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Proliferation and Differentiation of Unilocular Fat Cells in the Bone Marrow

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ABSTRACT. **Fat cells contribute not only to systemic lipid metabolism, but also to osteogenesis and hemopoiesis in the bone marrow. The present study represents the first attempt to culture mature unilocular fat cells of the bone marrow. Two methods devised in our laboratory were employed: one is the "ceiling culture method" that utilizes the floating property of the cells; the other, a three-dimensional collagen gel matrix culture that captures unilocular fat cells in the gel matrix. Using these methods, the proliferation of unilocular fat cells from the bovine metacarpal bone marrow was demonstrated. First, we confirmed the proliferative ability of unilocular fat cells derived from the bone marrow, using autoradiography to study ^{3}H-thymidine incorporation into the nuclei. The unilocular fat cells de-differentiated into fibroblast-like fat cells and then proceeded to proliferate. When they underwent a contact inhibition of growth, re-differentiation from fibroblast-like fat cells into unilocular fat cells occurred at a high rate. A specific enzymatic marker of the fat cell, α-glycerophosphate dehydrogenase activity related to lipogenesis, was then demonstrated in the cultured fat cells. We examined the functional reactivity of the fat cells by treatment with insulin and cyclic-AMP, and both lipogenesis and lipolysis were also confirmed in them.**

We concluded that unilocular fat cells from the bone marrow de-differentiated, proliferated and re-differentiated in culture. The present results may help to clarify the various functions of fat cells in the bone marrow.

Key words: bone marrow/adipose tissue/unilocular fat cells/culture/proliferation/differentiation

In recent years it has been recognized that the adipose tissue not only plays an important role in systemic lipid metabolism, but also in the production of various kinds of cytokines which exert a great influence on other tissues. Cell biological studies of fat cells in the bone marrow are prerequisite for elucidating the role of adipose tissue, compared with subcutaneous fat cells. Fat cells in the bone marrow have been considered to have five major roles of fat cells in the bone marrow (9): 1) a passive role, simply occupying excess space in the bone marrow cavity; 2) an active role in systemic lipid metabolism; 3) a localized energy reservoir in the bone marrow; 4) a direct contribution to the promotion of hematopoiesis (2, 14); 5) a direct contribution to the promotion of osteogenesis (17). In particular, the contribution to bone fracture healing and osteoporosis has attracted attention in the orthopedic field. Nuttal *et al.* have shown that "human osteoblast-like cells" derived from human trabecular bone were not only osteogenic, but were able to undergo adipocyte differentiation under defined culture conditions (18). In the adipose tissue, unilocular fat cells and preadipocytes exist as mature cells and immature cells, respectively. The former constitutes most of the adipose tissue and has a large lipid droplet in the cytoplasm. The latter is fibroblast-like and has minute lipid droplets. Only preadipocytes have been used as the material for cell biology in the literature (3, 4, 12, 19, 25, 31). Due to the fact that unilocular fat cells are generally considered to be in the terminal stage of differentiation and to be stationary cells which have lost their proliferative ability, they have not been studied in cell culture systems. In addition, conventional culture methods cannot be used because the unilocular fat cells

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Abbreviations: α-GPDH, α-glycerophosphate dehydrogenase; BrdU, bromodeoxyuridine; db-cAMP, N^6-2′-dibutyryl adenosine-3′-5′-cyclic monophosphate Na-salt.

float within the liquid. Some investigators have tried to culture unilocular fat cells, but their methods have been found to be too technically difficult to be repeated by other investigators (1, 22). We have devised new culture methods for subcutaneous unilocular fat cells and observed the proliferation and differentiation of the cells. In this study, we tried for the first time to culture unilocular fat cells from bone marrow using these methods and observed that the cells proliferated extensively. We describe here the cellular behavior of unilocular fat cells of the bone marrow, i.e., proliferation and differentiation, under culture conditions.

Materials and Methods

Isolation of unilocular fat cells

Adipose tissue from the bone marrow of metacarpal bones from 1- to 3-year-old calves was used for the cultures (30). To obtain unilocular fat cells, the adipose tissue was digested with a collagenase solution, according to Rodbell's method (23). The adipose tissue was chopped into small pieces and digested with a 0.2% collagenase solution at 37°C. After filtration and centrifugation of the digested cell suspension, unilocular fat cells were obtained in a thin, white, floating layer. To avoid contamination with stromal cells, we thoroughly digested the adipose tissue and repeated the pipetting, washing, and centrifugation of the dissociated fat cells three times. Centrifugation was performed at 700 rpm for 5 minutes at room temperature (20°C). With these procedures, unilocular fat cells were almost completely isolated without stromal cells such as preadipocytes, fibroblasts and endothelial cells.

Ceiling culture method

We devised a ceiling culture method to culture unilocular fat cells which floated in the liquid (26, 27). Fat cells obtained in the procedure mentioned above were incubated at 37°C in a medium consisting of equal volumes of Ham F-12 with 20% newborn calf serum (pH 7.2–7.4) and 50 μg/ml gentamycin. The concentration of glucose is high (200 mg/dl) in Ham F-12 medium. The flasks were completely filled with the medium. Initially, the flasks were turned upside down, and the cells floated up in the medium and adhered to the inner top

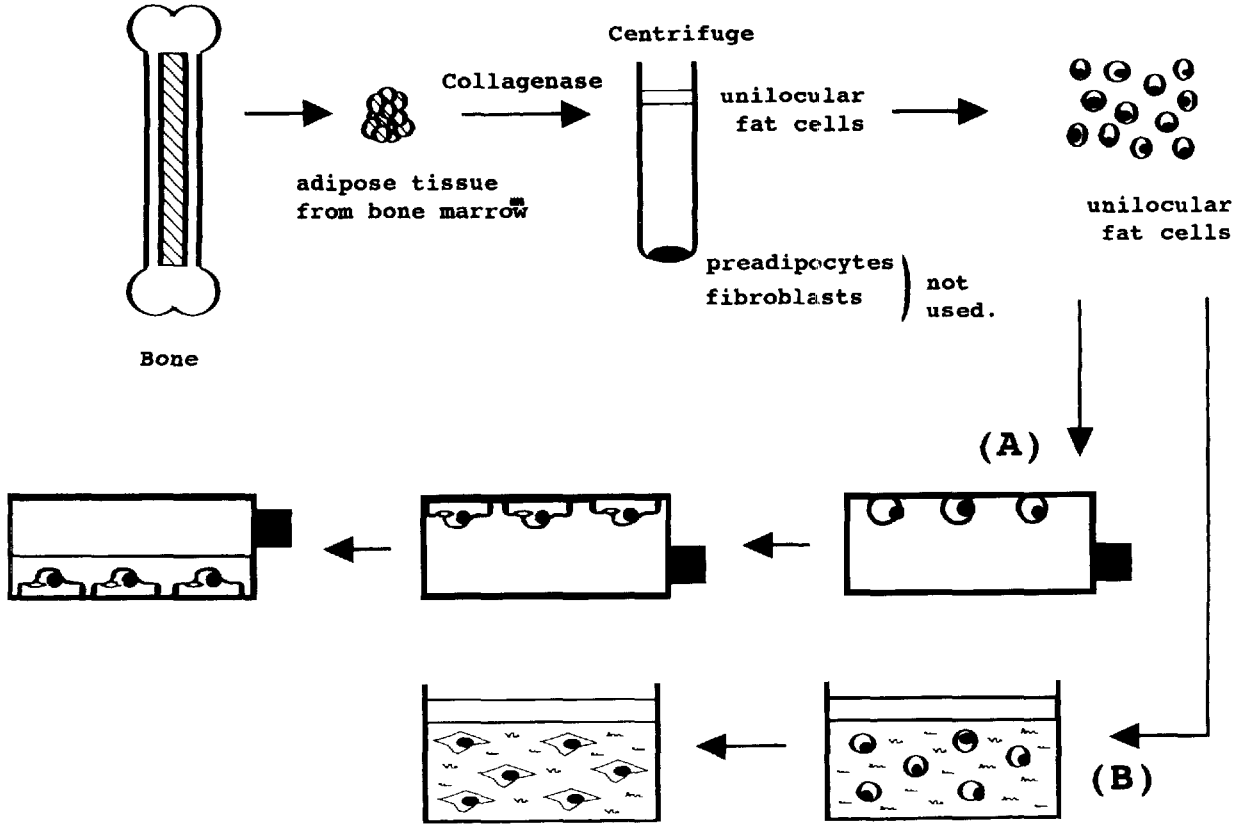


Fig. 1. The methods used for culturing unilocular fat cells. (A), Ceiling culture. The isolated unilocular fat cells were incubated in culture flasks. The unilocular fat cells floated to the inner top of the medium and adhered to the top surface of the flask. When the cells had firmly attached, the flask was placed upside down to allow regular observation. (B), Three-dimensional collagen gel matrix culture. The separated unilocular fat cells were embedded in collagen gel matrix and cultured.

surface (ceiling surface) of the flask. When the cells had become firmly attached, the flasks were turned so that the cells were on the inner bottom surface and could be easily observed. These procedures are shown schematically illustrated in Figure 1 (A).

Three-dimensional collagen gel matrix culture

This culture method was initiated by Elsdale and Bard (6). Briefly, eight volumes of acid-soluble type I collagen solution (Nitta Gelatin Co. Ltd., Osaka, Japan) were mixed with one volume of 10 × concentrated Ham F-12 (without $NaHCO_3$) and one volume of reconstruction buffer (2.2 g of $NaHCO_3$ and 4.77 g of HEPES in 100 ml of 0.05 N NaOH). This mixture, kept on ice, was mixed with the isolated unilocular fat cells. A two ml collagen solution containing 4×10^5 cells/ml was placed into a culture dish to form a gel. When the gel had hardened sufficiently, it was overlaid with 2 ml/dish of Ham F-12 medium supplemented with 20% newborn calf serum and 50 μg/ml gentamycin. These procedures are schematically illustrated in Figure 1 (B).

Chemicals added to cell cultures

The following chemicals were added to the media of some cultures. A Wilcoxon signed-ranks test was used to demonstrate that the data were significantly different. 1) 0.1 mIU /ml insulin (Sigma, St. Louis, MO, USA); 2) 10^{-4} to 10^{-3} M N^6-2′-dibutyryl adenosine-3′-5′-cyclic monophosphate Na-salt (db-cAMP; Seika-gaku Kogyo, Tokyo, Japan).

Examination of culture cells

Fat cells growing in culture were examined using the following procedure. 1) Phase contrast microscopy; 2) Oil-red-O/hematoxylin staining for triacyl-glycerol of lipid droplets in cells; 3) Enzyme histochemistry of α-glycerophosphate dehydrogenase (α-GPDH) according to Pearse's method (20, 21), α-GPDH being an essential marker of fat cells; 4) Labeling with ^{3}H-thymidine and autoradiography: after 24 hours of labeling with 74 kBq/ml of ^{3}H-thymidine (Amersham Japan, Tokyo, Japan) added to the culture medium, cultured cells on the ceiling surface of the flask or in collagen gels were fixed in 5% formalin solution. Cultured cells in the flask or deparaffinized sections were coated with Sakura NR-M2 emulsion (Konica Medical, Tokyo, Japan) and exposed for 4 weeks before development. As the control examination of the incorporation of ^{3}H-thymidine into DNA, cultured cells were treated with 100 mg/ml DNA-ase (Sigma) for 2 hours at 37°C after the labeling. Cells were then labeled with ^{3}H-thymidine in the presence of 0.01 M hydroxyurea. Cells containing more than 20 silver grains over nuclei were considered to be significantly labeled. For the routine examination of proliferation, bromodeoxyuridine (BrdU, 2 μl, 30 mg/ml) was added to 2 ml culture medium for 24 hours (10, 11). Cultured cells

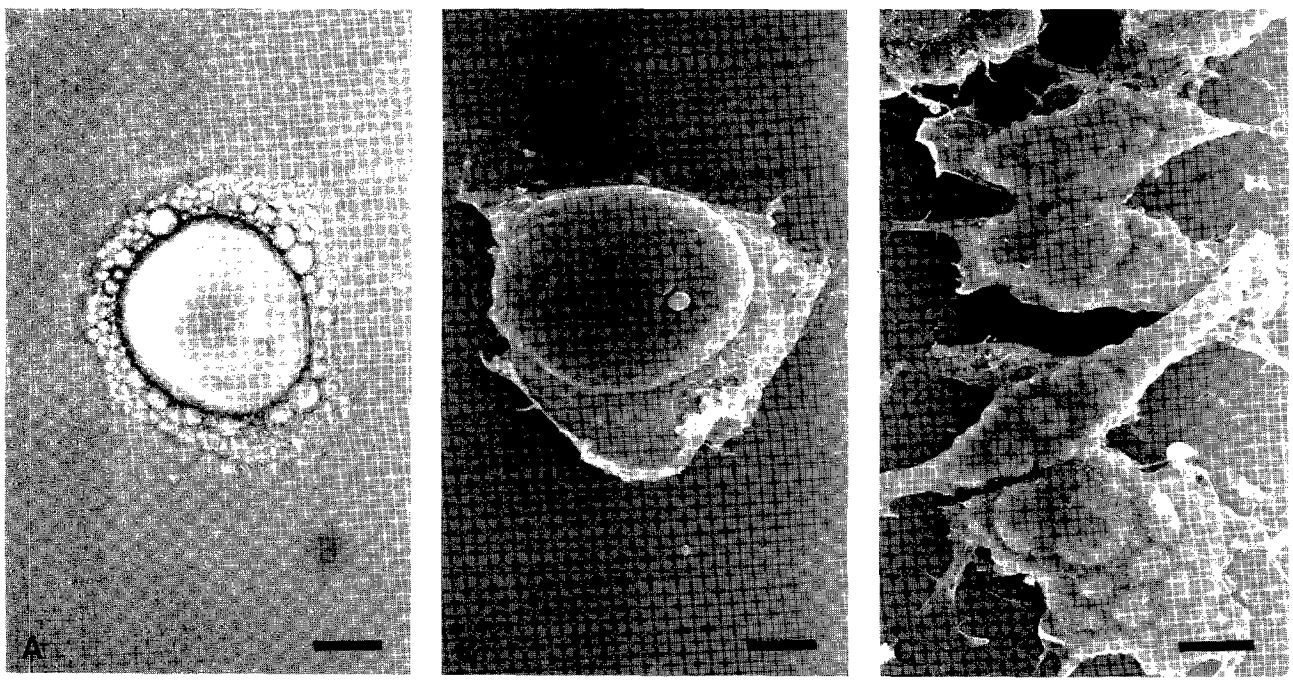


Fig. 2. A, Phase-contrast micrograph of a unilocular fat cell on the 4th day of culture. This cytoplasm has extended. The cell nucleus cannot be seen clearly in this figure. B, Scanning electron micrograph of a unilocular fat cell on the 4th day of culture. This cell adheres tightly. The adhered part of the cytoplasm has spread out and a large part of the cell is still spherical. C, Multilocular fat cells are seen here. Unilocular fat cells became these multilocular fat cells. This morphological change occurred within several days. Bar in A, B, C, 10 μm.

were immunostained with anti-BrdU antibody by the procedure of the BrdU kit (Amersham, Arlington Heights, VA, USA); 5) For determination of levels of glycerol in the medium, the enzymatic method of Eggstein and Kuhlman (5) was applied; 6) For scanning electron microscopy, cultured cells were fixed in 1.5% glutaraldehyde (in 0.05 M cacodylate buffer, pH 7.2), dehydrated in alcohol, critical-point dried with CO_2 and sputter coated with gold for 40 seconds. The specimens were observed using a JSM 5200 LV scanning electron microscope.

Results

Dispersed unilocular fat cells obtained from adipose tissue in bovine bone marrow were used for the primary culture. In culture flasks which were completely filled with medium, the fat cells floated to the top and adhered well to the inner top surface, i.e., ceiling surface, of the flask. This 'ceiling culture' method developed in our laboratory proved particularly useful for studies of unilocular fat cells in vitro. On day 3 of culture, unilocular fat cells adhered tightly to the ceiling surface of the flask at their cytoplasmic peripheries (15, 30) (Fig. 2). At this stage, cultured fat cells still contained a large lipid droplet at the center as well as many fine lipid droplets in the periphery. When the culturing was continued for 1 or 2 weeks, the morphology of the unilocular fat cells changed, such that they assumed the appearance of multilocular fat cells or fibroblast-like fat cells containing many fine lipid droplets; in other words, de-differentiation had occurred (Fig. 3). These cells then began cell division. Two types of cell division were recognized. One was the type in which the cultured unilocular fat cells divided into a unilocular fat cell that obtained a unilocular lipid droplet from the mother cell and a fibroblast-like fat cell, that is, two different daughter cells appeared. In the other type, the mother cell divided into two fibroblast-like fat cells after having changed from the unilocular fat cell into the multilocular fat cell containing intracytoplasmic lipid droplets. With increasing days of culture, these fibroblast-like fat cells derived from both types of cell division proliferated extensively (Fig. 4A). After these fibroblast-like fat cells had grown to confluence, they grew into multilocular fat cells containing enlarged intracytoplasmic lipid droplets (Fig. 4B). The intracyto-

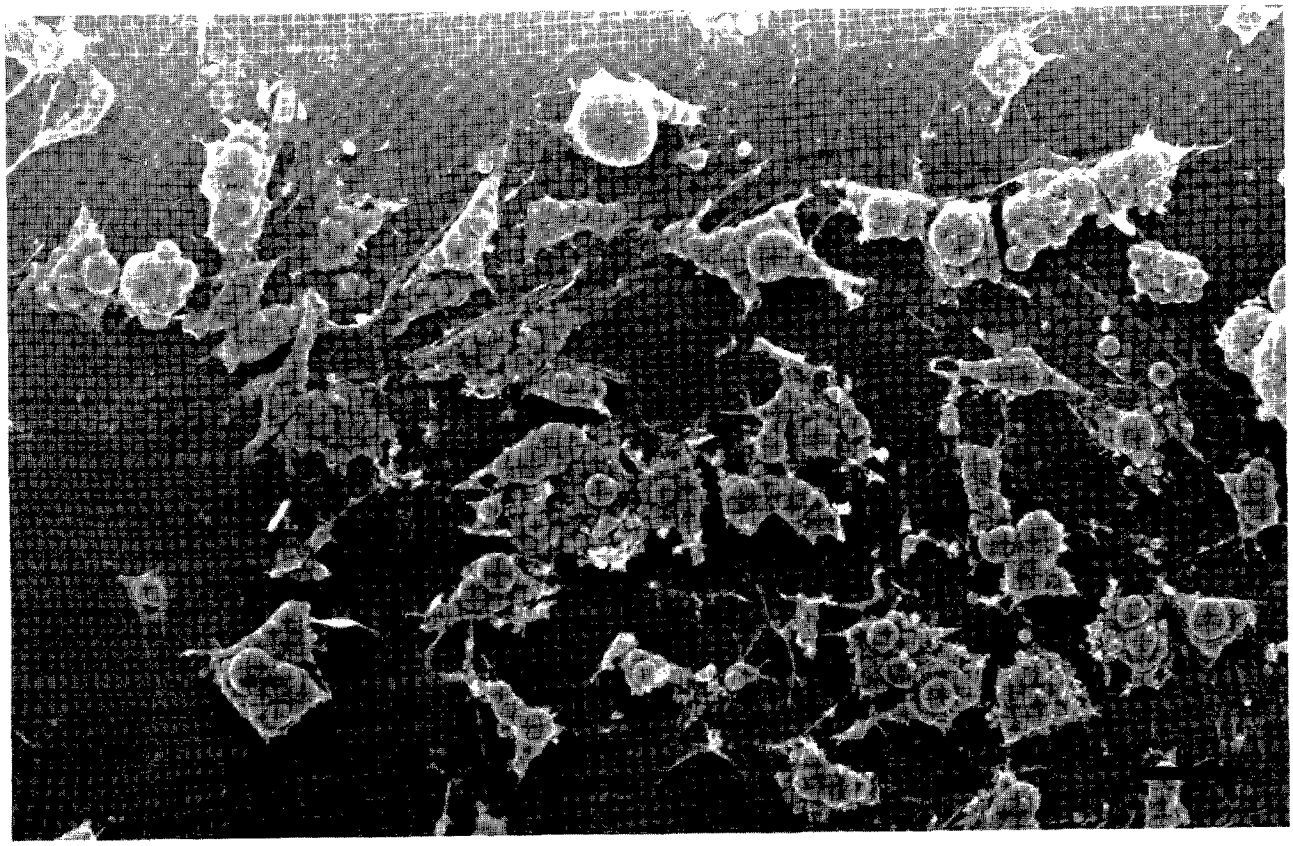


Fig. 3. Scanning electron micrograph of the cultured fat cells. After 2 weeks of culture, the morphology of the unilocular fat cells changed, so that they assumed the appearance of multilocular fat cells or fibroblast-like cells. Bar, 50 μm.

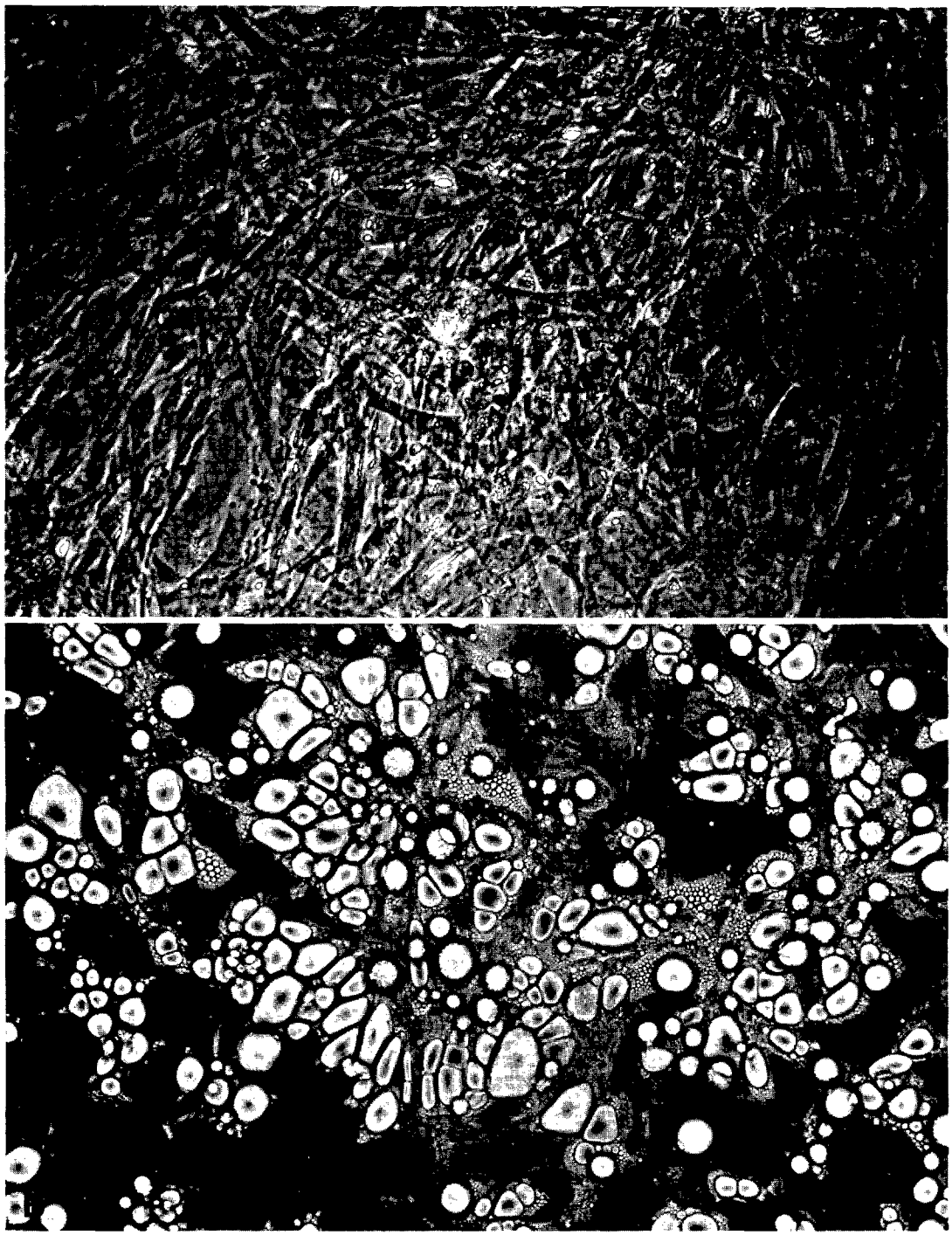


Fig. 4. Phase-contrast micrograph of the ceiling culture of unilocular fat cells at confluence. A, De-differentiated fat cells, such as the multilocular and fibroblast-like cells proliferated extensively. B, After these cells had grown to confluence, the number and size of the cytoplasmic lipid droplets increased, and unilocular fat cells reappeared among these cells. Bar in A and B, 100 μm.

plasmic lipid droplets increased in number and grew larger, finally differentiating into unilocular fat cells containing single lipid droplets. Next, we observed these findings by Oil-red-O/hematoxylin staining. Unilocular fat cells changed into fibroblast-like fat cells and proliferated. After these cells grew to confluence, they changed into multilocular fat cells, and finally into unilocular fat cells (Fig. 5). The lipid droplets, positive by

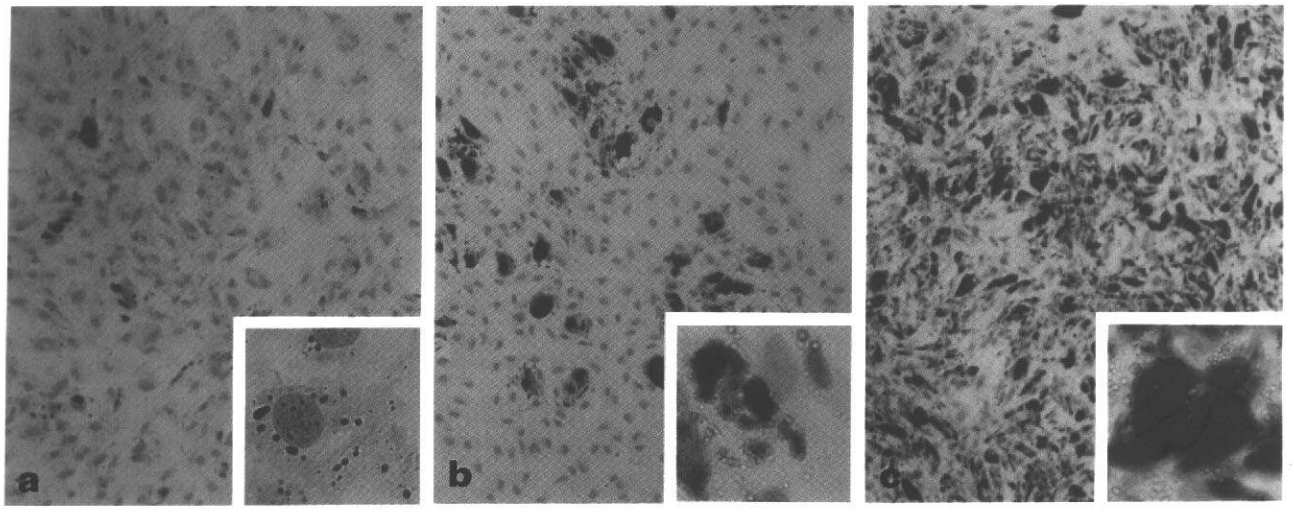


Fig. 5. a, b, c, After fibroblast-like fat cells had grown to confluence, they grew into multilocular fat cells containing enlarged intracytoplasmic lipid droplets. a, 3 days after confluence. b, 7 days after confluence. c, 10 days after confluence. Inset, higher magnification of the fat cell in a, b, c.

Oil-red-O staining, can be considered to indicate the differentiation of fat cells.

To confirm the proliferative ability of unilocular fat cells in the culture, we used autoradiography to study ^{3}H-thymidine incorporation in the cells on day 4 of culture (Fig. 6). The labeling index was $4.1\pm0.2\%$/24 hours in unilocular fat cells. The index of fibroblast-like fat cells was $4.3\pm0.3\%$/24 hours. We examined the bromodeoxyuridine (BrdU) incorporation rate, which had the same value as the ^{3}H-thymidine incorporation rate, to determine why these indexes were almost the same. The BrdU incorporation rate of the fibroblast-like fat cells on day 7 of culture was $9.8\pm2.0\%$/24 hours. The rate of the fibroblast-like fat cells on day 14 of culture was $22.8\pm3.1\%$/24 hours. The rate continued to increase day by day until these cells had grown to confluence. From these results we concluded that the cells on day 4 of culture had not reached the complete logarithmic growth phase.

In order to determine specific cellular functions such as lipogenesis, α-GPDH activity of fat cells related to lipogenesis was examined on day 7 of culture. Using histochemical procedures, α-GPDH activity was found to be positive in the cytoplasm (Fig. 7).

The addition of 0.1 mU/ml insulin to the medium for 7 days caused marked lipogenesis of fat cells; that is, the cultured fat cells enlarged and became rounder in conjunction with the increase in intracytoplasmic lipid droplets (Fig. 8). In the fibroblast-like fat cells, the nuclei moved to the center of the cell and the cytoplasm was fulled with similar-sized intracytoplasmic lipid droplets. These droplets were positively observed with Oil-red-O staining. The quantity of triacyl-glycerol in these cells increased from 12.8 ± 4.4 to 20.5 ± 4.4 μg /flask for 7 days, and the glycerol content, which is an index of lipolysis, in the medium increased from

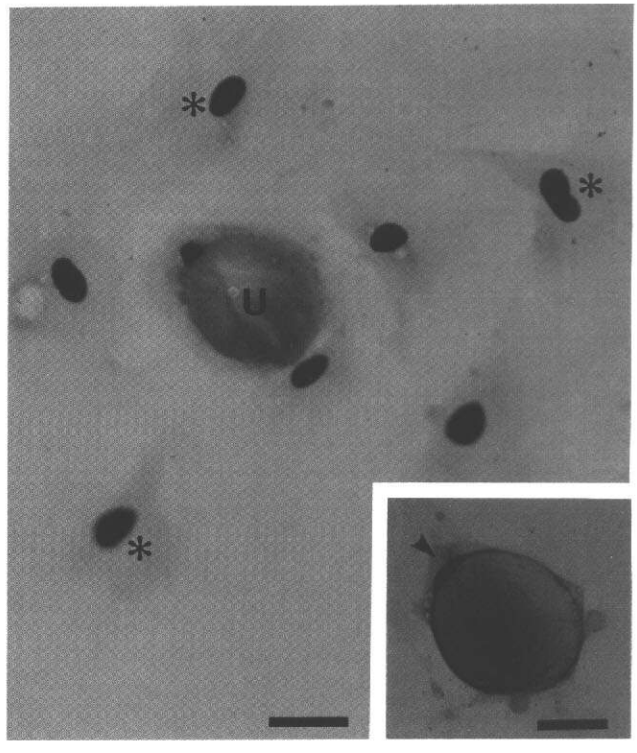


Fig. 6. Autoradiograph showing ^{3}H-thymidine incorporation. U, unilocular fat cell. *, fibroblast-like fat cells. Inset, note the grains (arrowhead) located over the nucleus of a unilocular fat cell. Bar, 20 μm.

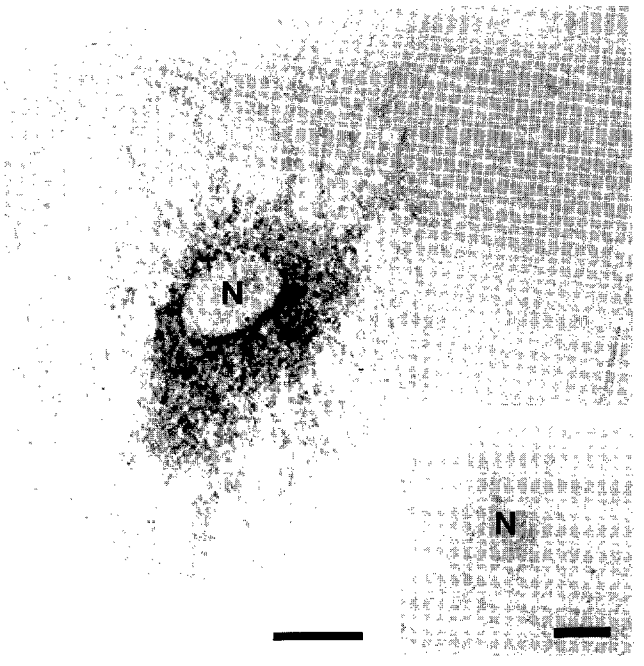


Fig. 7. Enzyme cytochemistry of α-glycerophosphate dehydrogenase. This enzyme activity was found to be positive in the cytoplasm. Not stained by hematoxylin for the nucleus. N, nucleus. Bar, 10 μm. Inset, negative control. This enzyme activity was found to be negative in the cytoplasm of fibroblasts. Hematoxylin stain. N, nucleus. Bar, 10 μm.

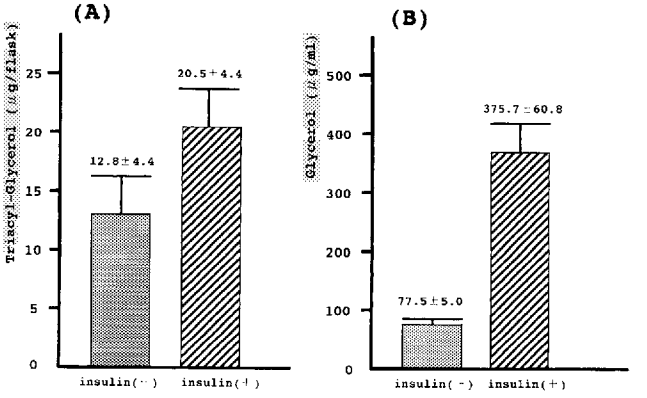


Fig. 9. Synthesis of triacyl-glycerol by cultured fat cells and glycerol content in the medium after stimulation of insulin. A, Quantity of triacyl-glycerol in the cells. B, Glycerol content in the medium.

77.5±5.0 to 375.7±60.8 μg/ml for 7 days (Fig. 9). These results indicated high acceleration of lipid metabolism.

The addition of 10^{-3} M db-cAMP caused lipolysis in the fat cells: the cultured fat cells retracted and the intracytoplasmic lipid droplets became finely granular. After 2 hours, the dispersion of lipid droplets was observed in the cytoplasm (Fig. 10). At the same time, the glycerol content in the medium increased from 80 to 100 μg/ml. In cultured fat cells, especially in the multilocular fat cells, finely granularized intraplasmic lipid droplets were observed, arranged along the radial cytoplas-

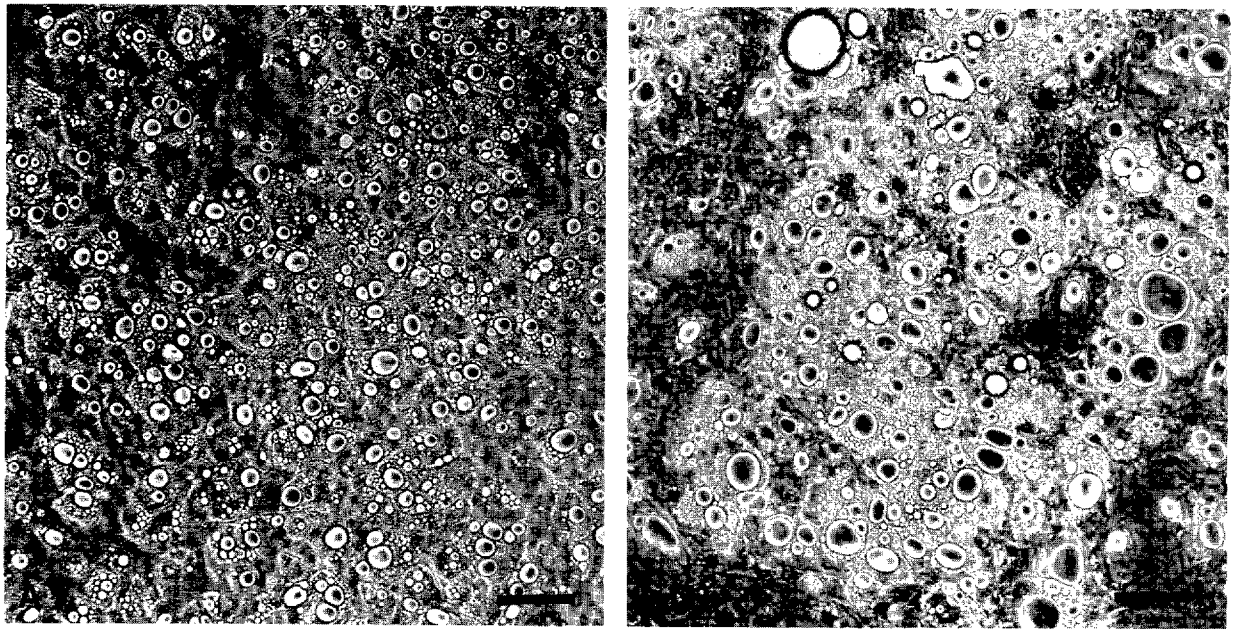


Fig. 8. Phase-contrast micrograph of cultured fat cells treated with insulin. A, Control (no treatment). B, Morphological changes of cultured fat cells caused by stimulation of insulin. Bar in A and B, 100 μm.

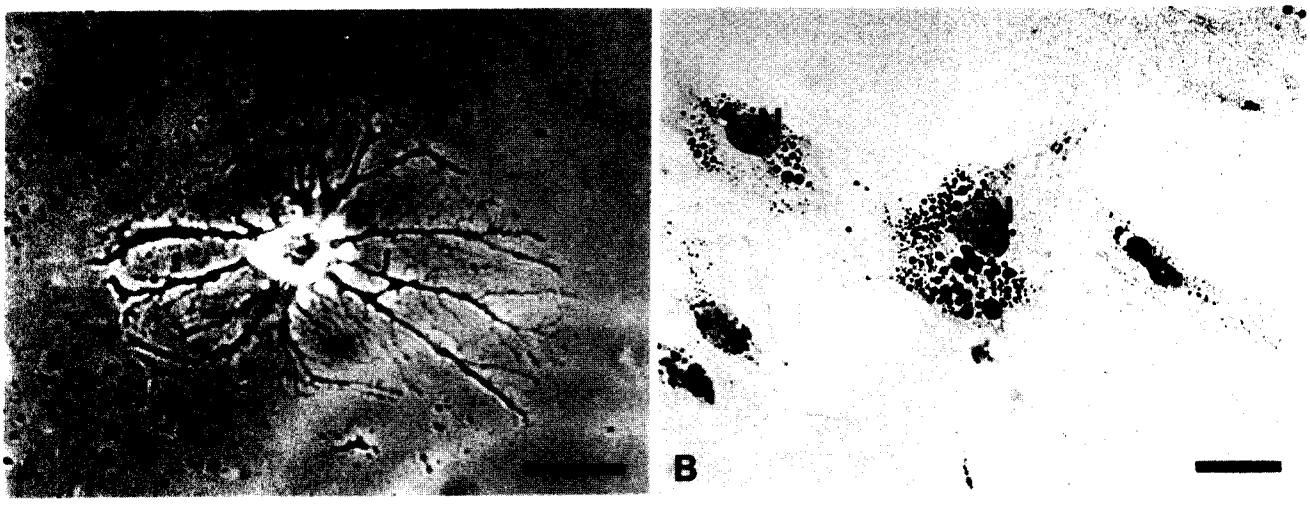


Fig. 10. A, Phase-contrast micrograph of a fat cell treated with dibutyryl-cAMP for 2 hours on day 7 of culture. The cell clearly retracted after the stimulation. B, The cell under the same conditions. The intracytoplasmic lipid droplets of the cell became finely granulated. When dibutyryl c-AMP was removed from the medium, these cells returned to their initial form within 6 hours. Oil-red-O/hematoxylin stain. N, nuclei. Bar in A and B, 20 μm.

mic processes.

We also observed the morphological and functional changes mentioned above utilizing three-dimensional collagen gel matrix culture (28). Freshly isolated unilocular fat cells were embedded and cultured in collagen gel matrix. Unilocular fat cells were evenly distributed in the absence of other types of cells, e.g., preadipocytes. Changes in the three-dimensional collagen gel matrix culture were similar to those observed in the ceiling culture. Within 24 hours after embedding, several fine lipid droplets appeared on the surface of the cells. This finding indicated that the fat cells remained viable in the collagen gel matrix culture. We obtained the same results for the collagen gel matrix culture as for the ceiling culture method. On day 7 of culture, the cytoplasm exhibited a spreading of the flattened cytoplasmic rim, although the cell bodies remained spherical. Fibroblast-like fat cells derived from unilocular fat cells proliferated extensively in the collagen gel matrix (Fig. 11). On day 7 of culture, we also confirmed the proliferative ability of unilocular fat cells with autoradiography using ^{3}H-thymidine. After these cells had grown to confluence, the number and size of the cytoplasmic lipid droplets increased and unilocular fat cells reappeared among these cells.

Discussion

This study is the first time that mature unilocular fat cells from the bone marrow have been successfully cultured using a ceiling culture method and a three-dimensional collagen gel matrix culture. Adipose tissue in the bone marrow of the bovine metacarpal bone was used as a cell source. By these methods, the proliferation of unilocular fat cells was demonstrated. First, unilocular fat cells de-differentiated into fibroblast-like fat cells and proliferated. When the proliferation stopped due to contact inhibition, i.e., density-dependent inhibition, morphological re-differentiation from multilocular fat cells into unilocular cells occurred. Secondly, we examined the functional reactivity of unilocular fat cells from the bone marrow. Insulin, a lipogenesis-enhancing factor, was added to the fat cells for examination of lipogenic potential, and c-AMP, a second messenger of lipolysis, was added to examine lipolytic potential. By treatment with insulin, intracytoplasmic lipid droplets in multilocular and fibroblast-like fat cells increased and enlarged, and the amount of intracellular triacyl-glycerol and the glycerol content in the culture medium were significantly increased. In contrast, when db-cAMP was administered, cells retracted and intracytoplasmic lipid droplets became fine. Glycerol content in the culture medium increased with time. On the basis of these results, we confirmed that unilocular fat cells in the bone marrow have lipogenic and lipolytic potencies.

Recently, obesity has been attracting increasing attention, and the cell biology of fat cells, the main element, has been actively studied (24). It has been clarified that the fat cells not only play the important role of lipid metabolism in the body, but also have a second function of producting and secreting various cytokines. However, these properties were primarily observed in the subcutaneous or intraabdominal fat cells,

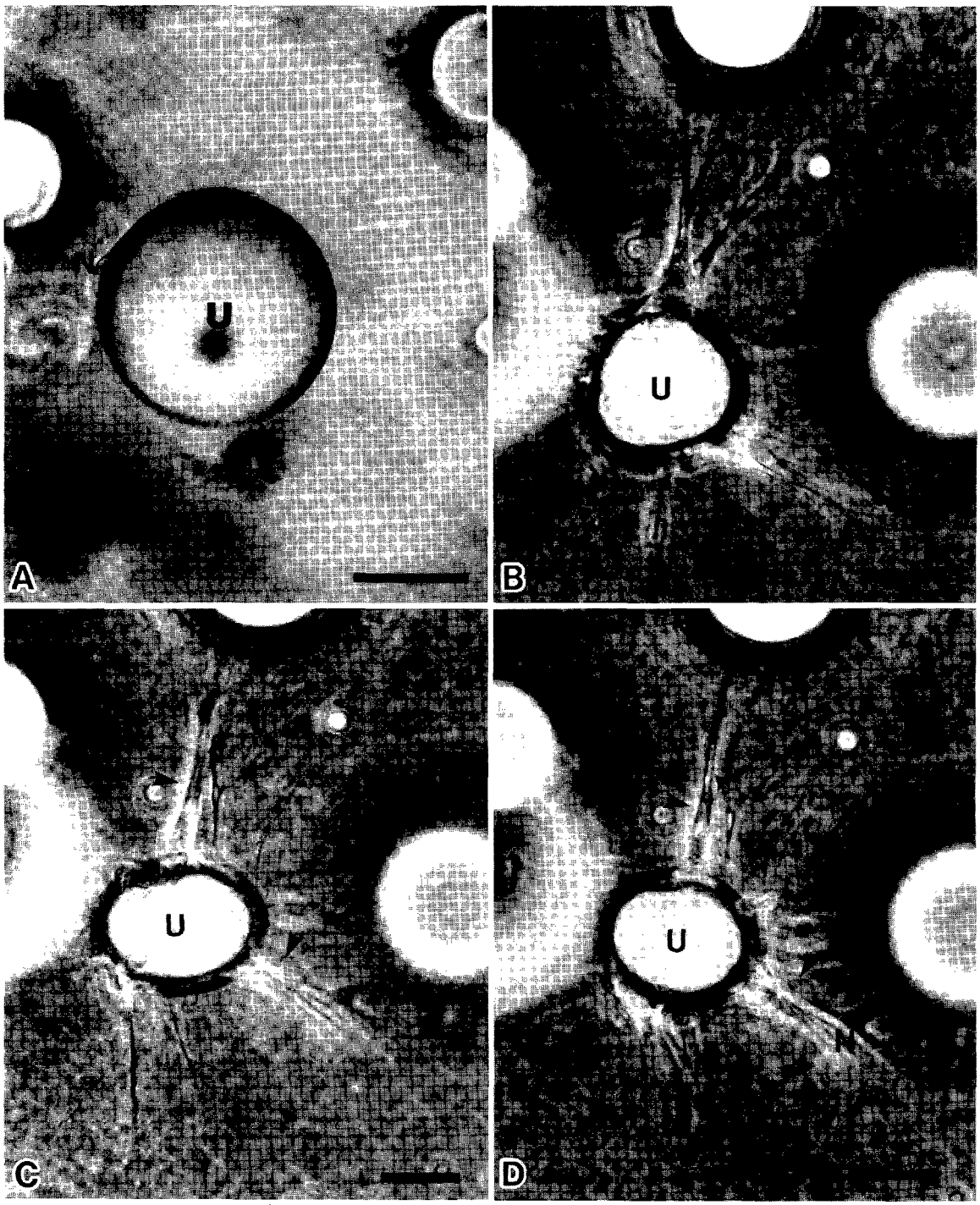


Fig. 11. Phase-contrast micrograph of cultured fat cells in the three-dimensional collagen gel matrix culture. This picture shows a time course of morphologic change of the individual cell. A, A unilocular fat cell (U) on day 1 of culture. Note the nucleus (N). B, C, and D, Fibroblast-like fat cells (arrowhead) were derived from one unilocular fat cell (U) here. The fibroblast-like fat cells extended slowly and the unilocular fat cell changed shape day by day. The nucleus (N) appeared in D. B, on day 7 of culture. C, 1 day after B. D, 2 days after B. Bar in A, B, C, and D, 20 μm.

and few cell biological studies on fat cells in the bone marrow have been reported. In contrast to subcutaneous or intraabdominal fat cells, the fat cells in the bone marrow may have a healing-enhancing factor at the time of bone fracture and may influence hemopoietic cells (16). However, little has been clarified to date. There are mature unilocular fat cells and immature fibroblast-like fat cells in the adipose tissue of bone marrow, and, to our knowledge, only immature cells have been used in most studies on adipocyte culture. As to why unilocular fat cells have never been used, two points can be ascertained. The first is that unilocular fat cells have been thought to have already completed their terminal differentiation and are assumed to have no proliferative potential. The second is that since the cells float in liquid, ordinary culture methods are not applicable. Therefore, we devised the following two methods for culturing unilocular fat cells. One is the ceiling culture method that utilizes the floating property which causes a great problem in routine cultures. The other is the three-dimensional collagen gel matrix culture. The advantage of this method is that cells can be observed under conditions close to those in vivo. Using these methods, studies from our laboratory have been reporting the proliferative potential of subcutaneous and intraabdominal unilocular fat cells during the past several years (8, 13, 26, 27, 29). In this study, we succeeded in culturing unilocular fat cells from the bone marrow using these methods.

The contamination of unilocular fat cells with stromal cells such as preadipocytes and fibroblasts is an important problem in our study. Because there is the possibility that contaminating preadipocytes in the culture have proliferated and differentiated, we thoroughly digested the adipose tissue. In addition, we tried to isolate unilocular fat cells by exhaustive pipetting. However, further objective evidence appears to be necessary. First, unilocular fat cells inoculated into a flask for ceiling culture were examined immediately with a phase-contrast microscope, and the incidence of contamination with stromal cells such as preadipocytes and fibroblasts was checked from the cell shape. Second, after digestion, some unilocular fat cells were collected, embedded in collagen gel matrix for 2 hours, fixed with formalin (of course, proliferation had not yet started), sectioned in paraffin, and the serial sections were carefully examined. In this examination, the basement membrane was positive by reticulin silver impregnation staining. This method is recommended by Fawcett (7), based on the fact that each adipocyte is surrounded by a basement membrane. We can distinctly detect each single unilocular fat cell by this staining method. In addition, S-100 immunostaining was performed for detection of preadipocytes. Preadipocytes are S-100 positive and their distinction from fibroblasts is necessary. In these two procedures, contamination of one preadipocyte or fibroblast was found among approximately 350 unilocular fat cells. These results indicated that the numbers of contaminating preadipocytes and fibroblasts are very small, and the contamination is not common to the overall culture. The findings in our study cannot be explained by only a few contaminating preadipocytes. As described, we demonstrated incorporation of ^{3}H-thymidine into the nuclei of cultured unilocular fat cells. In addition, we continuously observed the process whereby unilocular fat cells adhered and proliferated. On the basis of these facts, we concluded that unilocular fat cells proliferate showing morphological changes.

In conclusion, similar to our recent reports on subcutaneous and intraabdominal unilocular fat cells, unilocular fat cells from the bone marrow were demonstrated to have proliferating potency, and therefore, not to be cells that have completed their terminal differentiation and lost their proliferative potential.

A major difference between subcutaneous or intraabdominal unilocular fat cells and bone marrow unilocular fat cells is that the re-differentiation rate after proliferation is much higher in the unilocular fat cells from the bone marrow. The re-differentiation rate in the case of subcutaneous or intraabdominal unilocular fat cells is said to be 20–30%, whereas that of unilocular fat cells from the bone marrow was more than 70% in this study. This characteristic may be one reason for the rapid occupation of the bone marrow space when formed, due to rapid decrease in hemopoietic cells under various conditions. Nuttal *et al.* have shown that "human osteoblast-like cells" derived from human trabecular bone were not only osteogenic, but were able to undergo adipocyte differentiation under defined culture conditions. It is thought that the decrease in bone volume is compensated for by this mechanism. In addition, differentiation into light, floating unilocular fat cells appears favorable to other cells because the physical burden of the fat cells is minuscule in a liquid state in the bone marrow. Judging from the functional side of the production of various cytokines, the differentiation into unilocular fat cells is more adaptive. According to the reports to date, various cytokines are produced and secreted mainly by mature unilocular fat cells, and the production by immature fat cells has been shown to be trivial. Therefore, it can be inferred that fat cells have to be in the mature unilocular stage in order to exhibit their largest effect on hemopoiesis and osteogenesis. For these reasons, it is highly adaptive for mature unilocular fat cells to be able to de-differentiate into multilocular and fibroblast-like fat cells or re-differentiate into unilocular fat cells under various conditions.

In this study, we demonstrated that unilocular fat

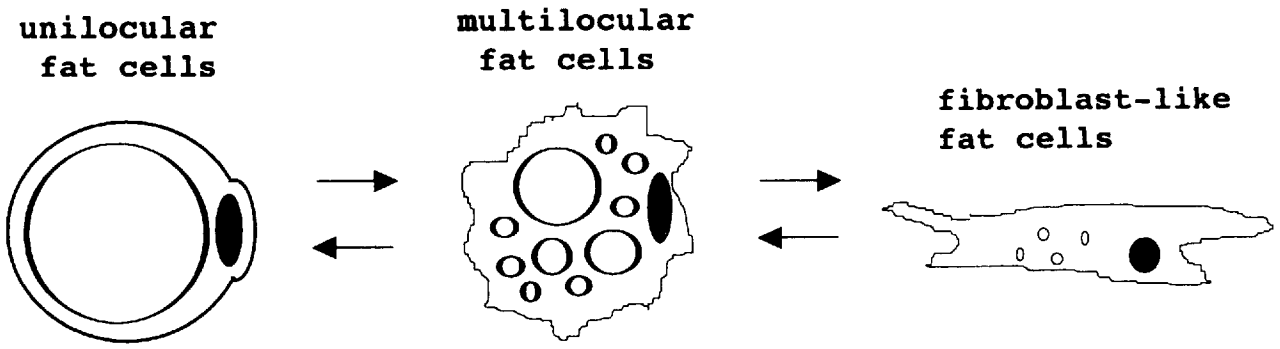


Fig. 12. Schematic drawing of the proliferation and differentiation of cultured fat cells in the present study. Unilocular fat cells de-differentiated through multilocular fat cells into fibroblast-like fat cells, and proliferated. After the proliferation, fibroblast-like fat cells re-differentiated through multilocular fat cells into unilocular fat cells.

cells from bone marrow de-differentiated, proliferated and re-differentiated in vitro (Fig. 12). In addition, the functional reactivity of fat cells was demonstrated. We hope to carry out further studies to clarify the significance and various yet unknown functions of bone marrow fat cells under conditions closer to those in vivo.

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