A simple culture method of fat cells from mature fat tissue fragments

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Abstract To obtain immature fat cells in vitro, we used a primary culture of undigested mature fat tissue fragments. The immature fat cells, i.e., fibroblast-like fat cells, proliferated extensively from the fat tissue and differentiated after reaching confluence. The process of differentiation was assumed by the development of intracytoplasmic lipid droplets and by the triglyceride content in the cells. Cellular differentiation was induced in high percentages (over 70–80%) of the cells in the medium containing high glucose concentrations (200 mg/dl) supplemented with 10–20% newborn calf serum. The intracellular accumulation of triglyceride was also enhanced by insulin administration. In these cells, a reciprocal relationship was observed between proliferation and differentiation. Fibroblast-like fat cells derived from mature fat tissue in this simple culture system are suitable for the study of the proliferation and differentiation of immature fat cells. —**Sugihara, H., S. Funatsumaru, N. Yonemitsu, S. Miyabara, S. Toda, and Y. Hikichi.** A simple culture method of fat cells from mature fat tissue fragments. *J. Lipid Res.* 1989. **30:** 1987–1995.

Supplementary key words primary culture • fibroblast-like fat cells • proliferation • differentiation

Adipocyte precursors, or preadipocytes, were discovered in the process of tissue culture of the stromal-vascular fraction of mature white fat tissue. There have been a number of studies concerning these cells (1–16). Preadipocytes are fusiform with fine lipid droplets in the culture condition. In morphological terms, therefore, they are also called fibroblast-like fat cells. They can be stimulated to proliferate and differentiate to exhibit intracytoplasmic triglyceride in the form of droplets, which reveal the morphological and enzymological characteristics of mature adipocytes. As mentioned above, preadipocytes were taken from the stromal-vascular fraction, which is the centrifugal sediment of digested mature fat tissue (4, 6). When this material is centrifuged, mature fat cells, or unilocular fat cells, are obtained at the uppermost layer; however, they were not used for tissue culture. We have cultured these unilocular fat cells by novel methods, namely, by the ceiling culture method (17, 18) and by collagen gel matrix culture (19). Through these culturing studies, it has been demonstrated that unilocular fat cells dedifferentiate to fibroblast-like fat cells, which in turn proliferate and redifferentiate to unilocular fat cells (18). Fibroblast-like fat cells derived from unilocular fat cells are not distinguishable from those derived from the stromal-vascular fraction with regard to the morphological and lipid or enzyme histochemical findings. In this study, mature fat tissue fragments were cultured without digestion and we obtained fibroblast-like fat cells which showed characteristically a high ratio of cellular differentiation. These cells were derived not from isolated unilocular fat cells or the stromal-vascular fraction but from undigested mature fat tissue. They were studied in terms of proliferation and differentiation in comparison with the preadipocytes derived from the stromal-vascular fraction. We also describe a new and simple method of culturing fibroblast-like fat cells taken from the mature fat tissue of newborn rats.

MATERIALS AND METHODS

Ceiling culture of mature fat tissue fragments and the routine culture of stromal-vascular fraction

Abdominal subcutaneous fat tissue was taken for culture from 3-day- to 4-week-old male Wistar rats. The fat tissue was chopped into very small pieces (0.2–0.3 mm in diameter) and centrifuged in buffer solution. Fragments were separated into two fractions: floating tissue and sedimented tissue. The floating tissue fragments consisting of mature unilocular fat cells were incubated with Ham F-12 (glucose concentration, 200 mg/dl) supplemented with 20% newborn calf serum at 37°C by the ceiling culture method (**Fig. 1**), which was described previously (17, 18).

Abbreviations: db-cAMP, N^6-2'-dibutyryladenosine-3'-5'-cyclic monophosphate Na-salt; BrdU, bromodeoxyuridine.

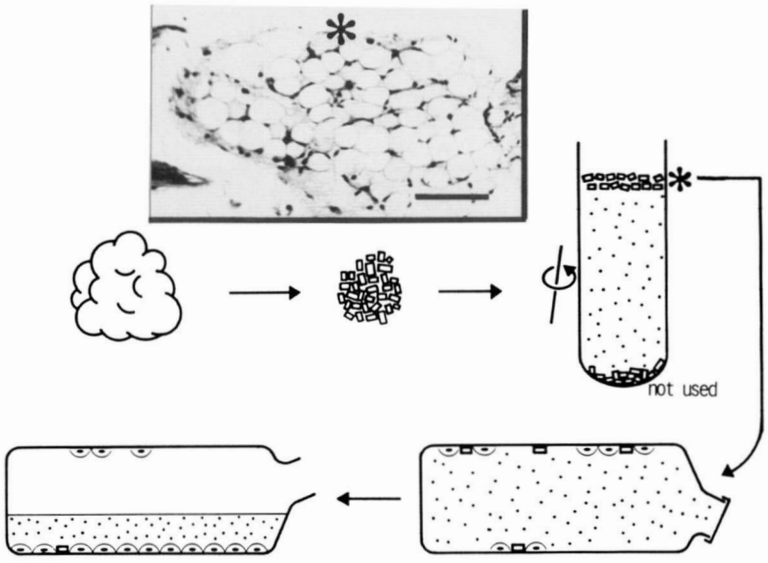


Fig. 1. Ceiling culture of mature fat tissue fragments. Mature white fat tissue was chopped into small pieces and centrifuged. Sediments were not used in this culture. Floating tissue fragments were incubated in culture flasks that were completely filled with medium. The fragments floated to the top of the medium and adhered to the top inner surface (ceiling surface) of the flask. When the fragments had attached firmly, the flasks were placed upside down to allow regular observation. Fibroblast-like fat cells proliferated extensively from the tissue fragments. Inset: histology of the floated tissue which consists for the most part of unilocular fat cells with a few stromal-vascular cells. Bar, 100 μm.

Briefly, tissue fragments were incubated in culture flasks (Lux 5325 or Nunc 170920) that were completely filled with medium. When the fragments had firmly attached to the inner upper surface, i.e., the ceiling of the flask, the flask was inverted to allow regular treatments. Some fragments sank in the medium and attached to the bottom surface; fibroblast-like fat cells proliferated from these fragments. However, these cells were not used in this experiment because of the low differentiation ratio (40–50%).

To obtain preadipocytes, cell isolation from abdominal subcutaneous fat tissue of newborn rats was performed by collagenase-digestion according to Björntorp et al. (4, 6). The isolated stromal-vascular cells were incubated with Ham F-12 supplemented with 20% newborn calf serum.

Chemicals and other substances added to cultured cells

The following were selectively added to the medium: *1*) 0.1–0.5 mU/ml insulin (Sigma); *2*) 10^{-4}–10^{-3} M N^6-2'-dibutyryladenosine-3'-5'-cyclic monophosphate Na-salt (db-cAMP); *3*) 10^{-5} M norepinephrine (Sigma); *4*) 10^{-4} M hydroxyurea (Sigma); *5*) fetal calf serum, calf serum, and horse serum; and *6*) serum-free medium (Ultrosera G from LKB and NU-serum from Collaborative Research Inc).

Examination of culture cells

The cultured cells were counted and examined by the following procedures: *1*) phase contrast microscopy; *2*) lipid histochemistry using oil red O stain and enzyme histochemistry of triglyceride lipase and glycerophosphate dehydrogenase; *3*) assay of triglyceride in the culture cells by the acetyl acetone method (20); cultured cells were harvested using a rubber policeman and were added to a solution of ethyl ether–ethanol for extraction of triglyceride; the extract was concentrated before the assay of triglyceride; *4*) immunohistochemical examination of bromodeoxyuridine (BrdU) uptake by cultured cells, using a monoclonal anti-BrdU antibody (21); and *5*) assay of glycerol in medium (22).

RESULTS

Fat tissue fragments became attached to the ceiling surface of the flasks on day 2 of culture, and some fibroblast-like fat cells appeared (**Fig. 2** and **Fig. 3**). On day 3–4 of culture, the cells began to proliferate extensively around the attached tissue. It was at this stage that the flask could be inverted without disturbing the culture process and the medium could be withdrawn to allow regular treatment. During the following week, most of tissue fragments detached and floated to the medium surface. However, this detachment no longer influenced the cells in culture. These fibroblast-like fat cells and scattered multilocular fat cells proliferated in dense arrangements until they became confluent (**Fig. 4**). Better results were achieved by using fat tissue taken from young rats 7–10 days of age. Consequently, we used only tissue from young rats in the following experiments. Supplementation of the medium

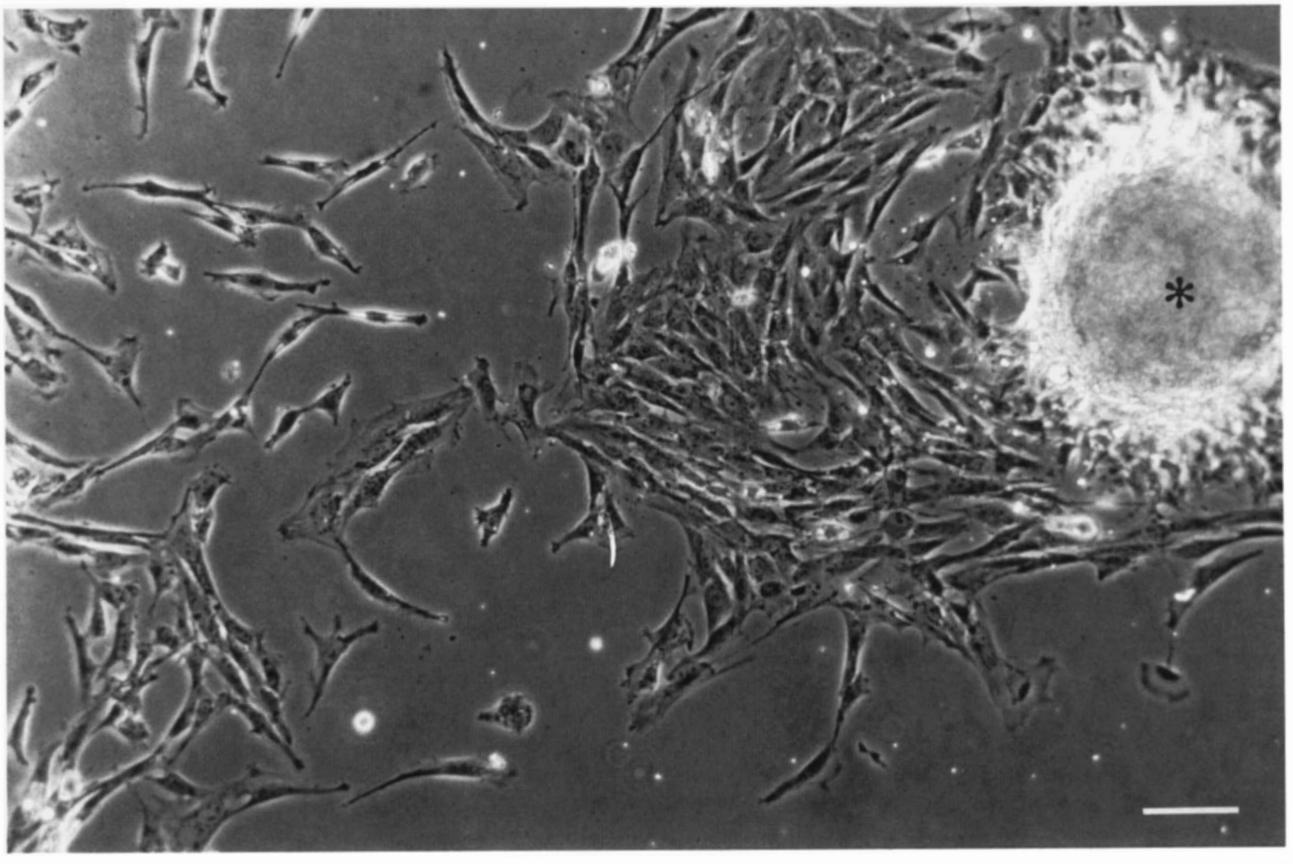


Fig. 2. The early stage (day 2–3 of culture) of ceiling culture of mature fat tissue fragments. A fragment of tissue (*, ca 0.3 mm in diameter) attached to the culture surface and fibroblast-like fat cells proliferated extensively from it. Phase-contrast micrograph. Bar, 100 μm.

with 20% newborn calf serum resulted in the best tissue attachment to the flask surface and cell growth, and also cellular differentiation. After cells grew from the tissue, the condition of 20% newborn calf serum was changed. The growth of cultured cells was examined under the conditions of 2, 5, 10, and 20% newborn calf serum, and insulin administration. The growth curve is shown in **Fig. 5** and the generation time was less than 2 days when 10% or 20% newborn calf serum was used. Insulin administration slightly accelerated the process of proliferation of the cells in culture.

After the culture cells had grown to confluency and proliferation was inhibited by cell-to-cell contact, the differentiation was observed first. This meant immediate identification of the cells in this culture system as fat cells. In these fibroblast-like fat cells, the intracytoplasmic small lipid droplets increased in number and grew larger until they became unilocular (**Fig. 6**). The lipid droplets were positive by oil red O staining and, in this study, this was taken as an indication of differentiation of the fat cells. In addition to the positive staining of lipid droplets there was histochemical evidence of glycerophosphate dehydrogenase and triglyceride lipase activities (Fig. 3, inset). The evaluation of cellular differentiation to mature fat cells was easily done by observing the intracytoplasmic contents of lipid droplets with a phase contrast microscope and oil red O staining, or by chemical assay of the intracellular triglyceride. The differentiation occurred in such a way that there were nodes that represented locally confluent patches. Unilocular fat cells were observed in the core of the nodular area, while there were multilocular fat cells in the periphery. The multilocular fat cells, too, became gradually unilocular, and the nodular areas fused together to form wide islands. The ratio of differentiated cells in the entire culture was over 70% on day 30 of culture under the best conditions (Ham F-12 medium supplemented with 20% newborn calf serum), and it was below 10% when the concentration of newborn calf serum was 2% (**Fig. 7**). In the presence of insulin, the ratio increased to 90% even with 2% newborn calf serum (**Fig. 8**). The counting of the number of differentiated cells was not always accurate, thus the triglyceride contents in culture cells was assayed (**Fig. 9**). The synthesis of triglyceride increased in a dose-dependent manner in medium supplemented with 2, 5, 10, and 20% newborn calf serum, and it

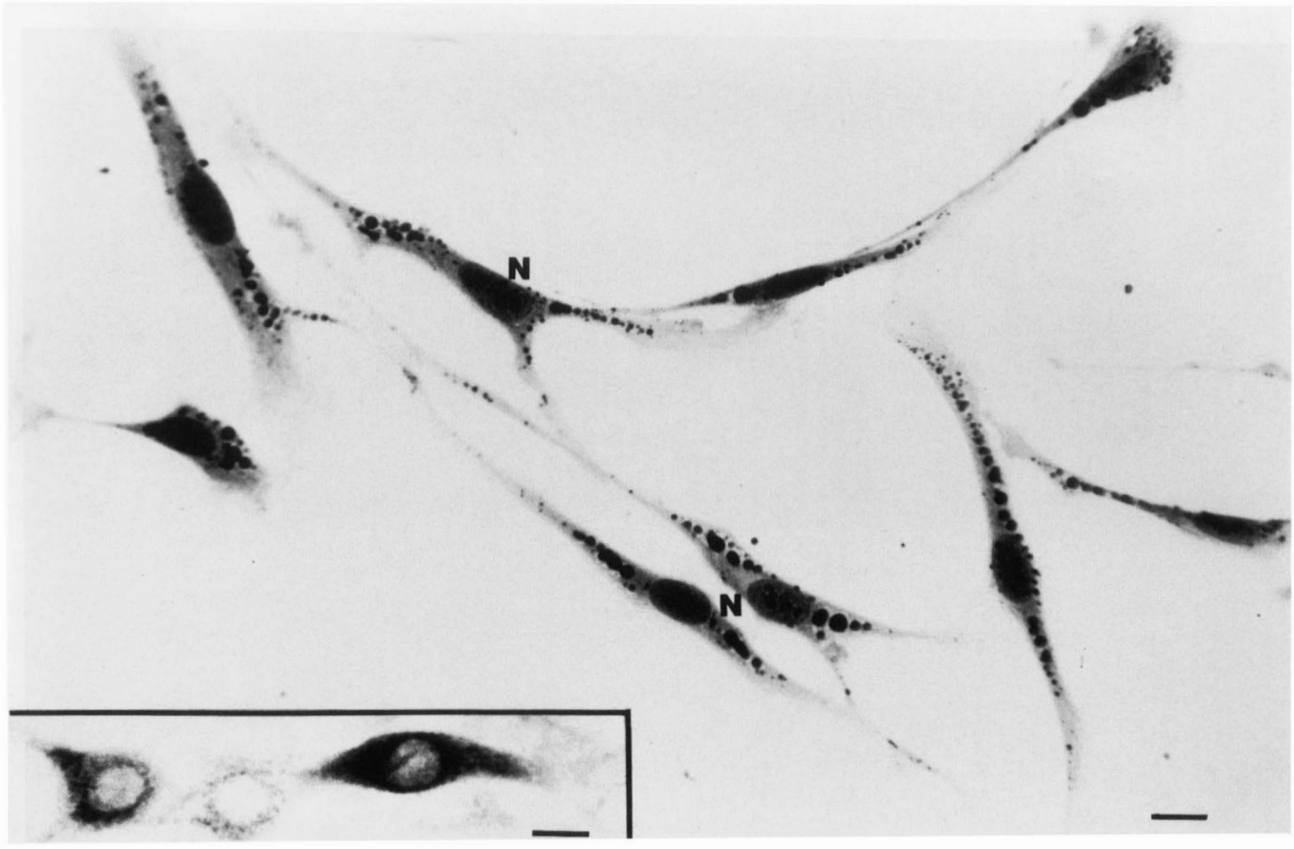


Fig. 3. High-power view of fibroblast-like fat cells in culture. These cells are long-fusiform with fine lipid droplets. Oil red O stain. N, nucleus, bar, 20 μm. Inset: histochemical activity of glycerophosphate dehydrogenase in these cells. Bar, 20 μm.

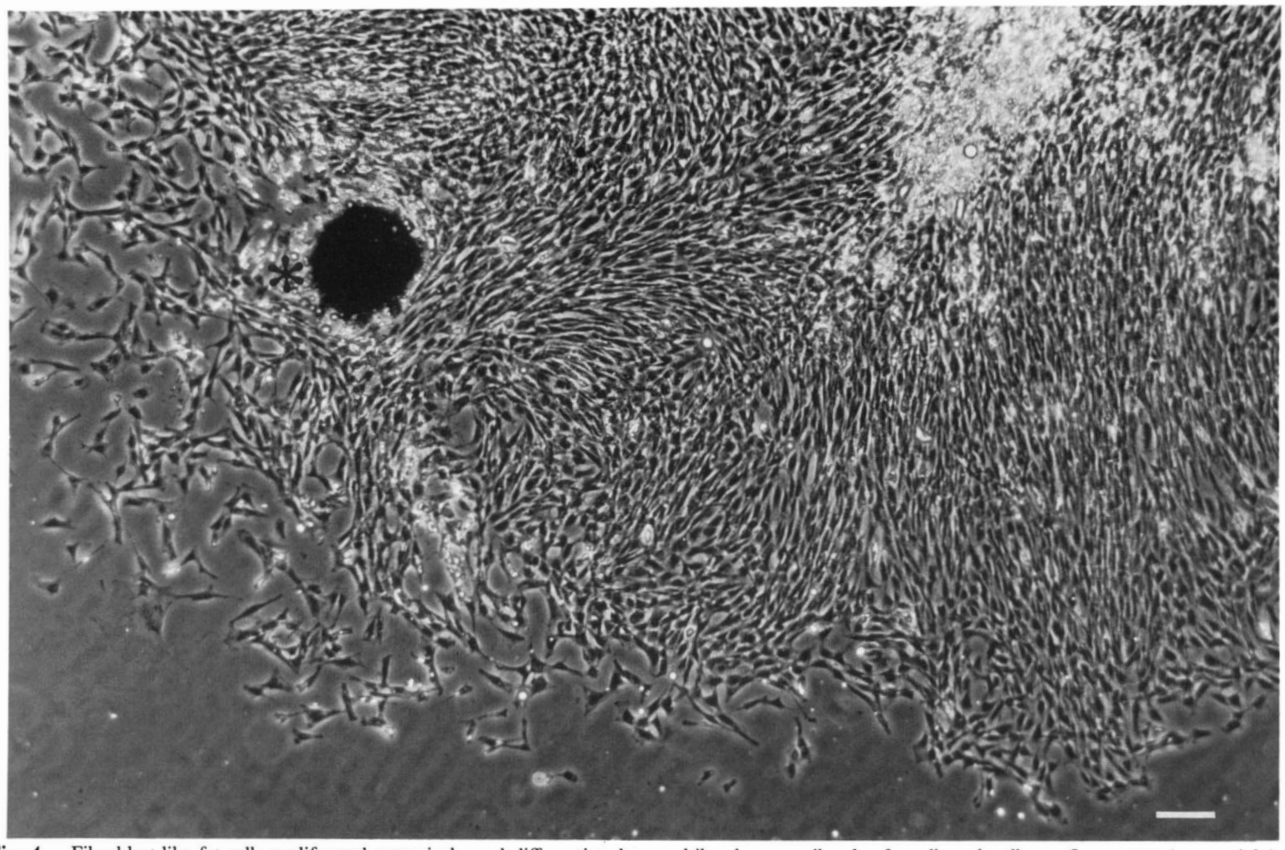


Fig. 4. Fibroblast-like fat cells proliferated extensively and differentiated to multilocular or unilocular fat cells at locally confluent area (upper right); *, detached tissue fragment. Phase-contrast micrograph. Bar, 100 μm.

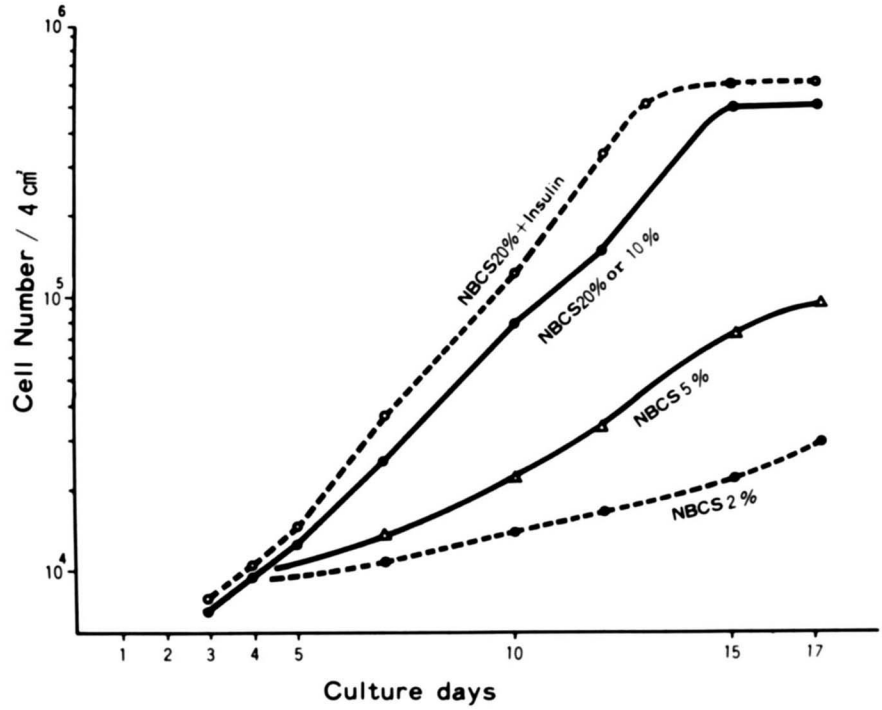


Fig. 5. Growth curve of fat cells in culture. Cells were derived from mature fat tissue fragments of a 1-week-old rat and were incubated with medium supplemented with 2, 5, 10, and 20% newborn calf serum (NBCS), and 20% NBCS with insulin (0.1 mU/ml).

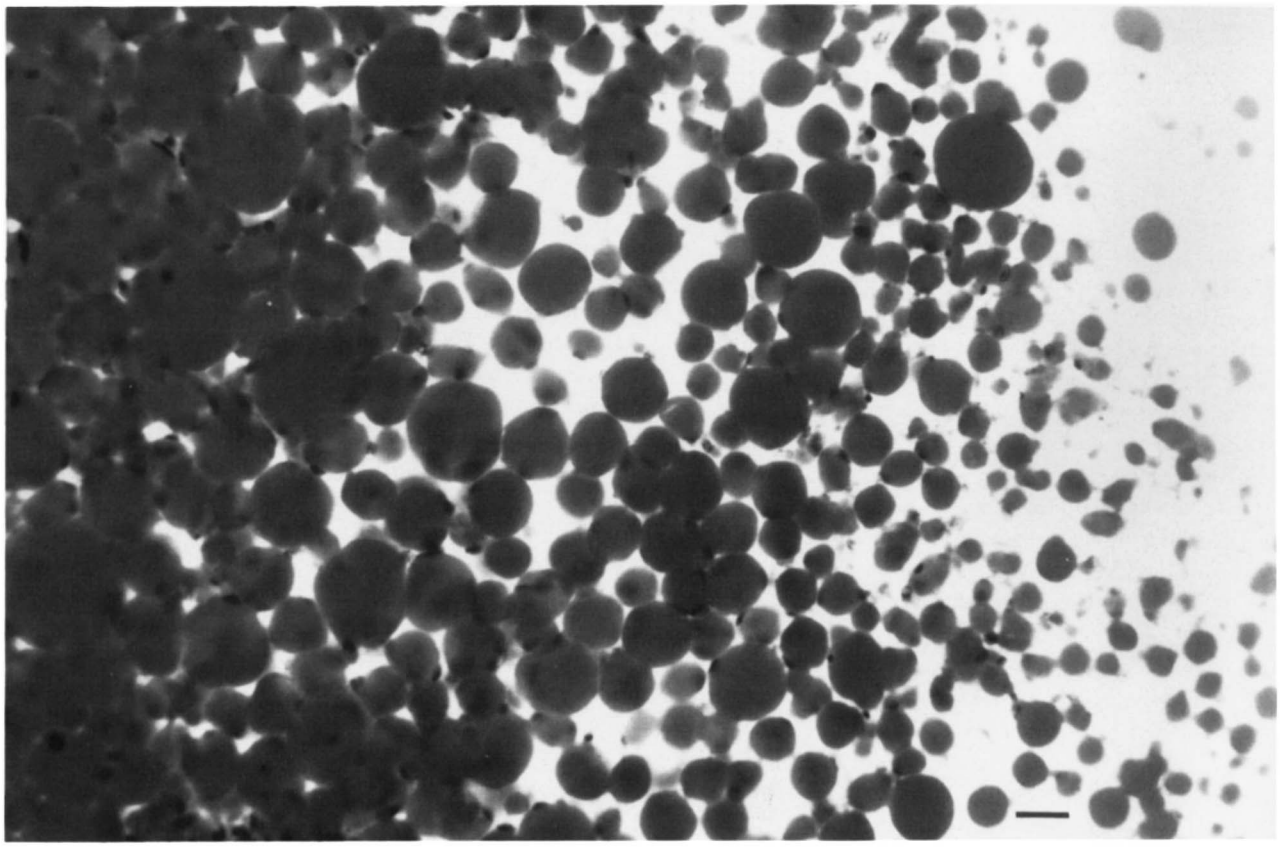


Fig. 6. At a locally confluent area, the fibroblast-like fat cells differentiated to mature (unilocular) fat cells. Large unilocular fat cells are observed in the core of the confluent area (left side). Oil red O stain. Bar, 50 μm.

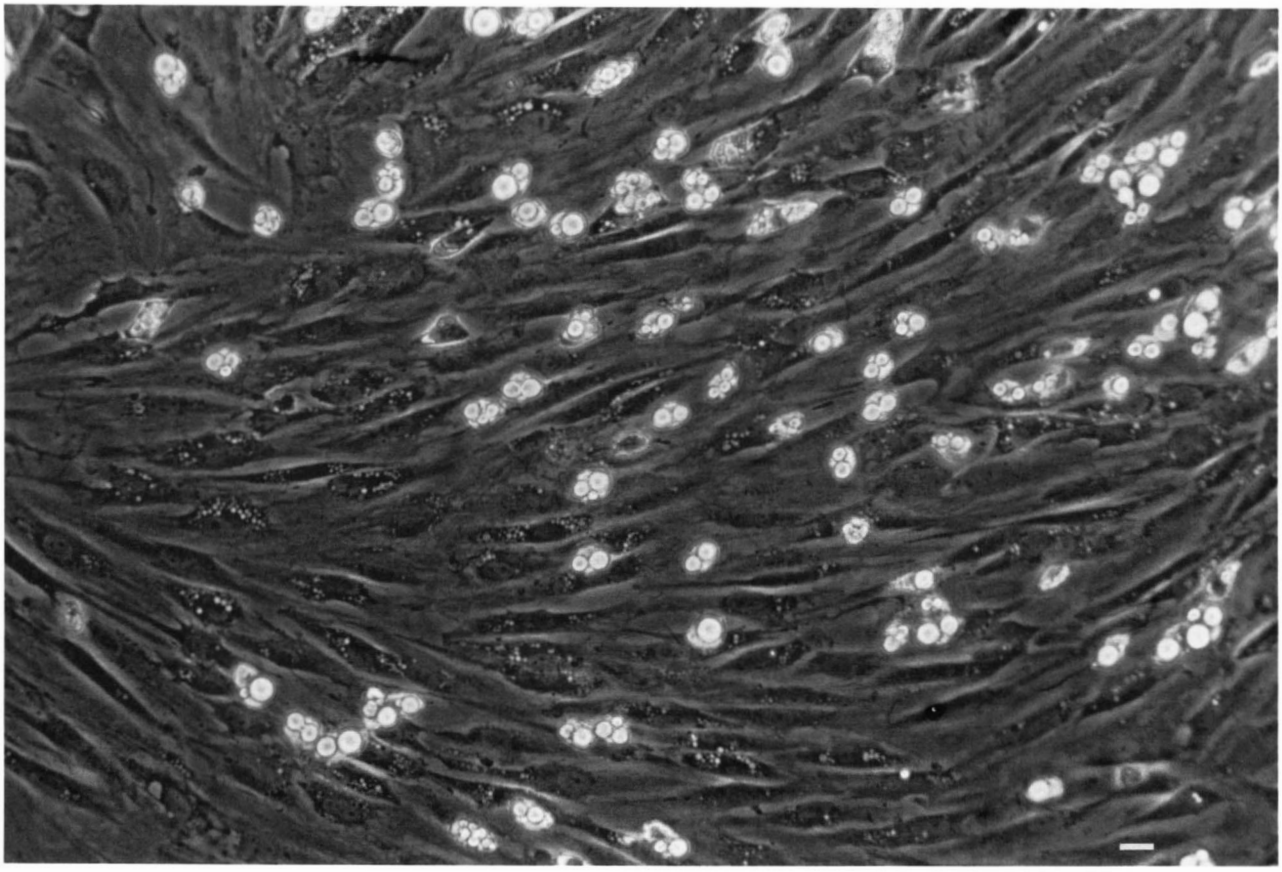


Fig. 7. Cultured fat cells incubated in medium (Ham F-12) supplemented with 2% newborn calf serum for 10 days. Large lipid droplets are seen in the cytoplasm, and this indicates cellular differentiation. The ratio of differentiated cells among culture cells is about 10%. Phase-contrast micrograph. Bar, 20 μm.

decreased when fetal calf serum was added to the medium. The glycerol concentration was higher in the presence of fetal calf serum than with newborn calf serum. These findings suggest that fetal calf serum had a higher concentration of lipolytic factors than newborn calf serum because approximately the same amount of insulin was present in both sera. By supplementation with horse or human serum, the triglyceride rapidly increased in the cultured cells, but most of them detached and floated up a week before maturation (Fig. 9). Insulin administration apparently accelerated the synthesis even in the presence of 2% newborn calf serum. In the preadipocytes derived from the stromal-vascular fraction of fat tissue, the ratio of differentiation in the number of cells was below 50% even under the optimal condition of 20% newborn calf serum supplementation, and the synthesis of triglyceride was low in comparison with the cells derived from mature fat tissue (Fig. 9).

We examined the lipolytic function of the differentiated culture cells by stimulating them with norepinephrine or db-cAMP. For example, 10^{-4} M db-cAMP or 10^{-5} M norepinephrine induced lipolysis of triglyceride in intracytoplasmic lipid droplets, resulting in cellular retraction. There was a concomitant increase in glycerol in the medium at the rate of 2–3 or 1.5–2 μmol/h per 10^6 cells, respectively.

The relationship between proliferation and differentiation of these culture cells was also examined. Lipogenesis in the cells occurred after the contact inhibition of proliferation brought about by confluency. This suggests a reciprocal relationship between proliferation and differentiation of these cells. This supposition was examined by uptake of BrdU by the culture cells. One hour after administration of BrdU, the labeling index was 15–20% in fibroblast-like fat cells and below 1% in the differentiated fat cells, i.e., in multi- and unilocular fat cells, respectively. In this study, 2000 cultured cells were counted (**Fig. 10**). Hydroxyurea, an inhibitor of cellular proliferation, completely inhibited the uptake of BrdU by cells, and 40–45% of the cells differentiated to mature fat cells in 5 days without becoming confluent. These experiments with BrdU and hydroxyurea support the supposition described above.

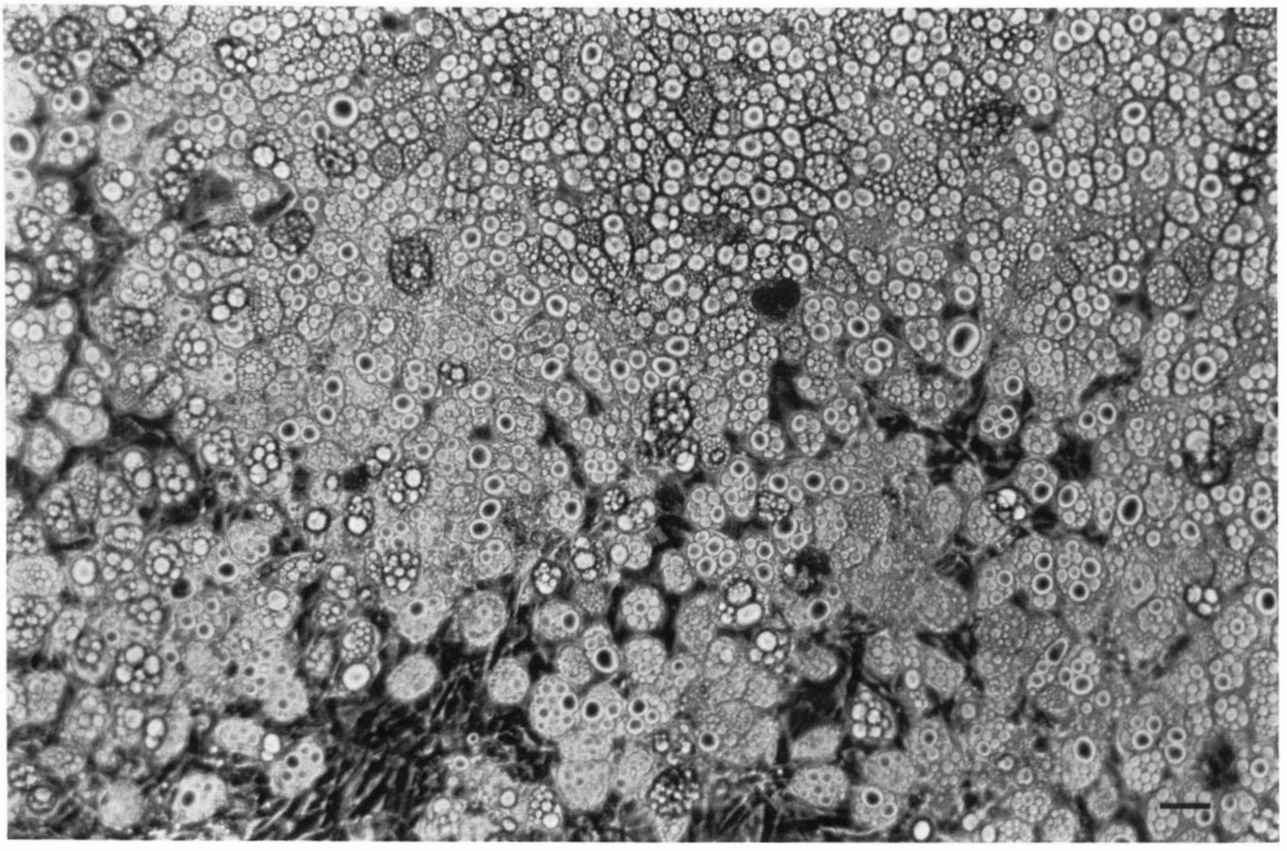


Fig. 8. Insulin (0.1 mU/ml) was administered to culture fat cells of Fig. 7. The ratio of differentiated cells increased to over 90% in 7–10 days. Phase-contrast micrograph. Bar, 30 μm.

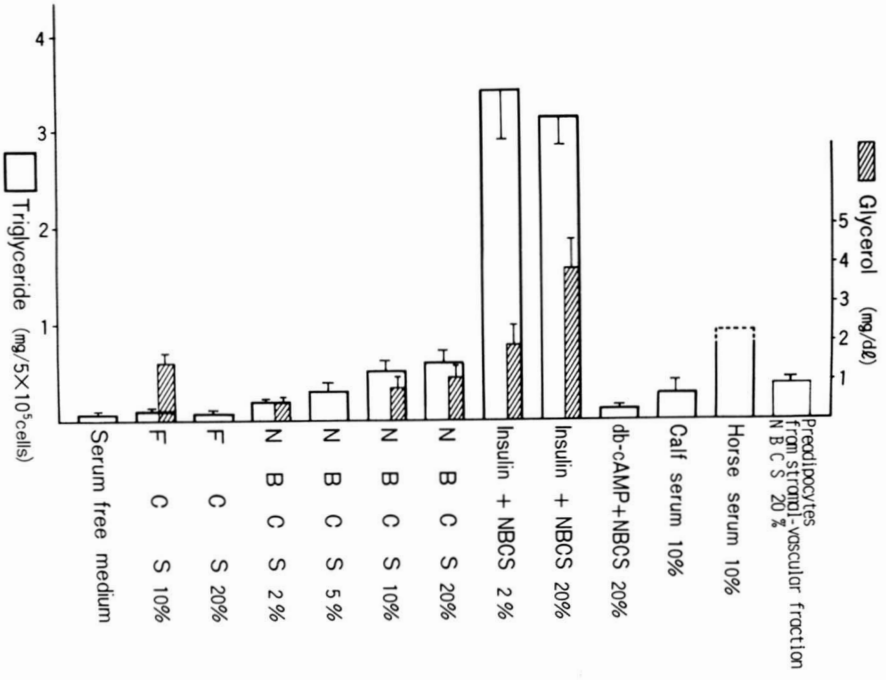


Fig. 9. Synthesis of triglyceride by cultured fat cells and glycerol concentration in the medium. Glycerol was measured after culturing for 3 days at the stage of confluence. Except for the last column, all cells were derived from mature fat tissue fragments. FCS, fetal calf serum; NBCS, newborn calf serum; db-cAMP, dibutyryl cyclic AMP. Administered insulin concentration, 0.1 mU/ml.

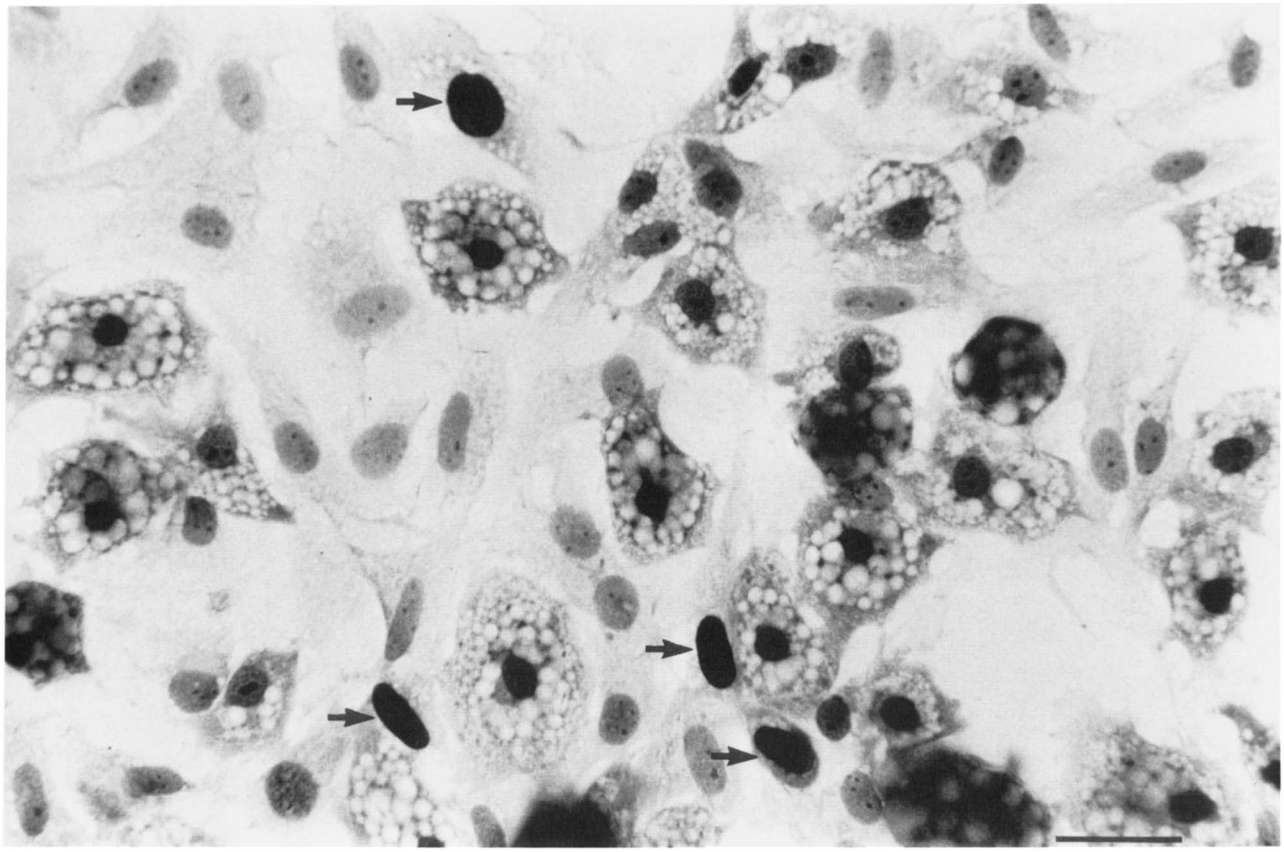


Fig. 10. Immunohistochemistry of bromodeoxyuridine (BrdU) by anti-BrdU antibody of culture fat cells. Four nuclei (→) of fibroblast-like fat cells that have a few lipid droplets show positive results, while nuclei of multilocular fat cells are negative. Bar, 30 μm.

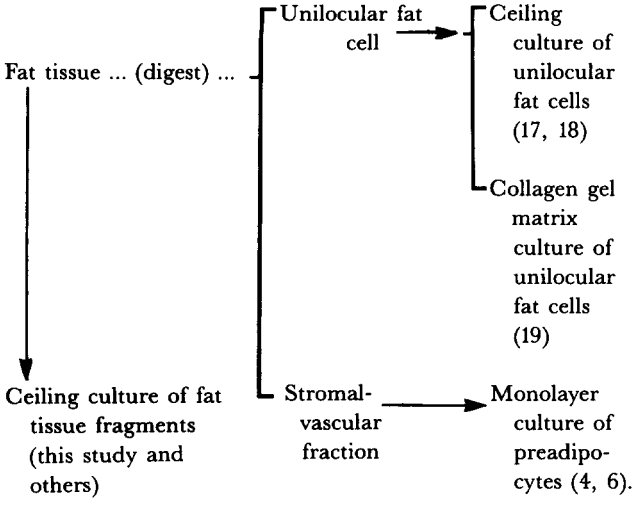
DISCUSSION

In this study, cells derived from mature fat tissue were cultured and examined in terms of proliferation and differentiation. They showed a fibroblast-like appearance with fine lipid droplets, and differentiated to mature fat cells that, in turn, exhibited the specific enzymatic activities of glycerophosphate dehydrogenase and triglyceride lipase as do fat cells. These cells also reacted to insulin, norepinephrine, db-cAMP, and other hormones. These characteristics allowed unequivocal identification of the cells as mature fat cells. These findings are similar to those with preadipocytes which are usually obtained from the stromal-vascular fraction of digested mature fat tissue (4, 11, 12, 15). We could not distinguish between the preadipocytes derived from the stromal-vascular fraction and fibroblast-like fat cells derived from mature fat tissue fragments.

Fibroblast-like fat cells derived from mature fat tissue offer unique features for biological study of fat cells. First, they are homogeneous and, therefore, the ratio of differentiation is high (over 70–80%). Second, the rate of differentiation is high. In general, the mature fat tissue-derived fibroblast-like fat cells began to differentiate in 7–10 days in the medium supplemented with 20% newborn calf serum. Finally, the culture method using undigested tissue fragments is common in the biological studies of cells from many other organs or tissues, e.g., skin, kidney, pancreas islets, and bone marrow. Ceiling culture of mature fat tissue fragments seems to be able to represent the cellular proliferation of the fat tissue in vivo. We have separately cultured stromal-vascular cells and unilocular fat cells after digestion of tissue with collagenase. However, these two cell types are considered to proliferate concomitantly in vivo. In the present method using fat tissue, these cells are cultured together. The origin of the fibroblast-like fat cells is not entirely clear, but there are two possibilities. It is conceivable that these cells arise through dedifferentiation of mature fat cells, or they arise from stromal-vascular cells in the tissue. In the former, very small fat cells (23) in the mature fat tissue are included. These cells are thought to be one of the origins.

We have previously described procedures for ceiling culture (17, 18) and collagen gel matrix culture (19) using unilocular fat cells, and these culture systems helped elucidate the processes of dedifferentiation and redifferentia-

tion of unilocular fat cells. In comparison with these previous culture systems, the method reported in the present study seems more practical for culturing mature fat tissue, simply because of the ease of the procedure. By this method, we can constantly obtain fibroblast-like fat cells that are nearly homogeneous. Mature fat tissue fragments can also be used for the collagen gel matrix culture system. The method for culture of fat cells is summarized as follows:



We wish to thank Mr. H. Ideguchi for his excellent technical assistance, Prof. K. Tohkaichi for editing the English text, and Prof. H. Sugimori, Prof. T. Hisatsugu, Prof. H. Watanabe, and Miss K. Naganuma. This study was supported by the Japan Foundation for Health Sciences and a Grant-in-Aid for Scientific Research in Japan (63570166).

Manuscript received 5 May 1989 and in revised form 28 July 1989.

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