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Growth and Maturation of Primary-Cultured Adipocytes From Lean and ob/ob Mice

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Abstract Stromal vascular cells from epididymal fat pads of lean and obese mice were cultured in a medium (α-MEM) containing fetal bovine serum (FBS) and cell replication followed for 11 days. In both types of cells, confluence occurred at 4–5 days, after which virtual growth arrest occurred in lean-mouse cells while replication continued, albeit at a slower rate in obese-mouse cells. Little or no lipid accumulation or glycerol-3-phosphate dehydrogenase (GPDH) activity was observed under these conditions. When a differentiation mixture consisting of insulin, corticosterone and isobutylmethylxanthine was added to the serum-containing α-MEM, a proportion of the lean-mouse cells accumulated triglycerides and GPDH activity increased significantly, indicating differentiation. By contrast, little or no differentiation occurred in obese-mouse cells. When cells grown in serum-containing α-MEM were transferred to a serum-free defined medium at confluence, extensive differentiation and maturation occurred in lean-mouse cells but not in obese-mouse cells. Similar experiments were conducted in cells isolated from the retroperitoneal fat pad. Although the growth pattern was similar to that of epididymal preadipocytes, the retroperitoneal lean- and obese-mouse cells differentiated more readily than epididymal cells, as shown by the GPDH specific activity. These data suggest that cells from obese mice are resistant to differentiation under conditions that support extensive differentiation in lean-mouse cells.

Key words: preadipocyte, adipocyte conversion, lipids, triglycerides, proliferation, ob/ob

The obese (ob/ob) syndrome in the mouse, the result of a mutation on chromosome 6, is characterized by hyperglycemia, hyperinsulinemia, hypercorticism, accelerated weight gain and marked obesity [Bray and York, 1971; Johnson and Hirsch, 1972; Herberg and Coleman, 1977]. Unlike other genetically obese mice, where only hypertrophy of the adipose cells is observed, the adipose tissue of ob/ob mouse is believed to be distinguished by a significant hyperplastic response [Johnson and Hirsch, 1972]. Increases in adipose cell number in vivo are a result of proliferation and subsequent differentiation of adipocyte precursor cells [Van, 1985].

A great deal of information on the development of adipose tissue has accrued from in vitro studies using cell culture techniques. Using preadipocyte cell lines, such as Ob17 and HGFu, derived from the epididymal fat pads of ob/ob or normal C57BL/6J mice, respectively [Négrel et al., 1978; Forest et al., 1983], and 3T3-L1 or 3T3-F442A cells, established from the mouse embryo [Green and Kehinde, 1975, 1976], it has been demonstrated that proliferation and differentiation of preadipocytes are coupled: i.e. growth arrest at confluence is followed by expression of early markers of the adipocyte phenotype (e.g., lipoprotein lipase and pOB24 mRNA), limited growth resumption, and finally terminal differentiation and expression of late markers (glycerophosphate dehydrogenase mRNA and activity, adipsin mRNA, and triglyceride accumulation) [for reviews, see Ailhaud et al., 1989, 1992a,b]. The establishment of adipogenic cell lines has proved invaluable for biochemical and molecular studies of adipocyte proliferation and maturation. However, ultimately, it is important to assess the growth characteristics of cells in primary culture.

The elevated circulating levels of corticosterone are thought to play and important role in the development of obesity in the ob/ob mouse [Bray, 1984]. Furthermore, the adipose tissue of the ob/ob mouse is characterized by an extreme resistance to the lipolytic action of catecholamines [Yen and Steinmetz, 1972; Bégin-Heick

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and Heick, 1977], an alteration in the hormonal regulation of adenylyl cyclase [Bégin-Heick, 1985] and a decreased abundance of G proteins (Bégin-Heick, 1990, 1992).

In vitro studies have identified various hormonal and growth factors important in the regulation of adipogenesis [for review, see Hausman et al., 1989]. Hormones, particularly insulin and corticosterone (or other glucocorticoids, such as dexamethasone), have been routinely used to trigger adipocyte differentiation in confluent cultures of 3T3-L1 and other cell lines [see Hausman et al., 1989], as well as preadipocytes isolated from fat deposits of rats [Schillabeer et al., 1989, 1990; Gaben-Cogneville et al., 1990].

The goal of the present study was to characterize the proliferation and differentiation of preadipocytes derived from fat tissues of normal lean (+/+) and obese (ob/ob) mice, to compare their ability to differentiate and to evaluate the effect of various differentiation programs on their ability to mature into lipid-containing adipocytes. Because adipose tissues from different anatomical sites are known to respond differently to hormones, some studies were carried out in parallel in epididymal and retroperitoneal tissues [Dijan et al., 1983].

We report that preadipocytes isolated from both epididymal and retroperitoneal fat pads of the ob/ob mouse exhibit an increased tendency to proliferate and a decreased ability to differentiate than the comparable cells from the lean (+/+) mouse under identical culture conditions.

MATERIALS AND METHODS

Animals

Male C57BL/6J ob/ob mice and their lean counterparts (+/+) were obtained from Jackson Laboratories (Bar Harbor, ME). They were kept in a temperature-controlled room (23 ± 1°C) with 12-h light cycles. The animals were fed chow and water ad libitum; they were used in the experiments at the age of 5–7 weeks.

Preparation of Preadipocytes and Cell Culture

Mice were killed by cervical dislocation and epididymal and retroperitoneal fat pads were excised aseptically. Preadipocytes were isolated as described by Shillabeer et al. [1990]. Briefly, the fat tissue was minced and digested with 1 mg/ml collagenase (Type IV, Sigma, St. Louis, MO) for 1 h at 37°C. The cells were filtered twice, once through a 250-μm mesh nitex filter (B and SH Thompson, Scarborough, Ontario, Canada) and again through a 25-μm mesh filter and a sample was counted using a Coulter counter (Coulter Electronics, Burlington, Ontario, Canada). The remaining cells were suspended in α-minimal essential medium (α-MEM, GIBCO BRL, Burlington, ON) supplemented with 10% fetal bovine serum (FBS, GIBCO BRL), penicillin, and streptomycin (S-α-MEM). Preadipocyte preparations were obtained from epididymal (E) and retroperitoneal (R) adipose tissue of lean (ln) and obese (ob) mice.

Measurements of Cell Growth and DNA Synthesis

Cells were seeded in 24 well plates (Nunc Plasticware, GIBCO BRL) at densities ranging from 8,000 to 35,000 cells per well for growth studies (corresponding to densities ranging from 4 to 20 × 10^3/cm^2) as described in the text. Growth was measured by counting cells on successive days, following trypsinization. DNA synthesis was assessed by measuring the incorporation of [^{3}H]thymidine (3 μCi/ml), as described by Gaben-Cogneville et al. [1990]. Cell were seeded at ~10,000 cells per well and the rate of replication and the incorporation of label were assessed in parallel samples.

Measurements of Differentiation

Cells were seeded in 24 well plates at a density of ~15,000 cells per well and grown in S-α-MEM until confluence. At confluence, the cells were exposed to S-α-MEM containing differentiation factors (DS-α-MEM). The factors used were 0.1 μM corticosterone, 0.01 μM insulin and 0.5 mM 1-methyl-3-isobutylxanthine (MIX) for 48 h. The medium was then changed for medium containing corticosterone and insulin but no MIX (HS-α-MEM).

In some studies, confluent cells grown in S-α-MEM were differentiated by culture for 5 days in the serum-free medium described by Deslex et al. [1987], which contained 1 μM insulin, 10 μg/ml transferrin and 200 pM triiodothyronine (SF-ITT), as described by Hauner [1990].

To evaluate the extent of differentiation, the cells were fixed in 10% formalin, stained with oil red O to show lipid droplets, and counterstained with 10% Giemsa. The cells containing visible fat droplets were counted and the results expressed as a percentage of the total number of

cells, in 6 randomly chosen fields per well. Glycerophosphate dehydrogenase activity (GPDH; EC 1.1.1.8), a late marker differentiation was measured according to the method of Wise and Green [1979].

RESULTS

Preadipocyte Replication

Since the seeding density of preadipocytes is an important factor in their ability to undergo growth arrest at confluence and to differentiate [Sztalryd and Faust, 1990], we measured the growth of preadipocytes derived from the epididymal fat pads of lean (E-ln) and obese (E-ob) mice after seeding at varying densities. Growth curves were calculated for cells seeded at 8,000–18,000 (Fig. 1A) or 20,000–35,000 cells per well (Fig. 1B) (corresponding to 4–10 × 10^3 and 11–20 × 10^3 cells/cm^2, respectively).

There were clear differences in the growth patterns of cells derived from E-ln and E-ob, at both seeding densities. In E-ln preadipocytes, there was an initial phase of logarithmic growth from day 1 until confluence at ~5 days, followed by growth arrest or a period of very slow growth from days 5–10. In E-ob preadipocytes, growth was virtually identical to that observed in the E-ln until confluence (day 5). However, unlike the E-ln, the E-ob preadipocytes continued to grow after only a transient (day 5–6) pause in growth. Doubling times calculated for each of these periods are shown in the inset to Figure 1. They were significantly different between cells of E-ln and E-ob origin for the post-confluent period (days 5–10). Comparable results were obtained whether cells were seeded at the low or high densities, except that in E-ln cells the period of growth arrest occurred slightly earlier at

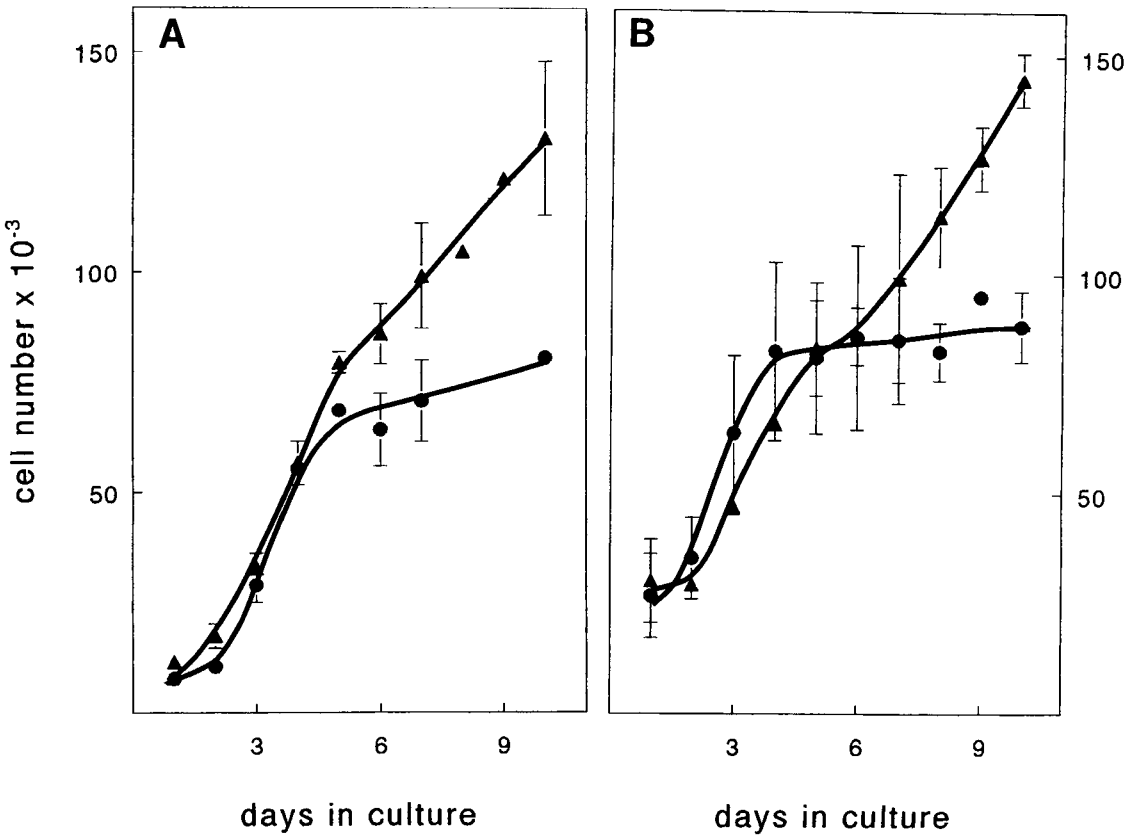

Fig. 1. Replication of epididymal pre-adipocytes. Stromal-vascular cells isolated from epididymal fat pads of lean (●) and ob/ob (▲) mice were plated at either 5–10 × 10^3 (A) or 11–20 × 10^3 (B) cells/cm^2 and maintained in S-α-MEM. Growth was followed by counting the cells on successive days for 11 days. The results represent means ±SE of 5 separate experiments. The rates of replication (in days) are given in the inset.

The column headed days refers to days in culture.

Days	+/+	ob/ob
1–5	2.5 ± 0.1	3.1 ± 0.6
5–10	26 ± 4.2	5.6 ± 0.7

the high seeding density (Fig. 1B). As a result of these discrepant growth patterns, the total number of E-ob preadipocytes per culture dish was 1.6-fold that of the E-ln by day 10 [$P < 0.01$ ($n = 5$)] at each seeding density.

[^{3}H]Thymidine incorporation and cell growth were measured in cells isolated from E-ln and E-ob fat pads (Fig. 2). Until days 4–5, [^{3}H]thymidine incorporation was similar in both groups. At confluence, there was a 75% decrease in incorporation of the label in the E-ln cells. This drop preceded the period of growth arrest or slow growth observed in parallel growth experiments. By contrast, [^{3}H]thymidine incorporation on a per cell basis was approximately 2.4 fold higher in E-ob than in E-ln preadipocytes. This difference parallels the continued growth of E-ob preadipocytes after confluence (Fig. 1A).

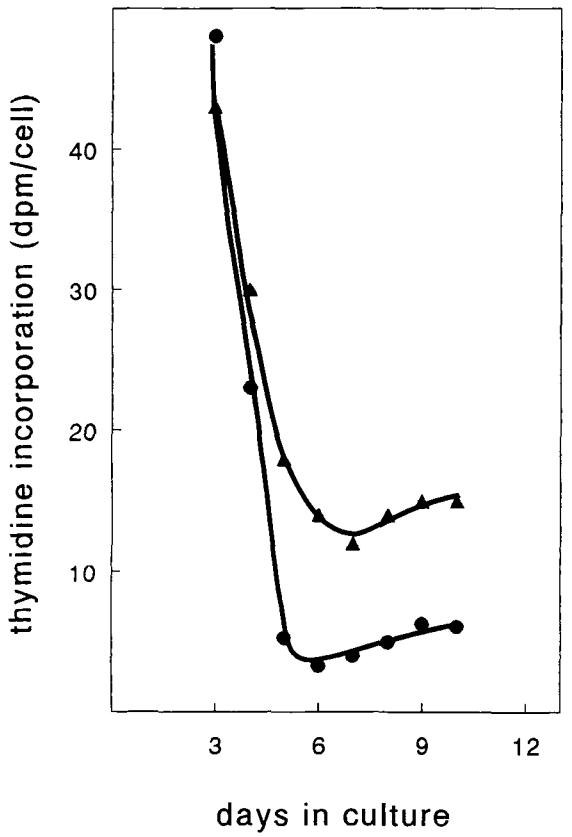

Fig. 2. [^{3}H]Thymidine incorporation in epididymal cells. Stromal-vascular cells isolated from epididymal fat pads of lean (●) and ob/ob (▲) mice were plated at a density of 0.4×10^4 cells/cm were pulsed with the labelled thymidine for 24 hours. The incorporation of label was assessed by counting duplicate samples. The data are from one experiment, representative of two such experiments.

Hormonally Induced Differentiation

To determine whether E-ln and E-ob preadipocytes are able to differentiate into mature adipocytes, despite their different growth characteristics, the cells were induced to differentiate by various treatments. Measurement of GPDH activity as well as assessment of lipid accumulation by Oil red O staining were used to assess the extent of differentiation. While both E-ln and E-ob preadipocytes could be induced to differentiate into cells containing numerous lipid droplets, both methods showed that E-ln cells differentiated to a significantly greater extent than E-ob cells (Table I). Because it is difficult to count post-confluent cells in situ with precision and because the oil red O method assessed only the presence of lipids but not the extent of accumulation, the GDPH measurements are believed to be a more accurate assessment of the difference between the two groups. The morphological appearance of the cells paralleled the GPDH activity. Figure 3A and B shows that control E-ln and E-ob (ie cells grown 5 days post-confluence in S—α-MEM showed little tendency to accumulate lipids (Fig. 3Aa, and 3Ba). Induction with the DS-α-MEM/HS-α-MEM caused some lipid accumulation in E-ln (Fig. 3Ab) but not in E-ob (Fig. 3Bb) cells.

Because the adipose tissue of the obese mouse is exposed to much higher levels of insulin and corticosterone in vivo than was the case in the DS-α-MEM, in some studies, confluent E-ob preadipocytes were cultured in medium containing levels of insulin and corticosterone comparable to the elevated levels present in the obese mouse circulation or, in medium containing high insulin (0.1 μM) and normal corticosterone. Neither of these manipulations increased the extent of differentiation in epididymal fat pads of obese mice (data not shown).

Effects of a Decrease in FBS on Growth and Differentiation

Since the epididymal preadipocytes of the obese mouse displayed an increased capacity for growth, we tested the effects of reducing the amount of FBS in the culture medium to 7% or 5% from 10%. Such a reduction was expected to diminish growth potential and, consequently, improve the capacity of the cells to differentiate. Cell growth and the extent of hormonally-induced differentiation were measured as described above. While 5% and 7% FBS decreased

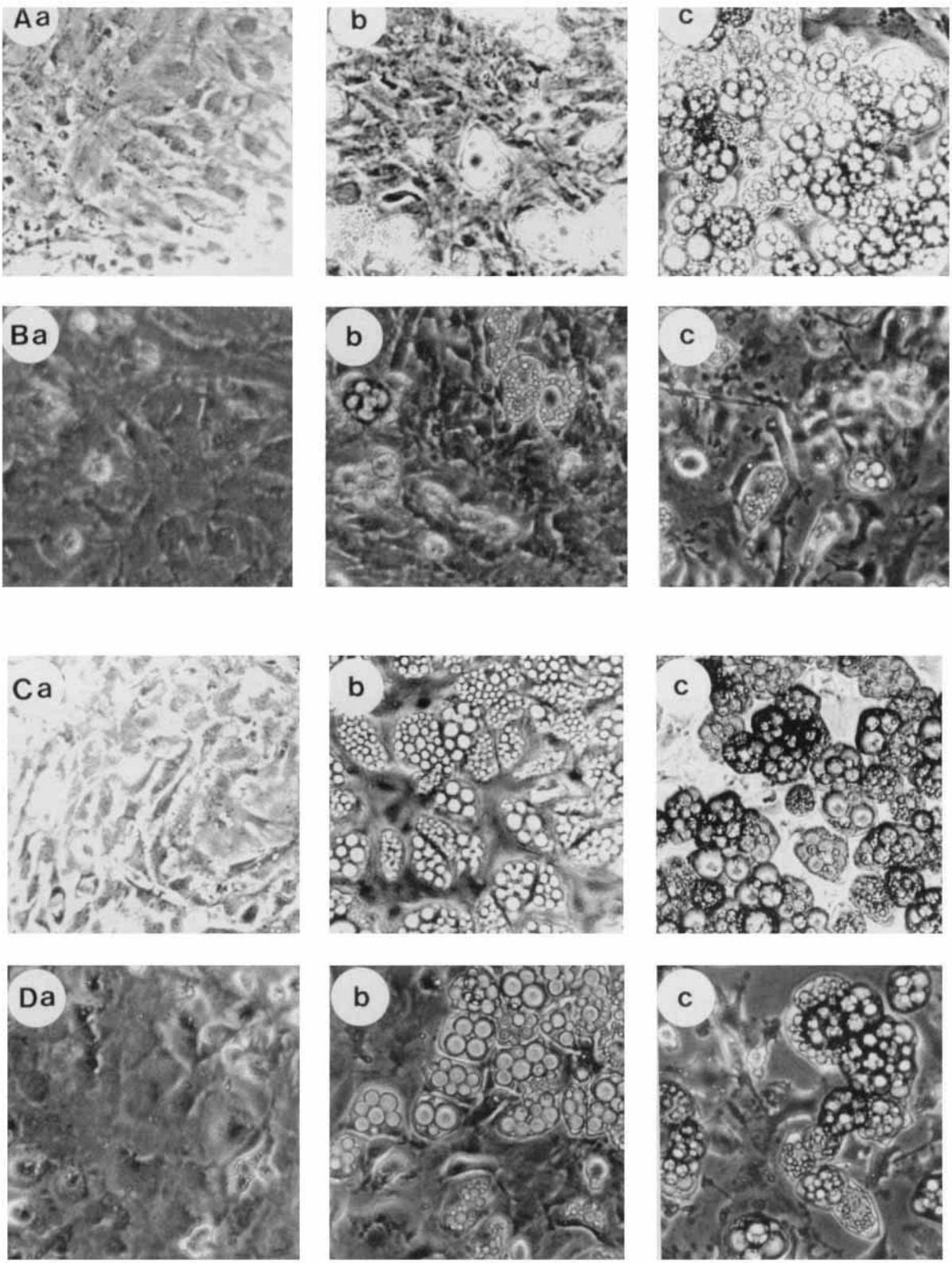

Fig. 3. Morphological appearance of stromal–vascular cells maintained under various culture conditions. Each set of photographs represents cells at 5 days post-confluence: a, control cells; b, cells exposed at confluence to DS-α-MEM followed by HS-α-MEM; c, cells exposed at confluence to SF-ITT medium. **A:** Epididymal cells from lean mice. **B:** Epididymal cells from ob/ob mice. **C:** Retroperitoneal cells from lean mice. **D:** Retroperitoneal cells from ob/ob mice. The morphological appearance of the cells correlates with the GPDH activities shown in Table I for epididymal cells and Table II for retroperitoneal cells. ×500.

TABLE I. Influence of Growth Conditions on Lipid Accumulation and Adipocyte Conversion in Stromal–Vascular Cells Isolated From Epididymal Adipose Tissue of Lean (+/+) and Obese (ob/ob) mice*

	Oil red O-stained cells (% total)		GPDH activity (mU/mg protein)	
Medium	+/+	ob/ob	+/+	ob/ob
S-α-MEM	1.6 ± 0.4 (9)	0.9 ± 0.2 (18)	5.0 ± 1.0 (6)	3.5 ± 1.2 (9)
DS-α-MEM (10%)	15 ± 6.1 (13)	5.0 ± 0.5 (24)	155 ± 13 (13)	7.2 ± 0.8 (14)
DS-α-MEM (5%)	nd	nd	185 ± 29 (4)	6.9 ± 1.4 (4)
SF-ITT	nd	nd	572 ± 241 (4)	23.4 ± 7.4 (8)

*The cells were grown to confluence in S-α-MEM and maintained 5 days post-confluence under the conditions specified; 5% and 10% refer to the concentration of FBS in the DS-α-MEM. Data are means ±SE for the number of samples tested. Details of the media used are described in the method section. For each type of measurement, the values were significantly smaller ($P < 0.05$) in E-ob than in E-ln, except in the S-α-MEM. The GDPH activity was significantly greater ($P < 0.05$) when cells were differentiated in the SF-ITT than in the serum containing media.

the number of cells in E-ob cultures by 41% and 23%, respectively, at day 10, the pattern of cell growth was not altered in that growth arrest also did not occur under these conditions (Fig. 4). By contrast, growth of E-ln preadipocytes was not reduced significantly by decreasing the concentration of FBS, at day 11, the cell count was 80 ± 8 vs 78 ± 3 × 10^3 cells/well for cells grown in 10 and 5% FBS, respectively.

Although dropping the FBS concentration in the culture medium by half diminished the rate of replication of E-ob preadipocyte, it had no positive effect on the ability of these cells to undergo growth arrest at confluence or to differentiate in response to hormonal induction, as measured by GPDH activity (Table I). These results suggest that while the proliferation of E-ob preadipocytes is sensitive to and is enhanced by FBS, the slower growth achieved at low FBS concentration is not sufficient to trigger the events leading to maturation into adipocytes.

Effects of Defined Medium

The serum-free defined medium, described by Deslex et al. [1987], was reported to increase differentiation in preadipocytes isolated from the epididymal fat pads of rats. We therefore tested its effect on the ability of E-ln and E-ob cells to differentiate, following growth to confluence S-α-MEM, as described by Hauner [1990]. This protocol significantly increased the ability of preadipocytes of both groups to differentiate (Fig. 3, cf. Ac and Bc to Ab and Bb, respectively). However, despite the low mitogenic potency of the serum-free medium, GPDH activity, was only stimulated 3–4-fold in E-ob cells, compared to 100 fold in E-ln cells (Table I), showing that, even under these conditions, adipose conversion does not occur normally. This suggests that cultured E-ob preadipocytes have lower adipogenic potential than E-ln preadipocytes. Taken together, these data suggest that E-ob preadipocytes in culture are resistant to differentiation stimuli that allow E-ln adipocytes to differentiate and mature.

Characteristics of Adipocytes From Retroperitoneal Fat Pads

The growth of preadipocytes from retroperitoneal fat depots of lean and obese mice (R-ln and R-ob) was also examined to determine whether the discrepant patterns of growth seen in the E-ob preadipocytes occurs in other fat tissues. In general, the growth pattern of retroperitoneal cells was similar to that observed for the epididymal cells (Fig. 5). As was the case for E-ob cells, the R-ob cells did not undergo growth arrest at confluence but continued to proliferate, albeit at a slower rate. The absence of growth arrest at confluence in cells derived from the obese mouse may therefore be a general characteristic of fat depots.

The ability of retroperitoneal cells to differentiate was also assessed. The data show that R-ln and R-ob cells showed some spontaneous (in the absence of added hormones) differentiation, once they reached confluence (Fig. 3, Ca and Da). Upon hormonal induction in SH-α-MEM, similar levels of differentiation were obtained in R-ln and R-ob cells as measured by GPDH activity (Table II) and by morphological assessment (Fig. 3, Cb and Db). Paradoxically, while the SF-ITT medium increased GPDH activity twice as much

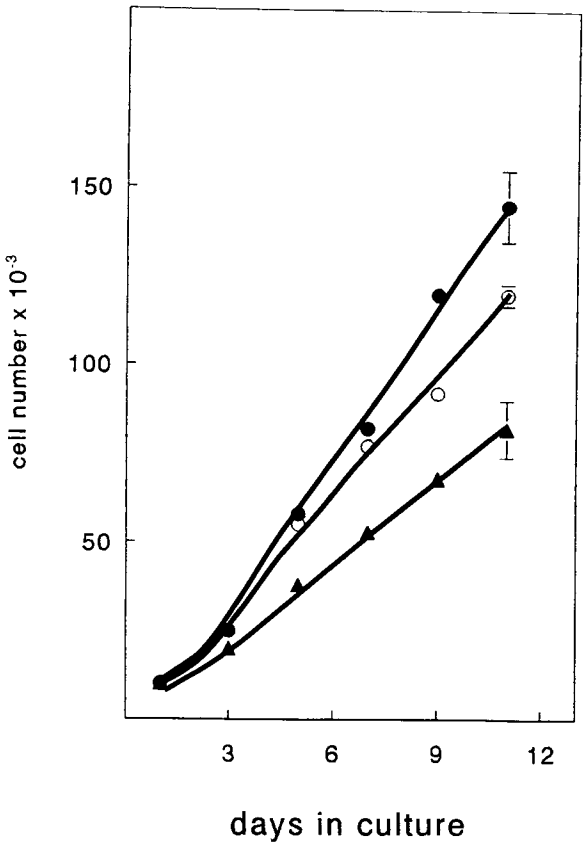

Fig. 4. Effect of serum concentration on the replication of epididymal pre-adipocytes from obese mice. Stromal–vascular cells isolated from epididymal fat pads ob/ob mice were plated at 10 × 10^3 cells/cm^2 and maintained in S-α-MEM containing either 10 (●), 7 (○) or 5% (▲) FBS. Growth was followed by counting the cells on alternate days for 11 days. The results represent means ±SE of 3 separate experiments.

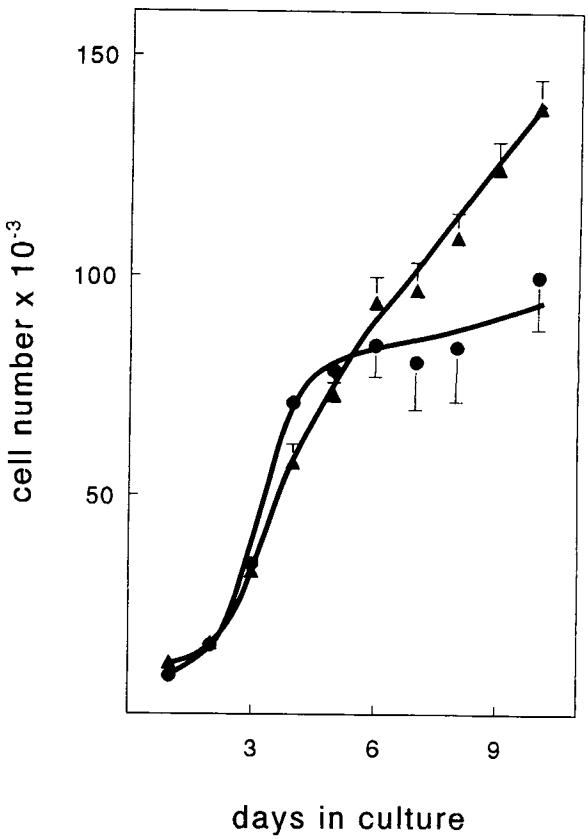

Fig. 5. Replication of retroperitoneal cells. Stromal-vascular cells isolated from retroperitoneal fat pads of lean (●) and ob/ob (▲) mice were plated at either 0.5–1.0 × 10^4 (A) or 1.1–2.0 × 10^4 (B) cells/cm^2 and maintained in FBS-containing α-MEM. Growth was followed by counting the cells on successive days for 11 days. The results represent means ±SE of 3 separate experiments.

as did the SH-α-MEM in R-ln cells, it did not increase GPDH of R-ob cells more than did the SH-α-MEM. Similarly, the lipid accumulation in R-ob cells was less (Fig. 3 Dc) than in R-ln cells (Fig. 3, Cc). These results suggest that different fat depots in the obese mouse have different differentiation programs and different requirements for hormones and other growth factors.

DISCUSSION

Proliferation and differentiation are considered to be coupled processes in preadipocytes, with growth arrest required to trigger commitment to the adipocyte phenotype and the limited growth resumption necessary for terminal differentiation [see Ailhaud et al., 1989, 1992a,b for reviews]. E-ln cells followed this differentiation programme, particularly when allowed to differentiate in SF-ITT medium. The absence of growth arrest upon confluence in E-ob cells was associated with a markedly reduced capacity to accumulate lipids and express GPDH activity (a marker for terminal differentiation). These data therefore support the generally accepted view.

Preadipocytes usually show a lower frequency of adipose conversion in serum-supplemented medium, because of its high mitogenic potency [Deslex et al., 1987]. Replacement of serum-supplemented with serum-free medium increases the frequency of adipose conversion in cultured preadipocytes derived from rat or human tissues [Deslex et al., 1986, 1987] or in clonal cells [Hauner, 1990]. Various factors (PDGF, TGF-β) in FBS have known antiadipogenic activities. For example, EGF has been shown to inhibit fat cell differentiation in vivo [Serrero and Mills, 1991] and in vitro [Serrero, 1987], and reduced EGF levels in the plasma of the obese mouse are suspected of being related to its abnormal adi-

TABLE II. Influence of Growth Conditions on Lipid Accumulation and Adipocyte Conversion in Stromal–Vascular Cells Isolated From Retroperitoneal Adipose Tissue of Lean (+/+) and Obese (ob/ob) Mice*

	Oil red O-stained cells (% total)		GPDH activity (mU/mg protein)	
Medium	+/+	ob/ob	+/+	ob/ob
S-α-MEM	6.7 ± 0.6 (4)	5.2 ± 0.7 (7)	96 ± 21 (4)	16.2 ± 4.2 (8)
DS-α-MEM	41.4 ± 6 (4)	56.6 ± 5.0 (8)	722 ± 100 (6)	561 ± 78 (13)
SF-ITT	nd	nd	1488 ± 228 (5)	443 ± 105 (6)

*The cells were grown to confluence in S-α-MEM and maintained 5 days post-confluence under the conditions specified. Data are means ±SE for the number of samples tested. Details of the media used are described in the method section.

pose tissue development [Serrero et al., 1993]. However, some investigators have reported various degrees of adipose differentiation using FBS-containing media by adding diverse combination of hormones and differentiation factors at confluence. It is clear that reducing the mitogenic potency of the culture medium, as shown by reduced growth, did not promote growth arrest nor improve the adipogenic potential of E-ob preadipocytes.

The finding that preadipocytes derived from E-ob fat pads accumulated only limited amounts of lipids under culture conditions that favor high levels of triglyceride accumulation in the E-ln cells is counter-intuitive in view of the almost unlimited capacity of the ob/ob mouse to accumulate triglericerides in adipose tissue. The expansion of adipose tissues in this animal is believed to be a result of both hyperplasia and hypertrophy [Johnson and Hirsch, 1972], suggesting that proliferation and differentiation are ongoing processes in vivo. Studies on the replication and conversion of Ob17 or Ob1771 [Forest et al., 1983] clonal preadipocytes suggest, however, that cells that originate from the obese mouse may have different requirements for growth and differentiation than cells that originate from normal mice. Thus, the SF-ITT medium first described by Deslex et al. [1986] was shown to support extensive differentiation in normal rat preadipocytes [Deslex et al., 1987] and in mouse 3T3 cells [Hauner, 1990].

The expression of the increased potential for proliferation and reduced potential of preadipocytes for adipose conversion in the obese took different forms in cells isolated from epididymal and retroperitoneal fat depots. Others have found that, in the rat, preadipocytes derived from fat depots of anatomically different regions differ with respect to their capacity for proliferation and differentiation [Dijan et al., 1983; Wang et al., 1989]. Preadipocytes from the perirenal depot have a greater number of clones with a higher frequency of replication and differentiation than those from epididymal fat and preadipocytes from retroperitoneal tissue differentiate to a greater extent than those from epididymal tissue [Grégoire et al., 1991]. Our observations are in harmony with these findings since both R-ln and R-ob preadipocytes differentiated more readily than those from the epididymal depot. R-ob cells, like E-ob cells, had a greater potential for growth, as shown by their failure to growth arrest at confluence. This failure to growth arrest at confluence is similar to the pattern reported for preadipocytes from massively obese humans compared to control subjects [Roncari et al., 1981], suggesting that enhanced proliferation may be common to other adipose tissues in the obese mouse and perhaps in other models of obesity. However, Shillabeer et al. [1990] showed that preadipocytes from several fat depots of the corpulent rat did not replicate as rapidly as those from normal rats. More work is clearly needed before the meaning of these observations is understood.

The preadipocyte population is heterogeneous, with various precursor subtypes having different replicative capacities depending on age, anatomic site, and other factors [Dijan et al., 1983; Wang et al., 1989]. It is possible that the content of immature preadipocytes and thus the potential for proliferation is greater in the epididymal fat pad of the obese mouse. The seeding density used in this study was relatively low in order to amplify any differences between adipose depots as well as between the lean and obese mice deposits, since increasing the seeding densities is known to decrease the differences in differentiation [Wiederer and Löffler, 1987] and proliferation [Sztalryd and Faust, 1990] between depots. It has been suggested that plating at high

densities results in seeding a greater number of cells at more advanced stages of differentiation, obscuring adipose conversion in the more immature cells [Sztalryd and Faust, 1990]. Since immature preadipocytes replicate more extensively than mature preadipocytes, it is possible that more immature preadipocytes were plated from the obese epididymal fat pad than from the same deposit of the lean mouse.

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