

Proliferation of unilocular fat cells in the primary culture

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Abstract Mature white fat cells (unilocular fat cells) have generally been considered to be in terminal differentiation and, hence, to have no proliferative ability. A new method, referred to as "ceiling culture," has been devised in our laboratory to culture unilocular fat cells in vitro. Under such culture conditions, the fat cells continue to exhibit specific functions of lipid metabolism and proliferate extensively. Intracytoplasmic lipid droplets did not inhibit division of the cells. There were two modes of proliferation of unilocular fat cells: "loculus-dividing" cell division, in which the single loculus of fat in the dividing cell was broken down into multiple droplets and distributed evenly between the daughter cells, and "loculus-preserving" cell division, in which the loculus in the dividing cell was minimally broken down and inherited with its shape preserved by one of the daughter cells with the other getting only a small number of fine lipid droplets. Such findings suggest that unilocular fat cells in mature fat tissue in vivo are probably capable of proliferation in such modes under some conditions.—Sugihara, H., N. Yonemitsu, S. Miyabara, and S. Toda. Proliferation of unilocular fat cells in the primary culture. *J. Lipid Res.* 1987. 28: 1038–1045.

Supplementary key words loculus • lipid droplets • tissue culture • proliferation • daughter cells

Mature white fat tissue consists of fat cells which have some hormone receptors and which are metabolically active. For example, the lipogenesis is accelerated by insulin (1–8) and the lipolysis is stimulated by norepinephrine, ACTH, TSH, or GH (1, 3, 4, 6–8). These fat cells typically have a large single lipid droplet and a peripherally located nucleus, hence are named unilocular fat cells. They have been considered to be in the terminal stage of differentiation and to lack proliferative activity (3, 4, 8). Culturing unilocular fat cells in vitro has been technically difficult due to their buoyancy in culture medium (3, 4, 8). Therefore, no in vitro description of proliferation has been available. This problem has been resolved by a new method designated as "ceiling culture," which we have recently reported elsewhere (9). Under the culture conditions, unilocular fat cells continued to exhibit normal functions such as lipogenesis and lipolysis. They also underwent morphological change to become multilocular or fibroblast-like fat cells which, in turn, proliferated further.

In this study, we describe the manner of proliferation of unilocular fat cells. Two modes have been observed: one, tentatively called "loculus-dividing," that is even; and another, called "loculus-preserving," that is lopsided. In the loculus-dividing mode, the single droplet of fat in the dividing cell is broken down into multiple or much finer droplets and distributed evenly between the daughter cells. In the loculus-preserving mode the droplet in the dividing cells is minimally broken down and inherited (with its shape preserved) primarily by one of the daughter cells while the other daughter cell receives only a small number of finer lipid droplets.

MATERIALS AND METHODS

Cell culture

Epididymal fat pads from 3-day-old to 2-week-old (male) Wistar rats and abdominal subcutaneous fat tissue from young female humans (13 to 21 years of age) were used for the primary monolayer culture. The fat tissue was chopped into pieces and then digested in a 0.2% collagenase solution. After filtration and centrifugation, unilocular fat cells were obtained as a thin white layer floating in the test tube. These fat cells were cultured by the "ceiling culture" method previously described in detail (9). Briefly, unilocular fat cells are incubated at 37°C in culture flasks that are completely filled with Ham F12 medium supplemented with 10% newborn calf serum. Newborn calf serum was more effective than fetal calf serum for the culture of fat cells. The incubated fat cells rise to the top of the medium and adhere to the top inner (ceiling) surface of the flask. When the cells have firmly attached, the flask is placed upside down to allow regular treatment. In order to culture only unilocular fat cells and avoid preadipocytes, fibroblasts, and other stromal cells, we digested the fatty tissue until the suspen-

Abbreviations: db-cAMP, N⁶-2'-dibutyryl adenosine-3'-5'-cyclic monophosphate Na-salt.

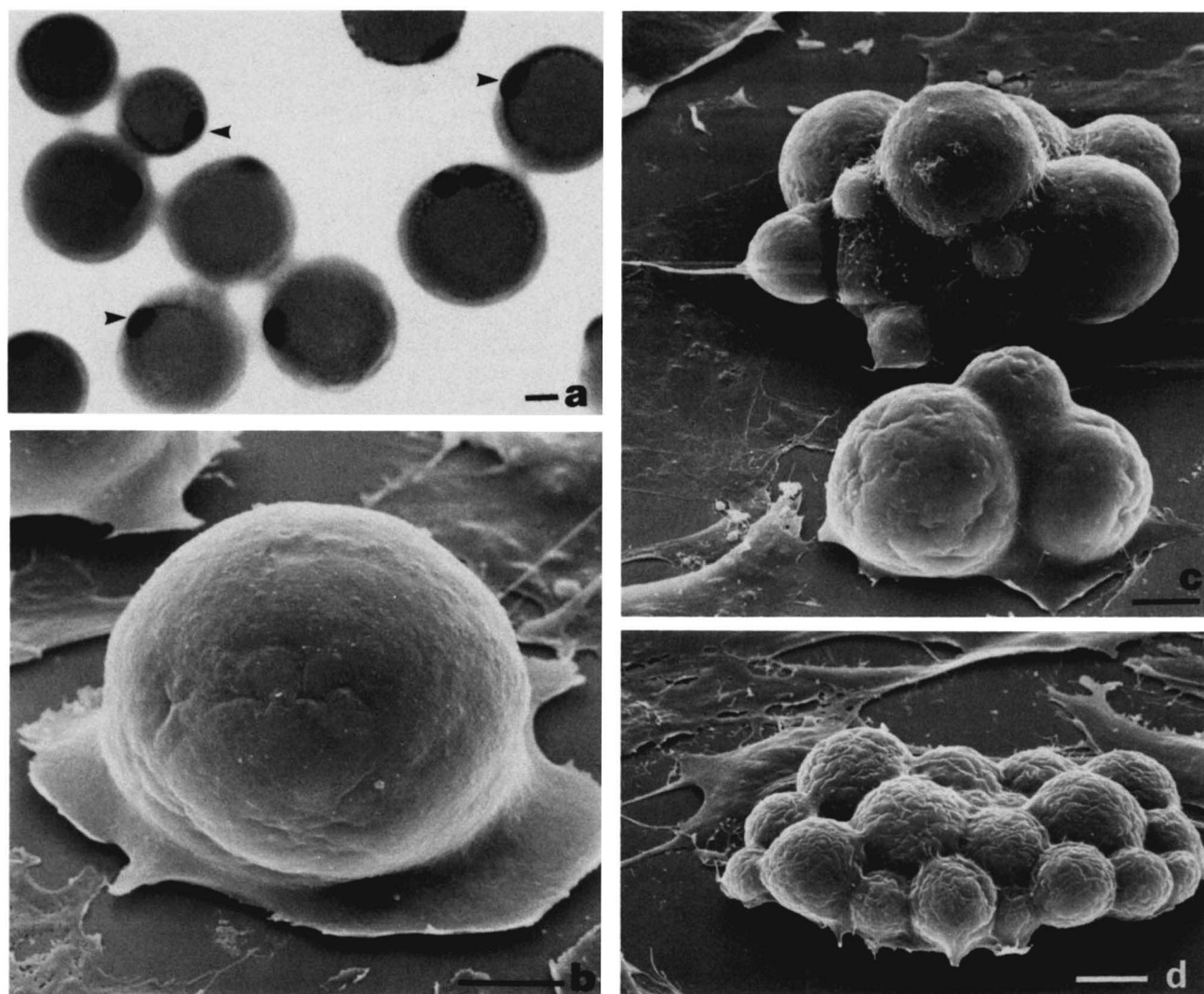


Fig. 1. a) Mature white fat cells containing unilocular lipid droplets in culture (unilocular fat cells). These cells were disaggregated by digesting the fatty tissue of young rats. On the second day of culture these cells adhered to the top inner (ceiling) surface of a flask that was completely filled with medium. One cell already has two nuclei; arrowheads, nuclei; oil red O / hematoxylin stain; bar, 10 μ m. b) Scanning electron micrograph of a mature white fat cell on the third day of culture. This cell can be seen to adhere tightly to the ceiling surface of the culture flask. The adhered part of the cytoplasm has spread out and a large part of the cell is still spherical; bar, 10 μ m. c) Two multilocular fat cells on the fourth day of culture. Unilocular lipid droplets have been divided into multilocular; bar, 10 μ m. d) One multilocular fat cell on the fifth day of culture. Lipid droplets are divided further than in the cells in Fig. 1c; bar, 10 μ m.

sion contained only single cells and repeated the pipetting, washing, and centrifugation of the dissociated unilocular fat cells three times. Moreover, on day 2 of culture, we carefully checked that all culture cells on the inner upper surface (ceiling surface) of the flask were unilocular fat cells (see Fig. 1a). A few preadipocytes that could not be eliminated through these procedures sank and adhered to the bottom surface of the flask. These preadipocytes were not used in this study.

Additions to the medium of some cultures were (final concentration): 0.1–1 mU/ml insulin; 10^{-6} – 10^{-5} M norepinephrine (L-arterenal bitartrate); 10^{-8} – 10^{-7} M ACTH (Cortrosyn, Organon-Daiichi Seiyaku); 2–10 mU/ml TSH;

20–100 ng/ml human GH; 10^{-4} – 10^{-3} M N^6 -2'-dibutyryl adenosine-3'-5'-cyclic monophosphate Na-salt (db-cAMP, Seikagaku-Kogyo); 10^{-8} – 10^{-7} M adenosine; 10^{-7} M N^6 -phenylisopropyladenosine; and 0.1–0.5 U/ml adenosine deaminase. These materials were administered from the first day of the culture for 2 weeks.

Examination of culture cells

Fat cells growing in culture were examined by the following procedures: lipid histochemistry using oil red O / hematoxylin staining and enzyme histochemistry of triglyceride lipase (10) and glycerophosphate dehydrogenase (11). For scanning electron microscopy, the cultured cells

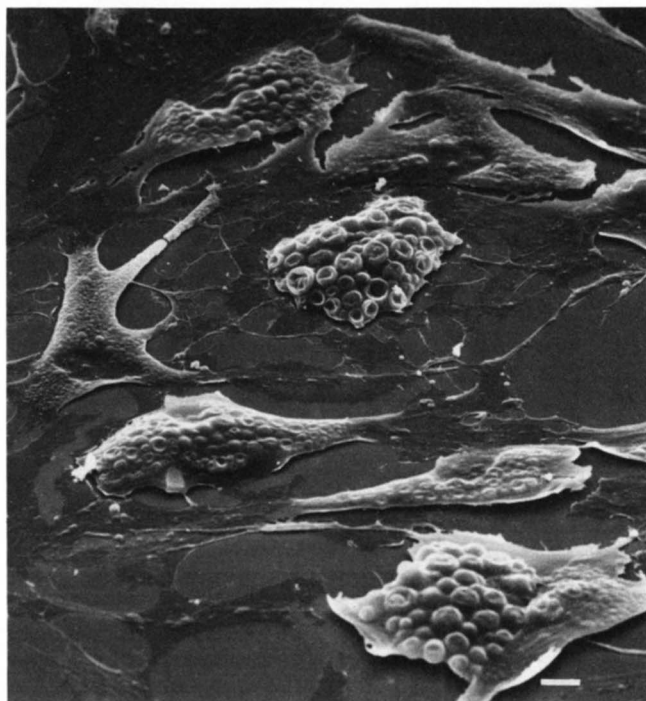


Fig. 2. Fibroblast-like fat cells in culture. These fat cells have many fine lipid droplets and proliferate extensively; bar, 10 μ m.

were fixed in 1.5% glutaraldehyde (in 0.05 M cacodylate buffer, pH 7.2), followed by dehydration in alcohol, critical-point drying with CO_2 , and sputter-coating with gold. The specimens were observed with a Hitachi S700 scanning electron microscope.

For the determination of glycerol in the medium, an enzymatic method (12) was adopted. Briefly, glycerol was reacted with glycerokinase, pyruvate kinase, and lactate dehydrogenase. The amount of NADH consumed in these reactions is stoichiometric with respect to glycerol. NADH was determined by its absorption at 340 nm.

RESULTS

Fat cells from newborn rats adhered well to the ceiling surface of flasks on day 2 of culture (**Fig. 1a**). On day 3 or 4 of culture, the unilocular fat cells changed their shape to hemispherical or almost flat (**Fig. 1b**). At this stage, these fat cells still had a large lipid droplet but also started to show much finer lipid droplets on the periphery: these cells are designated multilocular fat cells (**Fig. 1c and d**). Multilocular fat cells underwent cell division yielding fat cells of the same type or fibroblast-like fat cells containing only a few fine lipid droplets (**Fig. 2**). Such morphological changes occurred within several days of culture in the fat cells of newborn rats (3 to 14 days old), but it took 2 to 3 weeks for the human fat cells to reach a comparable stage. Better results were achieved using fat cells from these newborn rats than cells from rats over 2 weeks old. For example, it took more than 2 weeks for cells from these older rats to attach to the surface of the flask, and division of unilocular lipid droplets was observed only in low frequency in the usual culture conditions. Therefore, we limited the material used in this study to fat cells from rats that were 3 days old to 2 weeks old. The multilocular or fibroblast-like fat cells proliferated extensively until they became confluent.

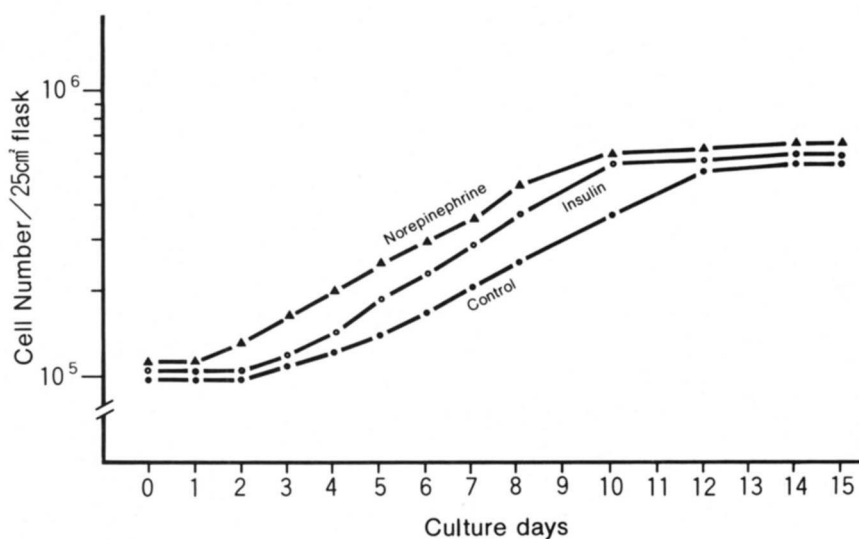


Fig. 3. Growth curve of mature fat cells in culture. Cells were derived from 1-week-old rats, and were treated with insulin (0.1 mU/ml) or norepinephrine (10^{-6} M) from the first day of culture.

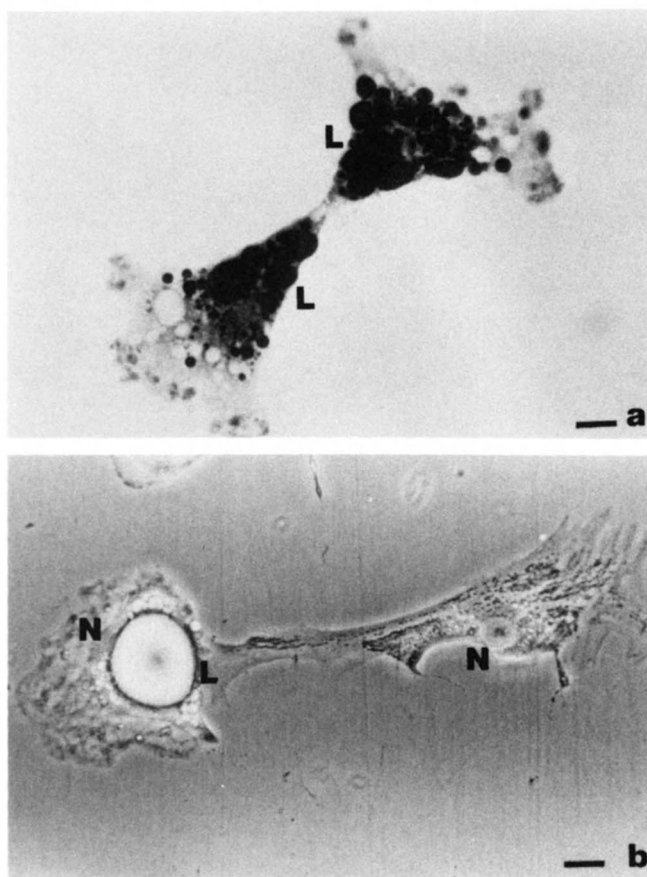


Fig. 4. a) Cell division of multilocular fat cells derived from a young female human. Lipid droplets are transferred equally to two daughter cells. This mode of proliferation of mature fat cells (unilocular fat cells) is "loculus-dividing" cell division; L, lipid droplets; oil red O / hematoxylin stain; bar, 25 μ m. b) Cell division of unilocular fat cells, derived from a young female human, after insulin treatment. This mode is "loculus-preserving" cell division. The unilocular lipid droplet is minimally broken down and is inherited, with its shape preserved, by one of the daughter cells, while the other cell has only a small number of fine lipid droplets; L, lipid droplets; N, nucleus; phase-contrast micrograph; bar, 25 μ m.

Their growth curves are shown in **Fig. 3**. At the proliferative stage, multilocular lipid droplets in a dividing cell were transferred more or less equally to the daughter cells (**Fig. 4a**). Lipid droplets in both types of proliferating fat cells contained triglyceride (stainable by oil red O) and triglyceride lipase activity and glycerophosphate dehydrogenase activity which helped identify the cells as fat cells.

The fat cells in culture maintained normal function as indicated by stimulation of lipolysis by norepinephrine, ACTH, TSH, GH, db-cAMP, or adenosine deaminase, and by lipogenesis stimulated by insulin, adenosine, or phenylisopropyladenosine. For example, in cells derived from 1-week-old rats (after becoming confluent), 10^{-5} M norepinephrine induced dispersion of the fat cell's intracytoplasmic lipid droplets and, at the same time, glycerol content increased in the medium at the rate of 3 μ mol/ 10^6 cells per 60 min. Upon addition of 0.1 mU/ml insulin to

the medium, the fat cells exhibited more numerous and larger-sized cytoplasmic lipid droplets.

The hormones and hormone-like substances mentioned above were added to fat cells in culture from the first day of culture, i.e., at the stage of cellular proliferation. When exposed to 10^{-5} M norepinephrine, the single lipid droplet of unilocular fat cells exhibited extensive division and turned into numerous lipid droplets of much smaller size within 3 days. At the stage of cell division, which subsequently occurred, these divided lipid droplets were transferred roughly equally to the daughter cells (**Fig. 4a**). This mode of division which is designated as "loculus-dividing" cell division was also observed within 3 weeks of culture in human fat cells. The daughter cells containing many fine lipid droplets proliferated extensively until they became confluent, indicating that the small lipid droplets do not inhibit cell division (**Fig. 3**). Cultures of unilocular fat cells stimulated by other lipolytic factors, e.g., ACTH, TSH and GH, also exhibited this mode of proliferation.

Administration of 10^{-4} – 10^{-3} M db-cAMP, however, inhibited adherence of unilocular fat cells when the administration started from the first day of culture. Although adenosine deaminase caused intracytoplasmic lipid droplets to divide very intensively, cell division did not take place in its presence. When insulin, a lipogenic hormone, was present in the culture of unilocular fat cells from the first day of culture, the "loculus-dividing" process was not observed, but an entirely different phenomenon took place in the proliferative process. When exposed to 0.1 mU/ml insulin, the single droplet in the unilocular fat cells grew slightly larger and several small lipid droplets appeared around the large droplet. At the stage of cell division, the lipid content was transferred to the daughter cells in a lopsided manner: one daughter cell received the large droplet and the other only several small droplets (**Fig. 4b**). Cells of the latter type are called fibroblast-like fat cells. This mode of proliferation of unilocular fat cells is referred to as "loculus-preserving" cell division and was observed within 4–7 days of culture in rat fat cells and within 3–4 weeks in fat cells taken from young human females. The fibroblast-like fat cells proliferated extensively (**Fig. 3**), while the unilocular fat cell (as a daughter of the initial dividing cell) underwent a second and sometimes third cell division of the loculus-preserving type. As with insulin, adenosine or phenylisopropyladenosine (which is the lipogenic factor) induced loculus-preserving cell division.

As described above, daughter fat cells proliferated extensively: doubling time was 2.5 days in fine droplet-containing fat cells of loculus-dividing cell division in the presence of norepinephrine (**Fig. 3** and **Fig. 5a**), and it was 2.0 days in fibroblast-like daughter fat cells of loculus-preserving cell division in the presence of insulin (**Fig. 3**, **Fig. 5b**, and **Fig. 6**). After fat cells of either proliferative mode had grown to confluency, and in a lipolytic factor-free condition, the number and size of the cytoplasmic lipid

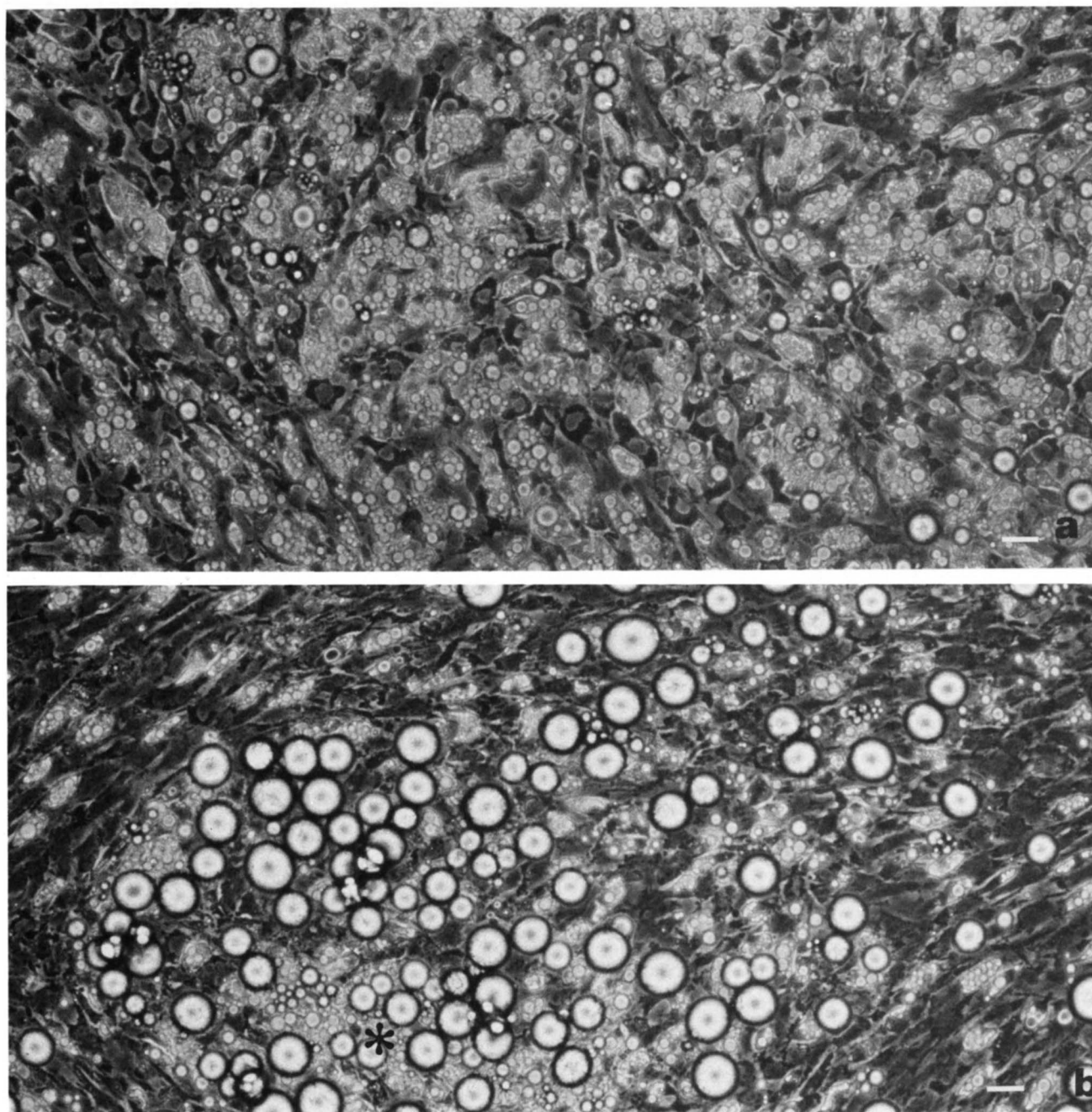


Fig. 5. a) Phase-contrast micrograph of a monolayer culture of mature white fat cells at confluence after norepinephrine treatment. Unilocular fat cells have turned to multilocular fat cells in this condition; bar, 50 μ m. b) Monolayer culture of mature white fat cells at the early stage of confluence after insulin treatment. Unilocular fat cells and fibroblast-like fat cells are intermingled as the result of locus-preserving cell division of unilocular fat cells. At a locally confluent area (*) the fibroblast-like fat cells have already changed to multilocular fat cells; bar, 50 μ m.

droplets increased and a large number of unilocular fat cells were observed. The addition of insulin enhanced such synthesis of lipid droplets (**Fig. 7**).

DISCUSSION

In vitro culturing of mature white fat cells, i.e., unilocular fat cells, has been extremely difficult. The authors have de-

vised a new culture method specifically for this purpose and have designated it "ceiling culture" (9). It has been demonstrated that unilocular fat cells will undergo delipidation and proliferation in vitro (9). In this report, the proliferative manner of the fat cells has been described in some detail. When cultured in Ham F12 medium supplemented with 10% newborn calf serum or 10% fetal calf serum (9), the large lipid droplet in the unilocular fat cell was divided into multiple or much finer droplets as depicted in **Fig. 8**

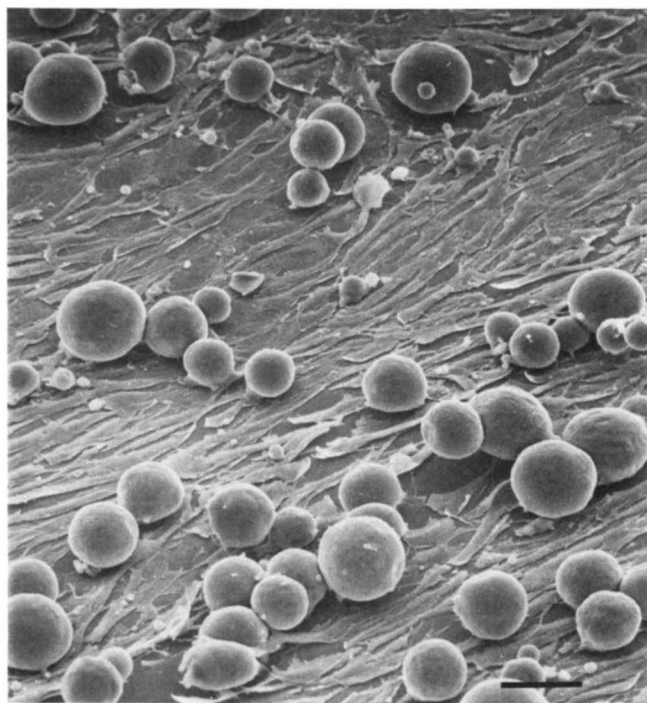


Fig. 6. Scanning electron micrograph of a monolayer culture of mature white fat cells at the early stage of confluence after insulin treatment (the same stage as Fig. 5b). Unilocular fat cells and fibroblast-like fat cells are seen in this figure; bar, 50 μ m.

(course A). This indicated that the presence of lipid droplets, per se, does not inhibit division of the fat cells. This process is referred to as the "loculus-dividing" cell division. Lipolytic factors such as norepinephrine or other lipolytic hormones, which all induce highly active division of lipid droplets, enhance proliferation of unilocular fat cells in this mode (course B of Fig. 8).

Insulin, which is a lipogenic or antilipolytic agent, is one of the major factors of obesity (13, 14), and the effects of insulin on fat tissue have been studied in functional terms. Although it has been assumed that insulin has some effect on fat cell proliferation (3, 4, 8, 15–17), no previous morphological demonstration has shown this to be true because of technical difficulties. Insulin-induced proliferation of fat cells has been demonstrated in vitro morphologically, for the first time, in the "loculus-preserving" mode of cell division in this study. Intracytoplasmic lipid droplets are not divided in unilocular fat cells at the proliferative stage, but this does not mean that cell division is inhibited. Lipid droplets are transferred from dividing to daughter cells in a lopsided manner, and the daughter cell that has received only a few small lipid droplets (referred to as the fibroblast-like fat cell), indeed proliferates more actively in the culture (course C of Fig. 8) (18–20) than either multilocular or unilocular fat cells. This fibroblast-like fat cell synthesized its own triglyceride in the form of lipid droplets when the proliferation ended due to the confluency as previously re-

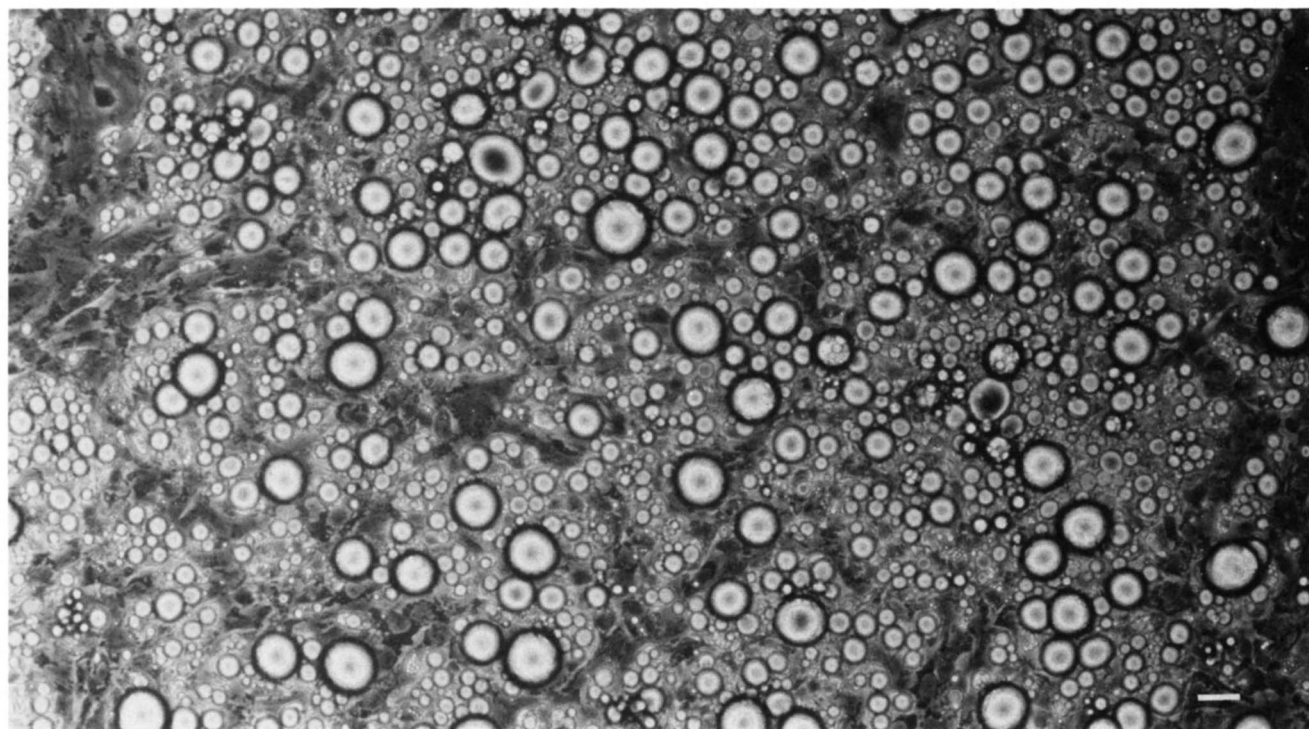
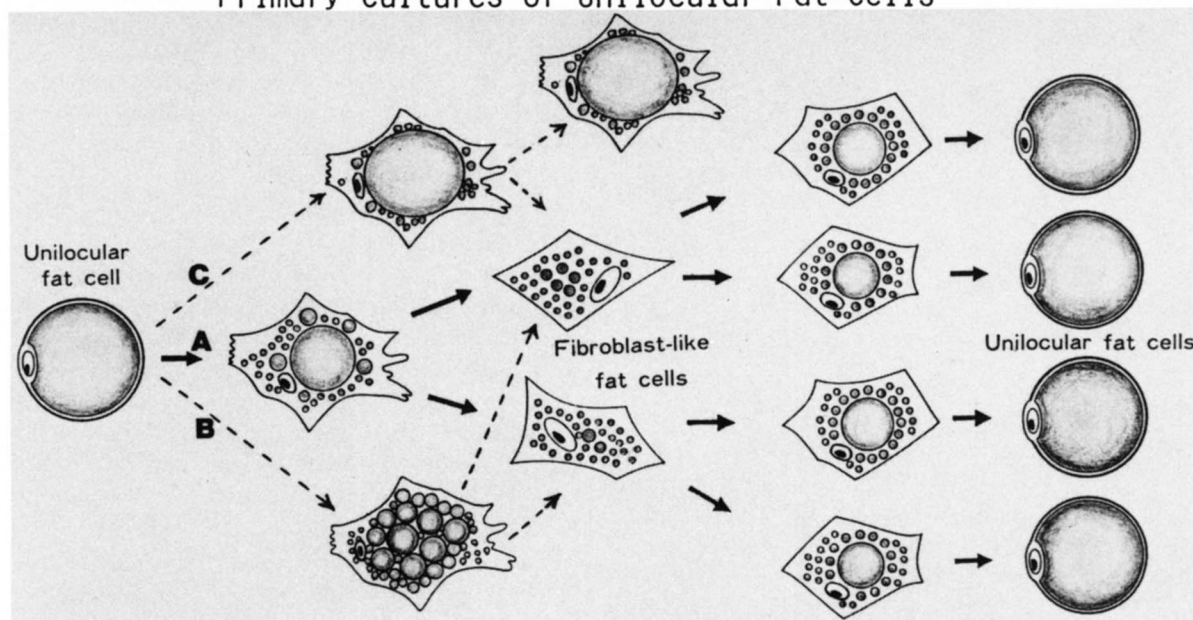


Fig. 7. Monolayer culture of mature white fat cells at the advanced stage of confluence after insulin treatment. Unilocular and multilocular fat cells are seen in this figure. Fibroblast-like fat cells observed in Fig. 5b are no longer seen in this stage.

Primary Cultures of Unilocular Fat Cells



Adhesion ————— Proliferation ————— Differentiation

Fig. 8. Summary of this study and schema of proliferation and differentiation of unilocular fat cells in primary culture. After supplementation with 10% newborn calf serum, unilocular fat cells become multilocular fat cells and, further, fibroblast-like fat cells, which differentiate to unilocular fat cells again. This mode is "loculus-dividing" cell division (course A). After administration of norepinephrine or other lipolytic hormones, active division of lipid droplets is caused and loculus-dividing cell division is enhanced (course B). After insulin treatment, the lipid droplet in the unilocular fat cell is transferred to daughter cells in a lopsided manner. This mode is "loculus-preserving" cell division (course C).

ported in the literature (1-4, 6, 8, 21). Demonstration of the proliferative process of fat cells in the "loculus-preserving" mode above helps elucidate, at least in part, the mechanism of insulin-induced obesity.

In vitro proliferation in the "loculus-preserving" mode is not specific to insulin-treated fat cells. We have observed the same mode of proliferation in the cell division of fat cells derived from adult human or adult rats in the absence of insulin. In the case of "loculus-preserving" cell division of fat cells derived from newborn rats or human adolescents in the presence of insulin, the division of lipid droplets is supposed to be inhibited by the antilipolytic effect of insulin. However, in the case of adult humans or adult rats, fat cells may have no functional ability to enable lipid droplets to divide because they have scanty cytoplasm in comparison to their large lipid droplets (9).

Ever since Rodbell's work (7) in this field, the cellular function of fat cells has been studied using isolated unilocular fat cells. However, another important aspect of fat cells, i.e., cellular proliferation, has not been well elucidated, largely because of the difficulty in devising a reasonable method of culturing fat cells. In our current study, we have offered some details of the manner of proliferation of fat cells

as well as showing that the presence of a large lipid droplet in the cytoplasm is not an inhibitory factor to cell division.

Bertrand et al. (22, 23) have emphasized that there is a change in the cellularity of adipose tissue through adult life, the so-called plasticity in fat cell number. This in vivo finding is compatible with our supposition concerning the proliferation of fat cells. Bertrand et al. (22, 23) showed that the fat cell number in the epididymal fat depots exhibited little change in rats from 6 to 27 months of age. In this regard, the epididymal fat cells of adult rats seem to be inappropriate as material for culture. We have successfully cultured the abdominal subcutaneous and intraabdominal fat cells of various ages, from newborn to adult. It is beneficial for the biological elucidation of obesity to culture in vitro the fat cells of these regions of the adult in variously modified culture conditions. ■

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