on the site where and the mechanism by which lipids are formed are indeed numerous ever since ALTMAN[1] first made his report on this subject. It has been the concensus of opinions that the deposition of lipids takes place on mitochondria judging from the optical microscopic observations by orthodox method of stained specimens or observations by the phase-contrast microscope[1,2]. Even in more recent observations by the electron microscope the majority of investigators hold the same interpretation[3,4,5]. However, the origin of intracellular lipid droplets may differ in each cell according to the different species or to the change in metabolism, and pathologically they may be divided into two groups : the exogenous lipids, the fat infiltration or lipophagia in which lipids or fatty acids enter into cells from outside[6,7,8], and endogenous ones, which will be further divided into two groups ; the degeneration form, the fatty degeneration or lipophanecrosis in a limited sense in which masked lipids appear as the results of cell degeneration, and the synthetic form, lipogenesis[6,7,8,9,10,11,12,13] in which lipids are synthesized from carbohydrates and other substances. And there is as yet no morphological proof to verify just in what part of the cell this lipogenesis takes place.

In our histochemical studies on lipid and carbohydrate metabolisms in adipose tissue, we have previously explored[6] into the mechanism of lipid synthesis from carbohydrates in brown fat cells, mainly enzymatic histochemical studies on the regulation mechanism. The purpose of this paper is to present the results of further electron-microscopic studies on the relationship between lipogenesis and the fine structure within the cell. The results incidentally offered some morphological evidences that the lipogenesis unlike commonly believed idea of taking place in mitochondria takes place in the ground substance adjacent to the endoplasmic reticulum.

MATERIALS AND METHODS

The observations have been carried out on the interscapular brown fat of normal mice, fasted mice, and those administered carbohydrates after the fasting. The animals belonging to the groups of hunger were fasted absolutely during 3 to 5 days, by which the lipid droplets were almost completely discharged from the brown fat cells. The animals belonging to the last group were injected glucose solution with the combined administration of the purified starch as diet after the just mentioned starvation period but not immediately before death, and materials were taken at the stages of 2, 6, 12, and 20 hours after the initiation of the carbohydrate administration. These materials were fixed in 1 per cent osmium tetraoxide solution (2% OsO_4 solution mixed with 0.1 M phosphate buffer of pH 7.4 in equal volume), washed with water, dehydrated with ethanol, and embedded in acrylic resin in the ordinary manner. Ultra thin sections were prepared with glass-knife and Shimazu K-type ultramicrotome ; and by removing resin and coating with collodion, these were observed under Hitachi HU-6 magnetic type electron-microscope at 50 kv.

OBSERVATIONS AND RESUTLS

Light microscopic observations of the brown fat tissue of both normal and starving mice : Brown fat cells of normal mice are gorged with numerous small lipid-droplets among which the nucleus is embedded in the center, and they are richly supplied with blood capillaries (Fig. 1). At the time of starvation the depot lipids are discharged, and at the extremely high and absolute starvation time they are all exhaused (Fig. 2). By administration of glucose or carbohydrates glycogen and lipids are synthesized in the cytoplasma and depot lipids do make their appearance except at the time immediately before death (for detail see Report 6).

Electron-microscopic structures of the fat tissue cells of mice : The cytoplasm of brown fat cells of mice under normal condition is filled with numerous mitochondria and big and small fat droplets (Figs. 3, 4). However, the empty space observable in Fig. 3 is artifact due to escapement of the droplets. Lipid droplets are found in the ground substance and not in mitochondria, and some of them are far smaller in size than mitochondria and some are almost equal or slightly larger than mitochondria. Mitochondria are of spherical or oval shape, and some of them are of columnar or irregular shape. Cristae mithochondriales run continuously in parallel line along the equatorial axis, and they are rather dense near both poles of the longitudinal axis. Again, some of mitochondria present

tripolar or multipolar irregular shape and likewise cristare present tripolar or multipolar arrangement in line with these poles. Such cases are quite frequently found in the starvation or in the cases later given carbohydrates (Figs. 6, 7, 8). Endoplasmic reticulum is relatively fine, presenting vesicular structures (Figs. 5, 8), being connected in strand formation. The nuclear membrane presents double membraneous structure, and nucleolus high in electron density can be recognized in the center of the nucleus (Fig. 3). The question whether or not fat cells possess cellular membrane peculiar to them has long been the point of conflicting opinions ; and among them the idea that fat cells lack in membrane has decidedly predominated[14]. However, from our studies as shown in Fig. 5, each fat cell possesses clear-cut membrane peculiar to it and distinctly apart from capillary membranes. Each fat cell contacts with one another at a portion of its cell membrane, but some of them contact themselves with the endothelial cell membrane of capillary blood vessels distributed among them. Capillary blood vessels are formed by thinly elongated cytoplasm of each endothelial cell, which, communicating with each other, forms a continuous capillary duct. Consequently both the inner wall and the outer wall of the duct are composed of cell membrane, and the intimate connection between the membranes of two endothelial cells can be observed (Figs. 5, 9). In between the inner wall and the outer wall there is fairly rich cytoplasm about 0.5 to 1.0 μ in thickness of endothelial cells, with a few small mitochondria. Endothelial cell nuclei can be recognized scatteringly. The space of capillary blood vessel contains erythrocytes or serous substance.

Observations on the process of lipid-formation in the fat cell by the electron-microscope

1) Electron-microscopic pictures of the brown fat of the mice on the 4th day of complete starvation (Figs. 5, 6). Lipid droplets are almost completely discharged from the fat cells, and numerous mitochondria fill up cytoplasm.

2) Electron-microscopic pictures of the brown fat of the mice two hours (Figs. 7, 8, 9) and six hours (Fig. 10) after glucose injection and carbohydrate administration on the 4th day of complete starvation. Cytoplasm is filled up with mitochondria and among these mitochondria a few extremely minute fat globules are scattered. Although it may be difficult to decide conclusively that these fat globules are products newly synthesized from glucose, in any event they are found independent of mitochondria but between these mitochodria, namely, at the site coinciding with endoplasmic reticulum or ground substance. There are some fairly large mitochondria, but no fat-globule formation is discernible among

them. However, in some cases which were in highly advanced starvation and extremely weakened, taken two hours after glucose injection, electron absorption density of mitochondria was generally high, and some granules that might be considered as the so-called osmophilic granules appeared. These granules were associated with mitochondria but they seem to have no direct relationship with still smaller lipid granules.

3) Electron microscopic pictures of the brown fat of the mice twelve hours after glucose injection and carbohydrate administration on the 4th day of complete starvation (Fig. 11). Although only a few fat globules can be seen as yet, both small and large ones are located independent of mitochondria. They are situated in the ground substance astride on the endoplasmic reticulum, and each of these fat globules seems to be in the process of growing into a larger globules (Fig. 11). It is assumed that these are not residual fat globules nor the globules that have infiltrated from blood but they are fat globules newly synthesized from glucose after its glucolysis within the fat cell[6,8]. It is difficult to conclude whether these globules are formed in the endoplasmic reticulum and migrate into its sack or into ground substance or just the reverse, namely, form in ground substance and attach themselves to endoplasmic reticulum, but it is assumed that they are in all probability formed in the ground substance adjacent to the endoplasmic reticulum. There is, however, no evidence to prove that such fat globules are being formed in mitochondria.

4) Electron microscopic pictures of the brown fat of the mice twenty hours after glucose injection and carbohydrate administration on the 4th day of complete starvation (Fig. 12). A quite considerable number of large and small fat globules can be observed. Smaller ones are far smaller than mitochondria while larger ones are bigger than mitochondria. The majority of these large fat globules seems to have grown to such size by each having a small globule formed around the endoplasmic reticulum; and some seem to have grown into larger ones by fusion of smaller globules. There is no picture suggestive of formation of fat globules in mitochodria or a new formation of such fat globules by fatty degeneration of mitochodnria.

DISCUSSION

For a long time in the field of pathology, phenomena such as abnormal increase in fat in the cell of the tissue or organ that physiologically possesses fat globules, or such as the appearance of fat globules in the cell where physiologically fat globules do not exist, are generally called

as fatty degeneration in a broader sense. Since these fats are derived by fat infiltration, fatty degeneration in a narrow sense, or by lipogenesis; and their origin differs according to the kind of each cell and to the change in metabolic process *in vivo*, their differentiation is, therefore, not so simple. In any event, such phenomena are usually brought about by the diminution in breakdown of lipids due to disturbances of oxidative process in the cell in contrast to the increase in the amount of lipids by infiltration into the cell due to an increase of lipids in blood, or to the increase in the amount of lipids synthesized within the cell. For example most of the lipids absorbed by intestine from food goes directly into fat cells and a portion of it into liver cells and other cells. Especially fatty acids of long chain (over C_{16}) seem to go directly or after various modifications in the liver to enter themselves into the adipose tissue[15]; and fatty acids (e. g. butyric acid) with a shorter carbon chain are mainly oxidized completely in the liver[16]. At the time of starvation depot lipids in the fat cell are discharged into blood and being carried mainly to the liver where they are deposited; and there they are burnt and thus utilized as the energy source[6]. In addition, fat globules increase in case when the oxidative process in the cell is disturbed by pathologic change in the cell or by disturbances in regulation by hormones and the autonomic nerves, or when the production of phospho-lipids is disturbed due to the lack of lipotropic factors. Fatty liver or what is known as the fatty degeneration of the liver is usually caused by such disturbances just mentioned. In some cases lipids appear by the unmasking of masked lipids[9] due to cell degeneration, namely, protein degeneration or by formation of lipds from intermediary metabolic products of proteins. The fats observed by many investigators who claimed that fat is produced either in mitochondria or from mitochondria, may be mainly fats under pathologic conditions such as fat globules infiltrating from outside into cells that were deposited on mitochondria or fat globules formed in the site of cell other than in mitochondria, that infiltrated into mitochondria, or masked lipid appearing at the site where mitochondria were degenerated. On the other hand it is well known that fat is synthesized from carbohydrates[6,10,11,12], and this synthesis takes place chiefly in the fat cell and partly in the liver cell, mammary gland, and others. Particularly the brown fat is one of the tissues in which carbohydrate and lipid metabolisms are most active, and hence in the case of a fairly advanced and complete starvation depot lipids are almost completely discharged. In the case which is still surviving by the aid of carbohydrate administration, the snythesis of glycogen and lipids promp-

tly takes place[6]. This seems to be one of the most suitable tissues for the precise observation of lipid synthesis from carbohydrates. From electron-microscopic observation in this experiment it may be said that aside from the fat-globule formation by pathologic fatty degeneration of mitochondria, the lipid synthesis has been morphologically proven to take place not in mitochondria but in the ground substance around the endoplasmic reticulum.

On the other hand in the past few years due to the advance in biochemical research it has been elucidated that biological oxidation of fatty acid takes place in purely isolated mitochondria[17,18,19,20,21,22] white acyl Co A[23] derivatives are oxidized step by step[24,25], and the acetyl Co A produced in the fatty acid cycle condensing with oxloacetate into citrate enters the TCA cycle, and its terminal oxidation is also performed by the respiratory enzyme system in mitochondria. In short it appears to be a certainty that the breakdown of fatty acids takes place in mitochondria ; but bio-synthesis of fatty acids being not so well understood, the fat synthesis like its breakdown had been thought to take place in mitochondria[24,26]. However, just recently PAPJAK and TIETZ (1955)[27], BRADY *et al.* (1956)[29], and LANGDON[30] (1957) made it clear by differential centrifugation of tissue homogenate that fatty acid-synthesizing enzymes exist in soluble supernatant fraction, and further they have deduced enzymologically that the fatty acid-synthesis takes place in soluble cytoplams by clarifying that during the course of the synthesis when the enzymes reduce α, β-unsaturated acyl Co A derivatives to the saturated counter part (trans-α, β-dehydroacyl Co $A \xrightarrow{+H_2}$ fatty acyl Co A), $TPNH_2$ plays an important rôle as an electron donor[29,30]. Likewise synthesizing enzymes of cholesterol[31] and glycerides[32], and various kinds of esterases have been verified to exist in microsome fraction by differential centrifugation, particularly in contrast to the existence of RNA in the submicrosome granule (Palade) of microsome, synthesizing enzymes of cholesterol and glycerides may exist in ergastoplasmic matrix (microsome membrane)[33]. Although it is dangerous to connect the results of the fractionation of cell components by differential centrifugation to intracelluar fine structure as they are, enzyme series of the endoplasmic reticulum and of the ground substance seem to be involved in their synthesis.

From these it may be deduced that fat is synthesized in the cell from carbohydrates in the following manners : On one hand glucose penetrating into the cell is synthesized to glucogen by enzyme series of the endoplasmic reticulum, or the ground subtance while on the other hand pyruvate produced by the action of glycolytic and glucolytic enzyme series ex-

isting either in the endoplasmic reticulum or in the ground substance, passing through acetyl Co A, a part of it is oxidized by the terminal respiratory enzymes in mitochondria on entering the TCA cycle, and in another part acetyl Co A condensing with acyl Co A forms β-ketoacyl Co A, and entering the fatty acid cycle, unsaturated acyl Co A is reduced to fatty acyl Co A by enzymes existing in the endoplasmic reticulum or in the ground substance, thus gradually fatty acids with longer carbon chain are synthesized. Again, glycerine is formed after glucolytic process or from triglyceride in food, and by the action of lipase that exists in the endoplasmic reticulum or in the ground substance neutral fat is synthesized from fatty acid and glycerine. Lipids with a little polarity are deposited there in the form of small fat globules due to the high surface tension in the liquid medium of the fat cell, and these globules as nuclei newly synthesized fat globules are added and thus each fat globule seems to grow up into a larger one. On fixing with osmium tetraoxide, although the fat-staining principle has not been sufficiently clarified yet, the sites where fat globules are located all show a high electron-absorption density due to osmium and thus they can be easily distinguished from other granules.

As for the breakdown of lipids, at first lipids are hydrolysed by lipase and other esterases existing in the endoplasmic reticulum or in the ground substance, and then by fatty-acid oxidative enzymes in mitochondria fatty acid undergoes oxidative breakdown, and finally entering the TCA cycle it is completely burnt by respiratory enzyme system in mitochondria. Most of the terminal respiratory enzymes of mitochondria are contained in cristae mitochondriales, but because they are also contained in the mitochondrial membrane[34], in the processes of fat synthesis and breakdown taking place outside mitochondria there is every possibility of enzymes in the mitochondrial membrane being utilized for such processes. In any case, it goes without saying that the correlation of various intracellular factors, such as mitochondria, endoplasmic reticulum, ground substance, and nuclei as well as the conditions of cell membrane are important, but for the breakdown of lipids enzymes of mitochondria play a leading rôle, while for its synthesis enzymes outside of mitochondria play the leading rôle. Namely, the morphological evidences with which we contend that the lipid synthesis takes place not in mitochondria but in the ground substance around the endoplasmic reticulum are also in agreement with recent enzymological findings on the isolation of cell components by differential centrifugation reported by many workers[27,29,30].

SUMMARY

With the purpose to elucidate morphologically the site where fat synthesis takes place in the cell, electron-microscopic observation has been conducted on the interscapular brown fat tissue of mice at various periods of carbohydrate introduction after starvation. By starving mice, the depot lipids in the brown fat have been discharged almost completely, and the carbohydrate introduction has caused the biosynthesis of lipids from carbohydrtates in the same tissue. Observations on the tissues proved that the lipogenesis in the brown fat tissue cells takes place in the ground substance keeping the intimate correlation with the endoplasmic reticulum but not in the mitochondria.

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EXPLANATION OF FIGURES

Fig. 1. A microscopic picture of the interscapular brown fat of a normal mouse. Paraffin section, hematoxilin-eosin stain (× 300).

Fig. 2. A microscopic picture of the brown fat of a mouse at the 3rd day of absolute hunger (× 500).

Figs. 3 and 4. Electron-microscopic pictures of the brown fat of a normal mouse (× 735 and × 22050).

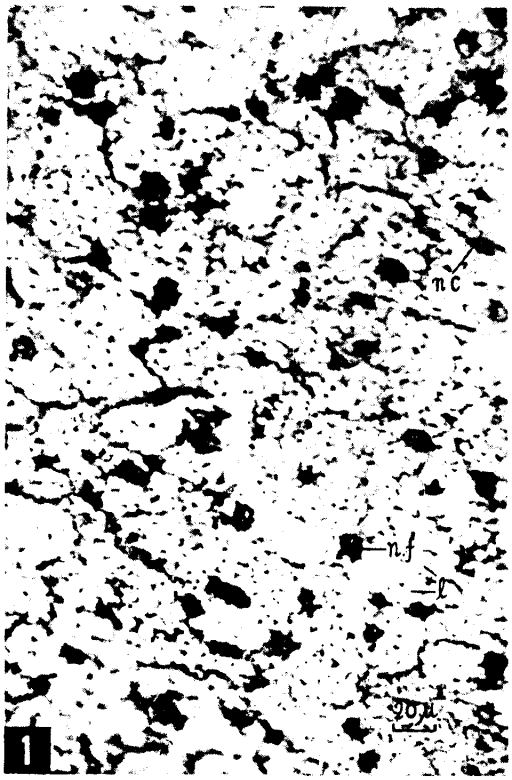
Figs. 5 and 6. Electron-microscopic pictures of the brown fats of the mice at the 4th day of the absolute hunger (× 7500 and × 12700). Lipid droplets are almost completely discharged from the brown fat cells.

Figs. 7, 8 and 9. Electron-microscopic pictures of brown fat cells of the mice injected glucuose solution with the combined administration of the purified starch as diet after 4 days' starvation (× 11700, × 19500 and × 6600). The materials were taken at the stage of 2 hours after the initiation of the carbohydrate administration.

Fig. 10. The one taken 6 hours after the same treatment as figures 7, 8 and 9 (× 7050).

Fig. 11. The one taken 12 hours after the same treatment (× 19050). Lipid droplets, probably newly synthesized from carbohydrates, are found in the ground substance being laid across or adjacent to the endoplasmic reticulum and not in mitochondria.

Fig. 12. The one taken 20 hours after the same treatment (× 21000). Lipid droplets, probably newly synthesized, are found in the regions between mitochondria and not in mitochondria.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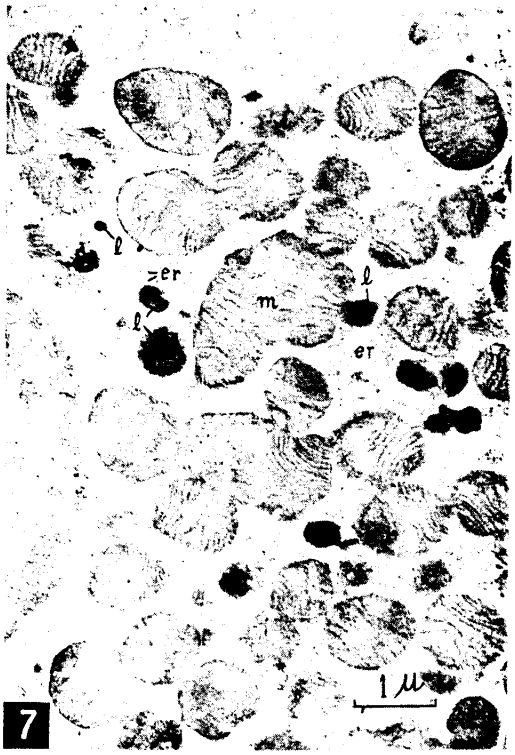

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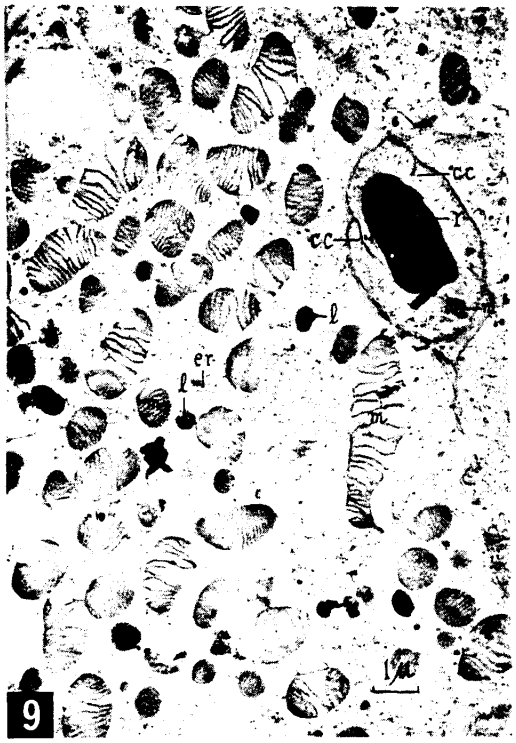
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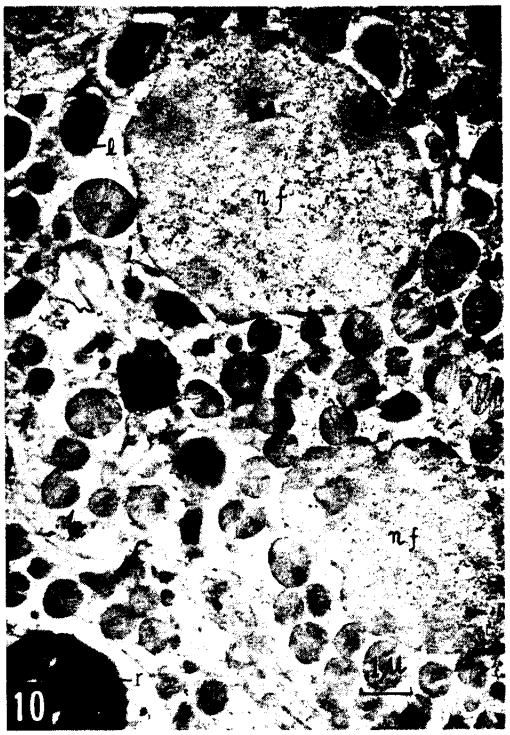
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