Unilocular fat cells in three-dimensional collagen gel matrix culture

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Summary Three-dimensional culture with collagen gel, developed recently for the in vitro study of some mammalian cells in a more physiological condition than a monolayer culture, was applied for a biological study of unilocular fat cells. Successfully embedded in the gel, the unilocular fat cells were shown to be able to keep their cellular function and actively proliferate. These findings confirm that unilocular fat cells do undergo proliferation under in vitro conditions as demonstrated in monolayer culture. —**Sugihara, H., N. Yonemitsu, S. Toda, S. Miyabara, S. Funatsumaru, and T. Matsumoto.** Unilocular fat cells in three-dimensional collagen gel matrix culture. *J. Lipid Res.* 1988. **29:** 691–697.

Supplementary key words tissue culture • proliferation • cellular function

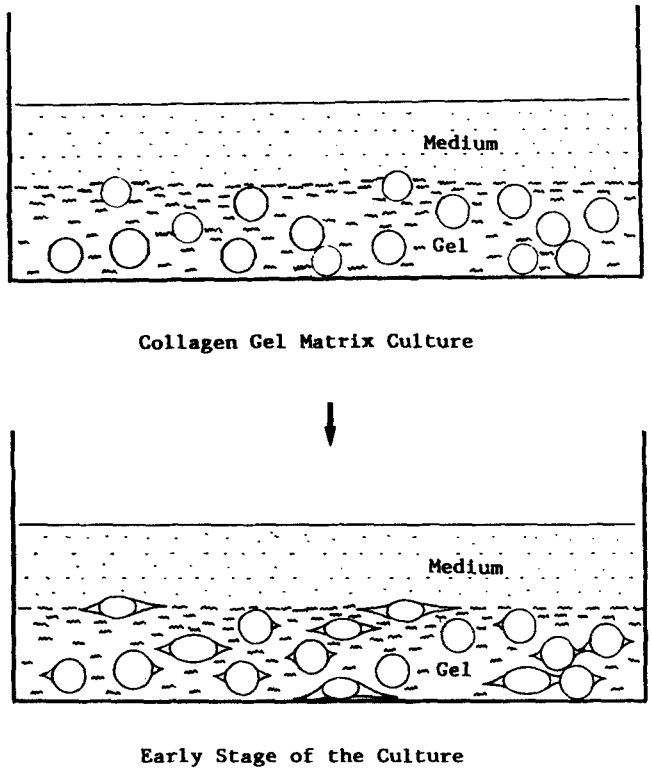

Fig. 1. Collagen gel matrix culture used for culturing unilocular fat cells. The separated unilocular fat cells were embedded in collagen gel (upper figure). At an early stage of the culture, some of the fat cells showed the extension of cytoplasm and subsequently proliferated (lower figure).

The establishment of a culture system of unilocular fat cells obtained from mature white fat tissue is a prerequisite for study of the cell biology and metabolism of fat cells. Mature white fat cells, i.e., unilocular fat cells, do not attach themselves to the bottom surface of a culture dish when incubated in medium. Instead, they tend to float on top of the medium because of their high lipid content. We described elsewhere (1, 2) a new method for culturing unilocular fat cells obtained from humans or rats. Let us briefly review this "ceiling culture" method, as it is called. Separated unilocular fat cells were incubated in completely medium-filled flasks; cells rose to the top of the medium and adhered to the inner upper surface (ceiling surface) of the flask; from then on culture flasks were maintained in the usual manner as in a monolayer culture. Unilocular fat cells thus cultured changed into multilocular fat cells or fibroblast-like fat cells, which in turn proliferated extensively. This may be called dedifferentiation.

Recently, culture work using extracellular matrix has been developed in the in vitro study of mammalian cells (3–5). Collagen matrix, in particular, proved to have critical influence on the growth and differentiation of some cultured cells (6, 7). Collagen was used either in the form of a thin film of dried collagen or as collagen gel. We have tried the former in the ceiling culture of unilocular fat cells without clear-cut effect except for a slight improvement of the adhesion in the culture. Another method was thus implemented for those fat cells by use of a three-dimensional collagen gel culture (the so-called collagen gel matrix culture). This system was suitable to catch unilocular fat cells, which otherwise tend to float to the surface of the culture medium.

In this study, the cell biological behavior of unilocular fat cells in the collagen gel matrix has been examined in terms of proliferation and cellular function. We also expected to confirm the finding that unilocular fat cells undergo dedifferentiation and proliferation in monolayer culture using ceiling culture.

MATERIALS AND METHODS

Fat cell separation

Materials used for culture were abdominal subcutaneous fat tissue from 3-day- to 4-week-old male Wistar rats and from young female human subjects (13–23 years old). The fat tissue was digested with collagenase solution ac-

Abbreviations: db-cAMP, N^6-2'-dibutyryl adenosine-3'-5'-cyclic monophosphate Na-salt.

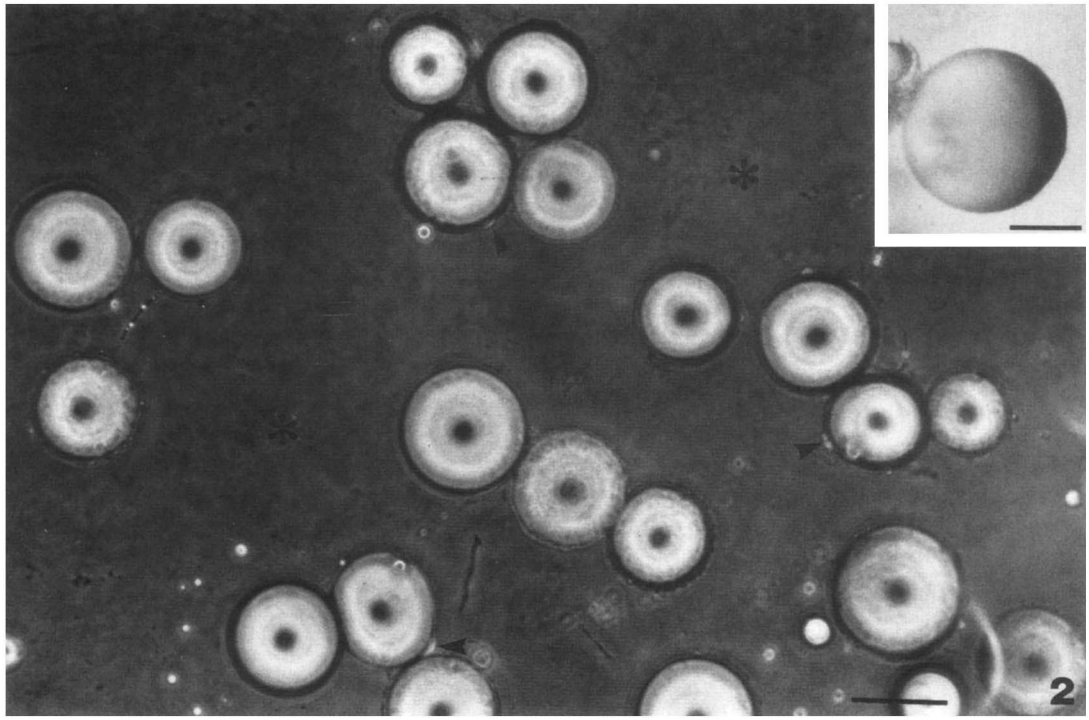

Fig. 2. Mature white fat cells containing unilocular lipid droplets (unilocular fat cells) in collagen gel matrix culture. These cells were separated by digesting the fatty tissue of young rats (2-week-old male rats). On the first day of culture these cells do not show the extension of cytoplasm. Arrowheads, nuclei; *, gel. Phase-contrast micrograph; bar, 40 μm. Inset: Nomarski differential-interference-contrast micrograph of a unilocular fat cell in collagen gel. Note the spherical appearance of the unilocular fat cell; bar, 20 μm.

cording to Rodbell's method (8), in order to obtain unilocular fat cells.

Three-dimensional culture using collagen gel (collagen gel matrix culture)

This culture method was initiated by Elsdale and Bard (3), and in this study we followed the procedures of Enami et al. (9, 10). Briefly, eight volumes of acid-soluble type I collagen solution 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). Collagen solution was obtained from Nitta Gelatin Co. Ltd., Osaka, Japan. This mixture kept in ice was mixed with separated unilocular fat cells. Two ml containing 2×10^5 cells/ml was placed into a culture dish (35 mm) and immediately warmed to 37°C to form a gel. Unilocular fat cells were thus embedded in collagen gel (**Fig. 1**). After 30 min, when the gel was strong enough, the gel was overlayered with 2 ml/dish of Ham F-12 medium supplemented with 10% newborn calf serum and antibiotics.

Chemicals added to culture cells

The following chemicals were added to the medium of some cultures: *1*) 0.1–0.5 mU/ml insulin (Sigma); *2*) 10^{-5} M norepinephrine (L-arterenal bitartrate, Sigma); and *3*) 10^{-4}–10^{-3} M N^6-2'-dibutyryl adenosine-3'-5'-cyclic monophosphate Na-salt (db-cAMP) (Seikagaku-Kogyo, Tokyo).

Examination of culture cells

Fat cells growing in culture were examined by the following procedures: *1*) phase-contrast microscopy and Nomarski differential-interference-contrast microscopy; *2*) lipid histochemistry using oil red O/hematoxylin staining and enzyme histochemistry of triglyceride lipase and glycerophosphate dehydrogenase; and *3*) enzymatic determination of medium glycerol content (11).

RESULTS

After unilocular fat cells spread in collagen gel, they were observed in each layer (about 2 mm in depth) in the gel (2 ml) of each culture dish (35 mm) (Fig. 1). The cells were completely spherical in shape, each with a peripherally located nucleus (**Fig. 2**).

In approximately 2% of cultured cells from 1-week-old rats on day 2, part of the cytoplasm of the spherical fat cell was observed to extend like an arrowhead and included some small lipid droplets. In some cells, the cytoplasmic

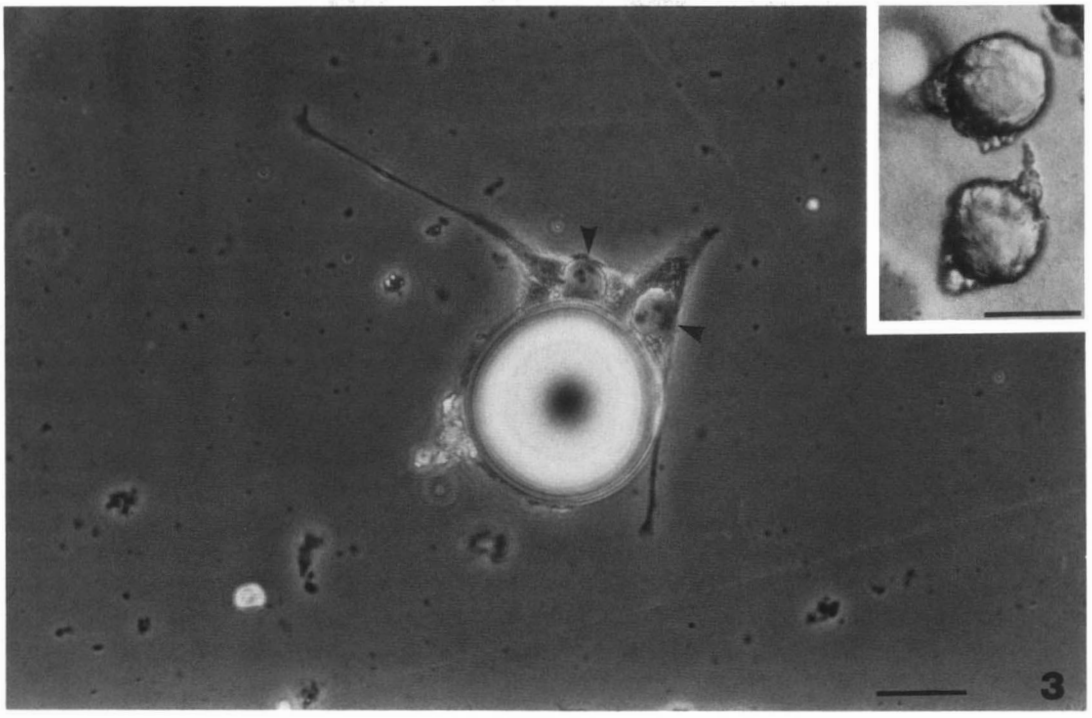

Fig. 3. A unilocular fat cell on the 4th day of collagen gel matrix culture. The material was same as that of Fig. 2. The extension of cytoplasm in three directions is seen here and the nucleus is already divided (arrowheads). Phase-contrast micrograph; bar, 20 μm. Inset: Nomarski micrograph of other cells showing the same appearance on the 4th day of culture; bar, 40 μm.

extension was in two or three directions on day 3 or 4 of culture (**Fig. 3**). In one week, unilocular lipid droplets were divided into multilocular droplets of variable sizes (**Fig. 4**). In these multilocular fat cells, the further extension of the cytoplasm occurred concomitantly with further division of lipid droplets. It was therefore assumed that fibroblast-like fat cells containing small lipid droplets grew among multi- or unilocular fat cells in the gel. Fibroblast-like fat cells also grew directly out of some unilocular fat cells through cell division, and this phenomenon was frequently observed in those cells that were treated with insulin or in the fat cells from adult rats and adult humans without insulin as described below (**Fig. 5 and Fig. 6**).

These findings of cultured fat cells, i.e., the cytoplasmic extension and cell division, were observed earlier in aggregated cell groups and later in a field of separated, non-aggregated cells in the gel. Cultured fat cells proliferated in all layers of the collagen gel, but most frequently in the uppermost layer, in which case they covered the upper surface of the gel. After fat cells had grown to confluency, the number and size of the cytoplasmic lipid droplets increased and a large number of unilocular fat cells were observed (**Fig. 7 and Fig. 8**). Addition of insulin enhanced synthesis of lipid droplets (Fig. 7).

The three types of fat cells in the culture, i.e., unilocular, multiocular, and fibroblast-like fat cells, contained triglyceride as demonstrated in their droplets stained with oil red O. Enzymatic activities of triglyceride lipase and glycerophosphate dehydrogenase helped identify these cells as fat cells. The fat cells in collagen gel matrix culture maintained the normal functions as demonstrated by stimulation of lipolysis by norepinephrine or db-cAMP, and by lipogenesis stimulated by insulin. Thus, in cells derived from 1-week-old rats at confluency, 10^{-4} M db-cAMP induced dispersion of intracytoplasmic lipid droplets, and at the same time, the glycerol content increased at the rate of 2–4 $\mu mol \cdot hr \cdot 10^6$ cells in the medium.

For the purpose of examination of the effect of insulin, the fat cells were exposed to 0.1 mU/ml insulin from the inception of culture. During cell division, the unilocular lipid droplets were minimally broken down in the presence of insulin. There was a daughter cell which inherited almost intact the shape and size of the droplet, and a second daughter cell with only a small number of fine lipid droplets, which was designated as the fibroblast-like fat cell above. We have referred to this mode of proliferation of unilocular fat cells as the loculus-preserving cell division (2). The fibroblast-like fat cells underwent extensive further proliferation. Following their growth to

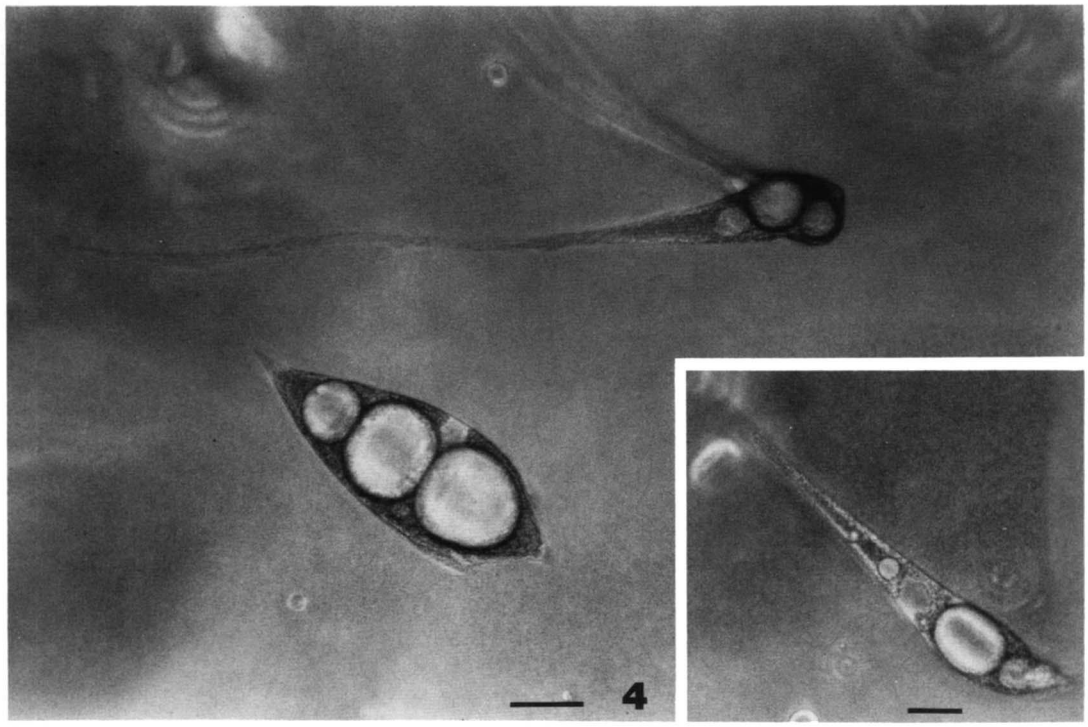

Fig. 4. Multilocular fat cells on the 6th day of collagen gel matrix culture. The material was same as that of Fig. 2. Unilocular lipid droplets were divided into these multilocular forms. Phase-contrast micrograph; bar, 20 μm. Inset: a spindle-shaped multilocular fat cell in the culture. This cell is in the advanced stage as the multilocular fat cell. Phase-contrast micrograph; bar, 20 μm.

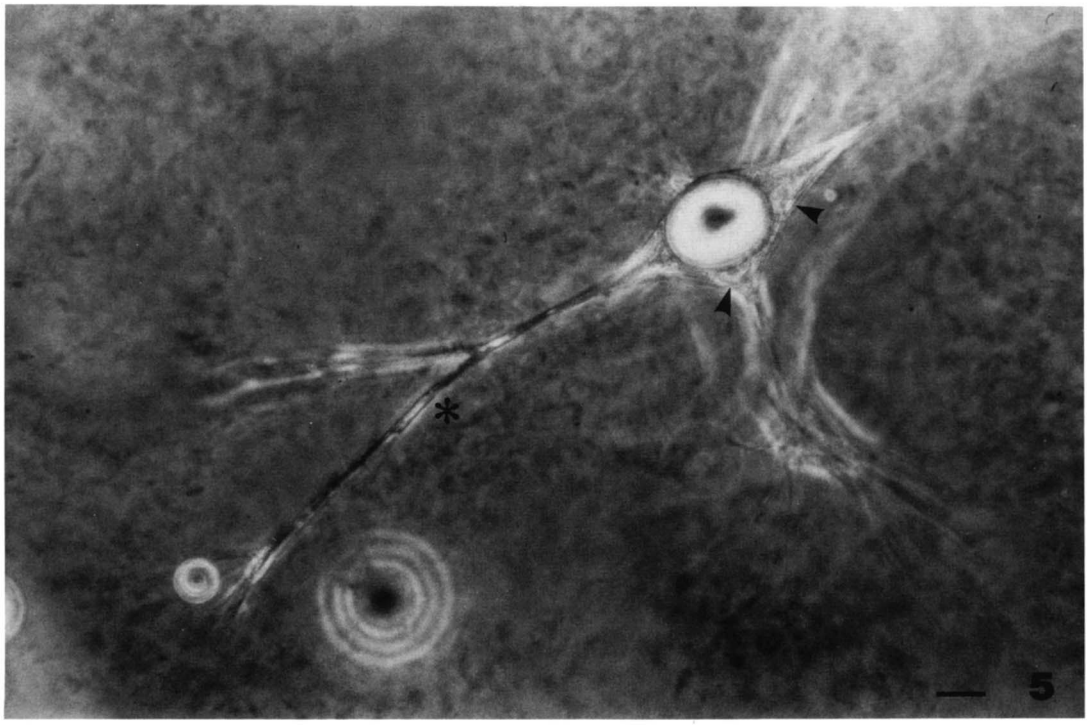

Fig. 5. A unilocular fat cell, derived from a young female human subject, in collagen gel matrix culture. This cell on the 6th day of culture has the extended cytoplasm in three directions; one part is particularly elongated in this figure (*); arrowheads, nuclei. Phase-contrast micrograph; bar, 40 μm.

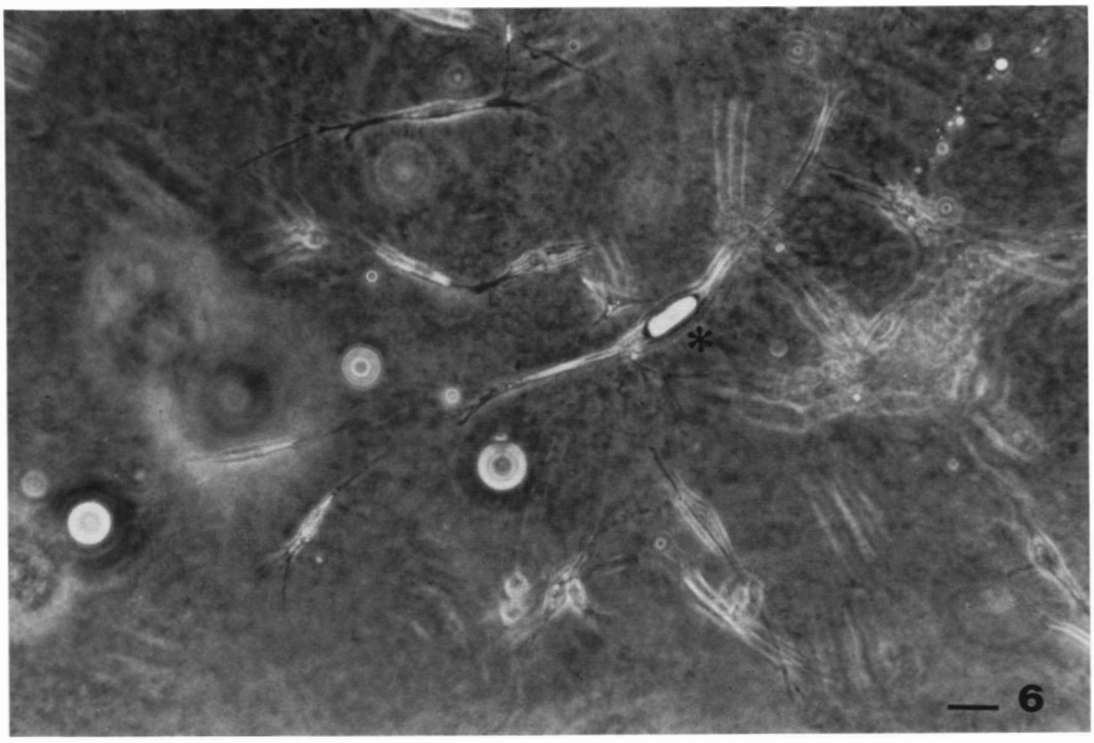

Fig. 6. The same cell as shown in Fig. 5, on the 12th day of culture. Fibroblast-like fat cells were derived from the unilocular fat cell (•), and the fibroblast-like fat cells proliferated extensively as observed. Phase-contrast micrograph; bar, 80 μm.

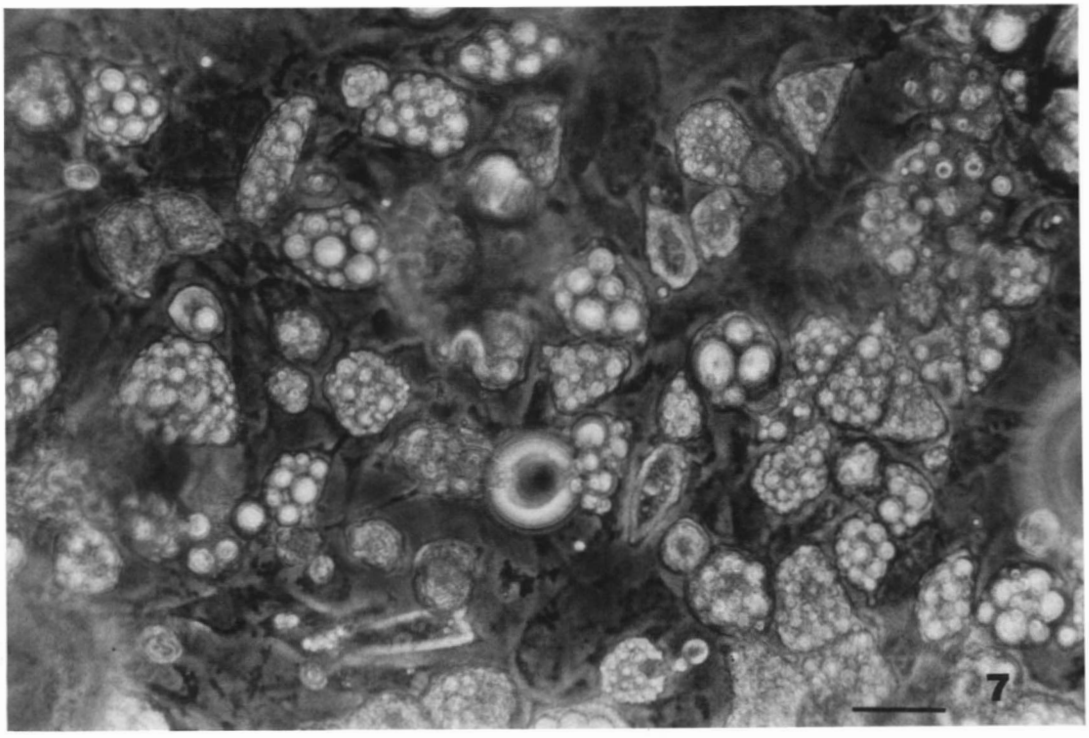

Fig. 7. The same culture material as shown in Figs. 2, 3, and 4. These cells on the 12th day of collagen gel matrix culture in the presence of insulin are at the early stage of confluence. Note the many multilocular and unilocular fat cells in the background consisting of fibroblast-like fat cells. Phase-contrast micrograph; bar, 40 μm.

confluency, the number and size of intracytoplasmic lipid droplets increased and a multitude of multi- and unilocular fat cells appeared in the presence of insulin (Fig. 7).

As stated above, cells in this study were from rats of 3 days to 4 weeks of age and from young female human subjects. In the experiment with rat-derived cells, the younger the rats, the better the results; active proliferation of the cells was obtained. With the ceiling culture method mentioned above and elsewhere (1, 2), unilocular fat cells derived from 3- to 4-week-old rats were not suitable as material for culturing because of their low frequency of attachment to the culture flask. In the collagen gel matrix culture in this study, however, those cells became multilocular or fibroblast-like fat cells in 2 or 3 weeks, and proliferated as actively as fat cells of younger rats. Human cells were also cultured in the collagen gel matrix (Figs. 5 and 6). In ceiling culture of unilocular fat cells obtained from young female human subjects, it took 2–3 weeks for those cells to attach and begin to proliferate. In the collagen gel, however, it required only 3–5 days before less than 1% of the cells began dividing and 7–10 days for less than 2% of cells to initiate the process. Once initiated, the proliferation was more active in the ceiling culture system than in the collagen gel matrix.

DISCUSSION

Since Rodbell (8) had succeeded in separating unilocular fat cells from mature white fat tissue, a number of biochemical studies have been carried out using separated unilocular fat cells. However, tissue culture of mature fat cells, namely, unilocular fat cells, was difficult, until recently, because of their buoyancy. The ceiling culture method, which we devised in our laboratory, has proved to be suitable for maintaining the proliferation and function of unilocular fat cells (1, 2).

In this study, another method of culturing unilocular fat cells, by use of collagen gel, was tried. One of the purposes was to confirm what had been observed in vitro in the ceiling culture of unilocular fat cells. Collagen, a major constituent of the extracellular matrix in vivo, was used as an environmental substrate for culturing mammalian cells for a sustained period of time. One way of utilizing collagen for culture is in the form of a thin film lining the surface of a dish or flask. This method was adopted in the study of preadipocytes that did not float in medium, and there have been some interesting reports (12).

The three-dimensional culture method using collagen developed by Elsdale and Bard (3) has been effective in

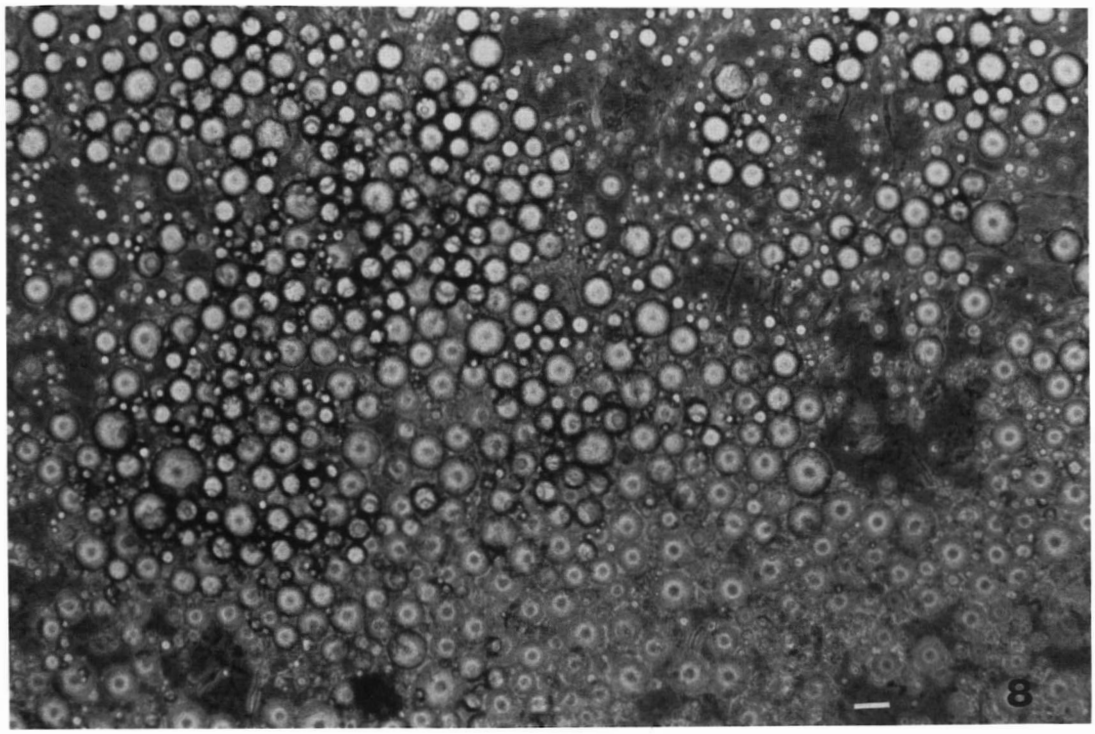

Fig. 8. Collagen gel matrix culture of unilocular fat cells, derived from 2-week-old rats, on the 28th day of culture. These cells are at the advanced stage of confluence. Many unilocular and multilocular fat cells are seen and fibroblast-like fat cells are scarcely observed at this stage. The focus of this figure is not perfect on all cells because these cells exist in three dimensions of the gel. Phase-contrast micrograph; bar, 40 μm.

their observation of the behavior of human fetal lung fibroblasts in a gel matrix. This method is also specifically useful in the study of differentiation of some epithelial cells, e.g., mammary epithelial cell in the morphogenesis of duct-like structure and casein production (6, 7, 9), and of thyroid follicular cells (13), as well as for observation of the migration of fibroblasts and melanoma cells (14). Our purpose in adopting this collagen gel matrix culture for the study of unilocular fat cells was, unlike the previous studies, to see whether we could successfully catch and fix floating unilocular fat cells in the gel. We did succeed in capturing the fat cells in a state active enough for them to continue functioning and perform cell division. Their cellular behavior in an environment which more closely approximates the in vivo condition was virtually the same as has been observed in the monolayer culture including the ceiling culture.

Collagen gel matrix culture thus has a possibility of serving as a useful method for the culturing of unilocular fat cells. It helped us confirm that unilocular fat cells do indeed undergo cell division and extensive proliferation under the in vitro conditions given above. The criticism that preadipocytes in the fatty tissue proliferated in the gel may be levelled against this study. However, the continuous observation of the behavior of cell division in unilocular fat cells shown in Figs. 2, 3, 5, and 6 should partly resolve this criticism. To the authors' best knowledge, application of this culture method in such a study as the present has never been tried before.

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