Studies on Human Adipose Cells in Culture: Relation of Cell Size and Cell Multiplication to Donor Age

FESTUS O. ADEBONOJO

Children's Hospital of Philadelphia, Rebound Health Center, Philadelphia, Pennsylvania 19146, and The University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19104

In an effort to test the adipose hyperplasia theory of obesity in humans, adipose cells, derived from anterior abdominal walls of human infants and children, were grown in synthetic medium (McCoy's 5A Medium) supplemented with 20% fetal calf serum. Adipose cells which became delipidized in culture were found to be capable of division and the rate and number of cell divisions was age dependent. Cells of infants under 1 yr of age and cells derived from early adolescent children divided to varying degrees in culture. Adipose cells from children aged 1–10 yr showed no cell division. Cell division was never observed in a lipid-laden adipocyte. Measurements of cell diameter showed that after the first year of life, cell size increased progressively with age. During the first year adipose cell size appeared to reflect the rapid hyperplasia of the first 3 mo, reaching smallest size at 3–12 mo but increasing thereafter.

Many theories have been proposed to explain the etiology of obesity. The glucostatic theory of Mayer (1,2) proposed that the body responds to the rate of glucose utilization and suggested that the arteriovenous glucose difference and its mechanism may be the key determinant. The lipostatic theory, proposed by Kennedy (3) suggested that obesity is a result of body's response to the total amount of fat. Another theory, championed by Miller (4) and Brobeck (5) proposed that the body responds to changes in internal temperature generated by food combustion which, in turn, controls the amount of energy stored and expended. None of these theories have survived as a single etiological explanation for obesity.

In recent years, there has developed increasing amount of evidence to suggest that most individuals with early onset obesity have total body adipose mass characterized by increased adipose cell number and cell size (6–14). This so-called adipocyte hyperplasia theory was first proposed by Hirsch and Knittle (6). The theory essentially postulates that the number of adipocytes in a given individual is related to fat cell multiplication in the last trimester of pregnancy and the first year of life, although as Knittle (10) and Mossberg (15) have noted, a second phase of adipose cellular hyperplasia is also possible. Brook (14) recently presented data to suggest that the adipose organ in man has a finite sensitive period during which the basic complement of adipose cells is determined between the thirtieth week of gestation and one year age.

Support for the adipocyte hyperplasia theory had been found earlier in animal studies by Hirsch and Han (11) who demonstrated that when Sprague–Dawley rats are over fed during the first 6 wk of life, the resultant obesity is characterized by increased fat cell number and size. After the 7th week, the fat depot increased exclusively by cellular hypertrophy. There was no cellular hyperplasia. When these animals were severely starved during the 6th week but normally fed thereafter, no

1 I thank the Joseph Stokes Jr. Research Institute of the Children's Hospital of Philadelphia for supporting this work.
effect on cell size or number was found. With prolonged starvation after the 15th week, greatly decreased cell size but normal cell number were found. Observations on obese human adults and from experimentally induced obesity in lean subjects suggest that late-onset obesity is characterized by increased cell size and normal cell number (6,9,10); conversely when obese subjects lose weight, the cell size decreases but there is no change in cell number (9,16) although Cheek (16) has reported one instance in which fat cell number also decreased with weight loss.

Although much is known about the adipose tissue and its contribution to obesity, many important questions remain to be answered. The use of tissue culture techniques has rarely been applied to the study of human adipocytes. Earlier animal studies suggest that this technique might be useful in the studies of human obesity. Dissociated fat cells from one to three day old rats, grown and maintained in monolayer cultures by Masters (17), demonstrated the ability to reaggregate into lobular islands similar to that of the mature parent tissue. Addition of insulin to the culture medium markedly enhanced lipogenesis in these cells. Sidman (18) had earlier demonstrated similar effects of insulin on rat adipocytes in tissue culture.

In humans, Ng et al. (19) reported the observation that adipocytes from obese adults rapidly increased in number in the first 4–6 days in culture and then stopped dividing, while cells from non-obese adults failed to increase in number with time. These cells were maintained in culture for 2 mo, but it was not stated whether the cells lost or retained their ability to survive in culture nor were there any visual proof that these cells grew in culture. Poznanski et al. (20) from the same laboratory, recently reported that stromal fibroblast cells from adipose tissue of human adults, termed preadipocytes by the authors, have the same doubling time (50 hr) as skin fibroblasts but distinctly different metabolic characteristics. These preadipocytes incorporated 5 to 10 times more labeled glucose into lipids and contained 2 to 5 times more intracellular glycerol than skin fibroblasts from which they are otherwise morphologically indistinguishable. Smith (21) has reported growing explants of human adipose tissue in culture and showed that insulin enhanced glucose uptake by adipose cells and that this effect was influenced by the size of the cells. Beyond a certain size (93 μm diameter) the insulin effect is markedly diminished.

This study was undertaken to establish, in the normal infants and children, the relationships, if any, between adipose cell size and adipose cell replicative behavior on the one hand, and the ages of the donor children on the other. Thus it might be possible to test the validity of the adipose cellular hyperplasia theory as it applies to postnatal adipose cellular multiplication.

MATERIALS AND METHODS

The technique employed to disaggregate and isolate human adipose cells from adipose tissue has been described elsewhere (22). It is based on a modification of the technique described by Rodbell in 1964 (23). The specimens of white adipose tissues used in this study were derived from anterior abdominal walls of 41 infants and children who were undergoing routine abdominal laparotomies. Usually 2–5 g of tissue was excised at the onset or at the end of the surgical procedure. The tissue was taken to the laboratory usually within 1–6 hr of excision and sliced into smaller pieces about 30–50 mg in weight and placed in 10 ml of McCoy's 5A medium modified (Microbiological Associates, Bethesda, Md) in 25 ml siliconized or plastic Erlenmeyer flasks. Ten milligrams of Collegnase (Worthington Biochemical Corp.) per gram of tissue was added and the mixture incubated in slow gyratory rotation (50–100 rpm) for 1 hr at 37°C.
Following incubation, the content of the flask was gently stirred and dispersed by gentle pipetting with siliconized glass or plastic pipettes. The cell suspension was centrifuged (IEC UV Centrifuged) in 15 ml plastic centrifuge tubes at 400xG (1300 rmps, rotar head diameter 30 cm). Fat droplets from ruptured cells and intact fat cells floated to the surface while stromal vascular debris formed a pellet on the bottom of the centrifuge tube. The cells were gently pipetted and resuspended in 10 ml of fresh McCoy's medium and recentrifuged as before. This step was repeated once more, after which the cells were plated in monolayers in 25 ml plastic culture flasks (Falcon Plastics). Using this technique only adipocytes are present in the supernatants. The cells are incubated at 37°C for 4 hr while allowing them to adhere to the bottom of the flask. Then they are gently covered with 2 ml of McCoy's medium which has been supplemented with 20% fetal calf serum, penicillin (60 units/ml) and streptomycin (60 units/ml). The cultures were incubated at 37°C in a gas phase of 95% air and 5% CO₂, pH 7.3, and left undisturbed for 5–7 days.

Subsequently, the medium was changed every 5 days and the cultures were observed morphologically under an inverted Nikon Microscope MS. At initial culture and at periodic intervals photomicrographs were taken on representative flasks with a Nikon Microflex AFM Automatic Camera. Cells which became lipid free and showed mitotic activities were subcultured according to the technique of Puck et al. (24). Cells were counted in a hemacytometer at the time of subcultures after the cells had been dispersed in new medium. The rate of cell multiplication was determined and expressed logarithmically (log₁₀) as a function of the ages of the donors of the cells.

The diameters of 100–200 cells in the initial photomicrographs were measured using a dial caliper (Mitutoyo SD Type Vernia Calipers) and mean cell diameter (micrometers, μm) with standard deviation was calculated for each tissue specimen.

RESULTS

Adipocytes, the largest cells in the human body, are generally spherical with considerable variations in sizes (Figs. 1A, B and C). Table 1 summarizes the data on the mean diameters of these cells. Between birth and 3 mo of age the mean cell diameter (60.6μm) was significantly larger than that in cells of children aged 3 mo–1 yr (49.0 μm). After 1 yr of age the mean cell diameter increased averaging 68.2 μm diam in the 1–5 yr olds and 73.6 μm diam in those over 5 yr of age. In two extremely obese children aged 5 and 15 years, the mean cell sizes were 108±56 μm and 120±51 μm respectively. These values were significantly higher than normal for children of these ages.

FIG. 1. Freshly isolated adipose cells: A: from a normal 14-mo old child (× 100). Note the variation in cell sizes. No cytoplasmic or nuclear material is discernible. B: from a somewhat moderately obese 8-yr old child (× 100). Note the large size and the moderate variation in sizes. C: from an extremely obese 5-yr old child (× 100). The cells are huge, but even these large cells vary in size in this field. The grids in the background (Figs. 1, 3, 4 and 6) are 50 × 50μm in size.
TABLE 1
Mean Cell Size in Normal Children (μm Diameter) by Age

| Age group   | N  | Mean cell size (μm diameter) |
|-------------|----|-------------------------------|
| Birth-3 mo  | 8  | 60.6 ± 14.8                   |
| 3 mo-12 mo  | 5  | 49.0 ± 12.1                   |
| 1-5 yr      | 16 | 68.2 ± 17.4                   |
| > 5 yr      | 12 | 73.6 ± 19.0                   |

In culture, after a quiescent period of 7–15 days, the spherical cells began to assume an oval shape (Figs. 2A, B and C). This change in shape occurred earlier (sometimes as early as 2 days) in cells from younger children and later in cells from older children. Shortly thereafter they spread out to assume a more fibroblast appearance but retain much of the intracellular lipid (Figs. 3A and B). In a few days, the intracellular lipid fragmented into small droplets and subsequently disappeared giving the cells (now called adipofibroblasts) an appearance microscopically indistinguishable from that of regular skin fibroblasts (Fig. 4). Cells from some of the older children tended to retain intracellular lipid granules for considerably longer periods (Figs. 5A and B), sometimes for as long as 60–90 days.

In cells derived from children under a year of age and from those 11–15 years of age, the spherical cells began to assume an oval shape. This change in shape occurred earlier in cells from younger children and later in cells from older children. Shortly thereafter the cells spread out to assume a more fibroblast appearance but retain much of the intracellular lipid. In a few days, the intracellular lipid fragmented into small droplets and subsequently disappeared giving the cells (now called adipofibroblasts) an appearance microscopically indistinguishable from that of regular skin fibroblasts. Cells from some of the older children tended to retain intracellular lipid granules for considerably longer periods.

FIG. 2. Oval adipocytes: A: from a 6 day old infant—5 days after culture was initiated. Many of the cells have become flattened and assumed oval early fibroblast appearance. A single large fat globule fills the entire cell, but some cytoplasmic material can now be identified (× 100). B: from a 2-mo old infant (× 50). Seven days after plating, the cells have become spindle-oval shaped with its central intracellular fat globule intact. Some cytoplasmic material can also be seen. C: from a 6 mo old 8 days after plating. The cells appear similar to those in B with single intracellular lipid globule. A few cells appear to have multilocular lipid globules and some cytoplasmic material visible (× 50).

FIG. 3. Adipofibroblasts with intracellular globules: A: from the 2 mo old shown in Fig. 2B, but 14 days after the culture was initiated (× 100). The cells now have the characteristic appearance of fibroblasts except that most cells still have a smaller but obvious single or bilocular intracellular lipid globule. Much cytoplasm and the nuclei can be seen. B: An adipofibroblast from the same culture of the 6 mo old shown in Fig. 2C, but 20 days after the culture was plated (× 100). A single small lipid globule occupying a central location can be seen. The nucleus and much cytoplasmic material can also be identified.
GROWTH RATE OF HUMAN ADIPOCYTES IN CULTURE

FIG. 4. Adipofibroblasts in which almost all the cells are now lipid free from the same culture as in Figs. 2B and 3A, but 30 days after initial culture was plated. Each cell has an identifiable nucleus (× 100). They are mostly spindle-shaped but some are stellate.

FIG. 5. A: Adipofibroblast from a 6 day old child. The cells have been in culture for 60 days and vitally stained with Oil-Red-O which darkly stains the intracellular lipids (× 100). The nucleus is obscured by the lipids. B: Adipofibroblasts from a 13-mo old child. The cells have been in culture for 90 days and are vitally stained with Oil-Red-O which darkly stains intracellular lipids (× 100). The nuclei can be identified. Long tails of some cells can be seen in the field and are filled with granules of lipids.

FIG. 6. Mitotic Adipofibroblasts: A: Many cells in the field have assumed a rounded appearance prior to dividing. Some of the smaller cells have just divided and will soon resume the fibroblast appearance (× 100). B: Shows a dividing adipofibroblast in the middle of the field. Other mitotic rounded fibroblasts can be seen and some are respreading after completion of cell division (× 100).

age, after intracellular lipids have disappeared, mitotic activities can be observed between 9–17 days after initial cultures. Mitosis was evident when the cells assumed a rounded shape, then divided (Figs. 6A and B). This phenomenon is also characteristic of dividing fibroblasts. After division, the daughter cells reassumed the fibroblast appearance. Cell division has not been observed in a lipid-laden adipofibroblast. Very little or no cell division was observed in cells from children aged 1–10 years, even after the cells had become totally lipid-free. Cell division time averaged 5–7 days in cells from the youngest infants and 7–10 days in cells from the older
children and adolescents. Figure 7 depicts the growth rate of these cells by age of donor. It shows that cell multiplication occurred more often and more times in cells of newborn infants than in cells of older infants and not at all in cells of those 1–10 yr old. A moderate amount of cell division was found in cells of 5 children aged 11–15 yr but not in cells of two children aged 16 and 17 yr respectively. The data shows, for example, that an adipocyte from a newborn has the potential, in culture, to multiply and produce $10^6$ cells, while one from a 6 mo old has less than a quarter of that potential. However, the data also shows that the cells of children during pre- and early adolescence also possess significant potential for cell division.

**DISCUSSION**

The adipose cell in man is said to be a mature cell which rarely, if ever, divides (9). However, in this study, adipocytes of young infants and preadolescent children, after losing intracellular lipids were found to have a significant multiplication potential in culture. It is unlikely that this potential is achieved under normal in situ circumstances. Other evidence suggests, and it is reasonable to assume, that the potential for hyperplasia of adipose cells starts in utero. Therefore, the importance of extending this work to fetal adipose cells is recognized.

There is evidence that fat cells continue to be formed after the first year of life (12–14). However, it is not known if any increase in cell number after the first year and before early adolescence is due to cell division by existing adipocytes or the transformation of anlage cells present in fat tissue. The observations of Poznanski et al. (20) in which stromal cells of adipose tissue (termed "preadipocytes" by the authors) behave biochemically more like adipocytes than fibroblasts which they morphologically resemble coupled with the finding in this study that lipid-filled adipocytes do not divide would suggest that adipocytes of older children are probably derived from existing anlage cells present in the stroma of adipose tissue. However, our findings also suggest that cell division contributes to the increase in cell number found during the early adolescent period and very significantly during the first year of life.

Under the circumstances of the tissue culture in this study, the adipocytes appear
to be growing under lipolytic conditions. That these were adipocytes was demonstrated by the earlier observation that de-fatted adipose cells could be induced to reaccumulate intracellular lipids under appropriate \textit{in vitro} conditions (25).

Adipose cell size appears to decrease in the post neonatal period but begins to increase after one year of age. The initial decrease in size is likely due to the fact that adipose cell number increases by cell multiplication most rapidly during the first 3 mo of life, slowing down considerably in later infancy. An increase in adipose cell size would be expected if cell multiplication ceases entirely or becomes markedly reduced while the cells continue their function of lipid-storage. Reduced cell size might also be anticipated during the adolescent period when additional cell divisions are potentially possible. But such reduction in cell size was not observed in the cells of the five children, aged 11–15 yr, whose adipocytes actively divided in culture. This may be due to the fact that the rate of lipid storage, during this period, was greater than the amount of potential cell division actually realized.

This study appears to lend support to the concept that adipose cells may have a finite sensitive period during which much of the adult complement of adipocytes making up the adipose mass are formed. That the critical periods are limited is suggested also by the findings that adipose cell multiplications in culture occur only in cells of infants and adolescents. Assuming that additional exogenous and endogenous factors of currently unknown nature, trigger the production of obesity in accordance with the adipose hyperplasia theory, then the periods when these factors are likely to result in obesity may correlate fairly well with the period when the adipose cells have the greatest potential to multiply, namely the early infancy and, to a lesser extent, the early adolescence. The nature of some of these exogenous factors remains to be elucidated. Whatever they may be, it should be possible to apply the technique of tissue culture described here to elucidate some of them.

REFERENCES

1. Mayer, J., Regulation of energy intake and the body weight: glucostatic theory and the lipostatic hypothesis. \textit{Ann. N. Y. Acad. Sci.} \textbf{63}, 76 (1955).
2. Mayer, J., Hypothalamic control of gastric hunger contractions as a component of the mechanism of regulation of food intake. \textit{Amer. J. Clin. Nutr.} \textbf{8}, 547 (1960).
3. Kennedy, G. C., The role of depot fat in the hypothalamic control of food intake in the rat. \textit{Proc. Roy. Soc. London} \textbf{140}, 578 (1953).
4. Miller, D. S., Mumford, P., and Stock, M. J., Gluttony: thermogenesis in overeating in man. \textit{Amer. J. Clin Nutr.} \textbf{20}, 1223 (1967).
5. Brobeck, J. R., Neural regulation of food intake. \textit{Ann. N. Y. Acad. Sci.} \textbf{63}, 44 (1955).
6. Hirsch, J., and Knittle, J. L., Cellularity of obese and non obese human adipose tissue. \textit{Fed. Proc.} \textbf{29}, 1516 (1970).
7. Bjorntorp, P., and Sjorstrom, L., The number and size of adipose tissue fat cells in relation to metabolism in human obesity. \textit{Metabolism} \textbf{20}, 703 (1971).
8. Stern, T. S., Batchelor, B. R., Hollander, N., Cohn, C. K., and Hirsch, J., Adipose cell size and immunoreactive insulin in obese and normal weight adults. \textit{Lancet} \textbf{ii}, 948 (1972).
9. Bray, G. A., Davidson, M. B., and Drenich, E. J., Obesity: A serious symptom. \textit{Ann. Int. Med.} \textbf{77}, 797 (1972).
10. Knittle, J. L., Obesity in childhood: A problem in adipose tissue cellular development. \textit{J. Pediatr.} \textbf{81}, 1048 (1972).
11. Hirsch, J., and Han, P. W., Cellularity of rat adipose tissue: Effects of growth, starvation, and obesity. \textit{J. Lipid Res.} \textbf{10}, 77 (1969).
12. Brook, C. G. D., Lloyd, J. K., and Wolf, O. H., Relation between age of onset of obesity and size and number of adipose cells. \textit{Brit. Med. J.} \textbf{2}, 25 (1972).
13. Salans, L. B., Cushman, S. W., and Weismann, R. E., Studies of human adipose tissue: Adipose cell size and number in non obese and obese patients. \textit{J. Clin. Invest.} \textbf{52}, 929 (1973).
14. Brook, C. G. D., Evidence for a sensitive period in adipose cell replication in man. *Lancet* ii, 624 (1972).
15. Mossberg, H. O., Obesity in children: A clinical prognostical investigation. *Acta Paediatr.* 35, Suppl. 2 (1948).
16. Cheek, D. B., Insulin, early cell growth and excess adipose tissue. *Obes. Bariatric Med.* 2, 190 (1973).
17. Masters, E. M., Monolayer cultures of brown fat cells. *Proc. Soc. Exp. Biol. Med.* 119, 44 (1965).
18. Sidman, R. L., The direct effect of insulin on organ cultures of brown fat. *Anat. Rec.* 124, 723 (1956).
19. Ng, C. W., Poznanski, W. J., Borowiecki, M., and Reimer, G., Differences in growth in-vitro of adipose cells from normal and obese patients. *Nature (London)* 231, 45 (1971).
20. Poznanski, W. J., Waheed, I., and Van, R. Human, Fat cell precursors: Morphological and metabolic differentiation in culture. *Lab. Invest.* 29, 570 (1973).
21. Smith, U., Morphologic studies of human subcutaneous adipose tissue in vitro. *Anat. Rec.* 169, 97 (1971).
22. Adebonojo, F. O., Monolayer cultures of disaggregated human adipocytes. *In Vitro* (In Press) (1974).
23. Rodbell, M., Metabolism of isolated fat cells I: Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* 23, 375 (1964).
24. Puck, T. T., Cieciura, S. J., and Fisher, H. W., Clonal growth in vitro of human cells with fibroblastic morphology: Comparison of growth and genetic characteristics of single epithelioid and fibroblast—like cells from a variety of human organs. *J. Exp. Med.* 101, 148 (1957).
25. Adebonojo, F. O. Synthesis and storage of lipids by cultured adipocytes of a human neonate: Effects of sera from obese and non obese human adults. *Biol. Neonatorum* 23, 366 (1973).