Influence of Anatomic Site and Age on the Replication and Differentiation of Rat Adipocyte Precursors in Culture

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ABSTRACT Using a propagating cell culture system of adipocyte precursors from 70–400-g rats, we explored the possibility that regional variations in properties of adipose tissue may reflect site-specific characteristics intrinsic to the cells, rather than extracellular influences. Initially, studies were made of the nature of the fibroblastlike cells from perirenal adipose tissue stroma. Using colony-forming techniques, it was shown that these cells were adipocyte precursors; each confluent colony that was derived from a single cell displayed differentiated adipocytes. This characteristic was evident in cells from rats of all ages and persisted during secondary culture. At all ages of rats studied, perirenal cells replicated more rapidly than epididymal precursors, e.g., for 179-g rats the population-doubling times were 19.3±0.7 vs. 25.5±1.2 h (means±SEM, $P < 0.03$). With aging of the rats, the replication rate of their perirenal cells decreased progressively. Under clonal conditions, the colony size distribution of both perirenal and epididymal precursors revealed heterogeneity in their capacity for replication, perirenal cells showing greater proliferation. These also differentiated more extensively by morphologic and enzymatic criteria. Age and site had effects that persisted through many cell generations; however, high-fat feeding had no perpetuating influence. The dissimilar properties of perirenal as compared with epididymal precursors may reflect differences in regulation of gene expression. The data are also compatible with a later development in embryological life of perirenal tissue. We suggest that the composition of the adipocyte precursor pool is an important determinant of the growth of adipose tissue that occurs in response to a nutrient load. Interregional or interindividual variation in composition may explain regional and individual differences in fat accumulation.

INTRODUCTION

Regional variation in adipose tissue growth is an intriguing yet unexplained aspect of the biology of this tissue. Despite the abundant information that is available concerning the mechanisms that subserve lipid storage and mobilization in adipocytes, there is little understanding of why adipose tissue accumulates more readily in certain anatomic sites rather than others and why this distribution is affected by hormones such as glucocorticoids and estrogens. The limited information that is available suggests that adipose tissue samples from different sites display disparities in lipolysis and in fat cell number, two factors that are obviously fundamental to the determination of the mass of a given adipose depot.

Pronounced differences exist between human subcutaneous and omental adipose tissue in the lipolytic response to catecholamines; these differences probably represent variations in alpha receptor content or function in fat cells derived from the two sites (1). Indeed, alpha-1 receptors are only identifiable in human omental fat tissue and are entirely absent from human subcutaneous depots (2). Significant regional variations in the extent to which adipocyte number changes in response to stimuli such as lipectomy and/or fat feeding are also evident. In rats, inguinal lipectomy followed by high-fat feeding results in specific enhancement of adipose growth in the subcutaneous areas (3). In rodents with genetic obesity syndromes or in whom obe-

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sity is induced with high-fat diets, adipocyte hyperplasia develops more readily in specific fat depots (4, 5). These data, while derived from a variety of experimental approaches, lead us to suggest that differences in behavior of adipose tissue from various regions may reflect fundamental, site-specific characteristics intrinsic to cells, or cell clones. The disparate properties intrinsic to cells might result in regionally different modes of cellular communication including direct transmission of chemical messengers between contiguous cells as well as paracrine and autocrine mechanisms. Using a propagating cell culture system of adipocyte precursors (6, 7), we explored the possibility in the present study that regional differences in adipose tissue growth reflect intrinsic cellular properties, rather than locational disparities in such external influences as blood or nerve supply, or ambient temperature. Our experimental system allowed observations of replication and differentiation of cell mixtures and clones not only in primary culture, but also many generations removed from the animal, and hence free of external influences that might apply in vivo. Studies were made of the effects of site of origin, and of animal age, weight, and diet on replication rates and on differentiation of adipocyte precursors from perirenal and epididymal depots.

METHODS

Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories Canada Inc., St. Constant) were kept in air-conditioned (20–23°C) quarters for 3–5 d and fed with rat Purina chow (Ralston Purina Co., St. Louis, MO) before any experimental procedure. The initial weights of the animals were in the 70–400-g range, and varied with the particular experimental protocol. For experiments evaluating effects of diet, rats were kept one per cage; in the other studies, two rats were maintained in one cage.

For the studies on the influence of diet, a 55% fat diet (5793-M-1 Ralston Purina Co.) was used. Lard (55%) was substituted for corn oil by the company to facilitate administration and consumption of the diet. This diet will be referred to as "high-fat." The control "low-fat" diet (5793-M-2 Ralston Purina Co.) contained 10% corn oil. All animals were fed and provided tap water ad lib. Animals were killed by cervical dislocation.

Isolation of adipocyte precursors. Adipocyte precursors were isolated from epididymal and perirenal adipose tissue and cultured by methods similar to those previously reported in references 6 and 7. To summarize the procedure and specifying the modifications, the fat pads were digested at 37°C with a 1.5 mg/ml collagenase preparation Type II, Sigma Chemical Co., St. Louis, MO), in Hanks' balanced salt solution, pH 7.4, and in the presence of 1.5 mg/ml bovine serum albumin (Fraction V, Sigma Chemical Co.) for 30–45 min (until complete disaggregation of tissue occurred). After filtration through a 25-μm pore nylon sieve (Nitex CH325, Thomson Band SM, Montreal, Canada) the cell suspension was centrifuged at 800 *g* for 10 min. The cells contained in the pellet were then plated on 100-mm Petri dishes.

Primary culture and cloning of adipocyte precursors. For primary culture, the cells were allowed to adhere for 12 h in alpha minimal essential medium buffered with 15 mM Hepes (Gibco Laboratories, Grand Island, NY), supplemented with 10% fetal bovine serum (Gibco Laboratories), penicillin (100 U/ml), and streptomycin (100 μg/ml). After 12 h, the floating cells, mainly erythrocytes, were removed by extensive washing with phosphate-buffered saline (pH 7.4). The adherent cells morphologically resembled fibroblasts and are subsequently referred as "fibroblastlike" cells. They were used as the source of cells in all subsequent studies. In the clonal experiments, the fibroblastlike cells were detached with 1 mg/ml trypsin-0.2 mg/ml Na_2EDTA, and an aliquot of cells was counted with a modified Neubauer hemocytometer after staining the nuclei with crystal violet. A period of 12 h was sufficiently long to allow adherence of cells, but was too brief for appreciable replication as indicated by negligible incorporation of radioactive thymidine into DNA. Thus, at this time, the number of isolated adipocyte precursors should have been similar to that occurring in vivo. Cloning was only carried out with these cells in primary culture.

After they were counted, cells were diluted with fresh growth medium and plated at a calculated inoculum of 1 cell/2.5 "microwells" (96 wells/plate, 0.28 cm^2/well, Linbro Scientific Co. Inc., Dublin, VA). When cloning was carried out to determine replicative potential, the cells were kept undisturbed for 14 d in the previously described growth medium. The medium was then decanted, the colonies were stained with 10% Giemsa, and the cell number was estimated through a phase-contrast microscope with the aid of a grid.

Secondary culture of adipocyte precursors. Cells were grown for 3–4 d in primary culture after which the floating cells were removed and the adherent cells were detached with trypsin and subcultured. For determination of population-doubling time, ~30,000 cells were plated on each 35-mm Petri dish, and counted daily for 4 d. The population-doubling time was then calculated from the linear segments of semilogarithmic plots.

Differentiation of adipocyte precursors in secondary culture. After primary culture, ~50,000 perirenal or epididymal precursors were inoculated per 2-cm^2 well (Costar, Data Packaging, Cambridge, MA) in multiwell plates and allowed to reach confluence without any change of medium. The medium was then removed completely, and a "collagen gelation mixture" was overlaid on the cells. This mixture included a preparation enriched in collagen derived from rat tail, and plasma-derived serum prepared from Canadian Red Cross citrated plasma (8). Aliquots (0.8 ml) of the gelation mixture were overlaid on the cells of each well and after gel formation 1 ml of alpha medium containing additional plasma-derived serum and 1 μg/ml bovine insulin (Sigma Chemical Co.) were added. The gels, along with the adherent cells, were detached from the wells with a Pasteur pipette, washed, digested with collagenase, and the released cells were counted in a hemocytometer.

Differentiation was assessed by light-microscopic examination and by determination of *sn*-glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) activity at different times relative to confluence. The morphological criterion of differentiation was the appearance in the cytoplasm of large lipid droplets. Other studies have established the importance of glycerophosphate dehydrogenase activity as a marker of fat cell differentiation (9). In the assay, after detaching the gels, cells were disrupted by sonication (three intervals of 30 s at 50-kilocycles and 0–4°C) with the tip of a Biosonik III Sonifier, (Bronwill Scientific, Rochester, NY) in 50 mM Tris-HCl (pH

7.5), 0.25 mM sucrose, and 1 mM Na_2EDTA. After centrifugation at 48,000 *g* for 1 h, aliquots of the supernate were assayed at 27–28°C with a recording Gilford Spectrophotometer, model 2400-S, by a method reported in reference 9. The total volume of each reaction mixture was scaled down to 0.2 ml. Conditions of linearity and optimal concentrations of substrates and cosubstrates were observed. One unit of enzyme activity is defined as the oxidation of 1 nmol of NADH/min.

Differentiation of adipocyte precursor clones in primary culture. To determine the capacity for differentiation in perirenal precursor clones, the clones were isolated as previously described. After several clones had reached confluence, the growth medium was removed and the collagen gelation mixture was overlaid on the cells, followed by medium supplemented with plasma-derived serum and bovine insulin. The supplemented growth medium was changed every 4 d. 2–3 wk after adding the collagen gelation mixture, the degree and frequency of differentiation were estimated by using light microscopy.

RESULTS

Differentiation of adipocyte precursors. Previous studies have demonstrated that human and rat adipocyte precursors will differentiate into mature elements in cell culture (7). In the present study, an attempt was made to determine the fraction of rat fibroblastlike cells that have the capacity to form differentiated fat cells. In these experiments, perirenal tissue was taken from 180-g rats and the fibroblastlike cells studied in primary culture under clonal conditions. Under these conditions, each colony was derived from one precursor. The cells were allowed to replicate to confluence, after which differentiation was promoted with the collagen gelation mixture, insulin, and plasma-derived serum. Of the cells inoculated, on the average one-third grew rapidly enough to produce confluent clones after 2–3 wk. As exemplified by the colony shown in Fig. 1, by morphological criteria all confluent colonies contained differentiated fat cells. Further, when the precursors were propagated in secondary culture, they retained the capacity for adipocyte differentiation. Figures documenting this process are shown later in the text. Since the cells that grow rapidly in primary culture are adipocyte precursors and since rapidly growing cells must increasingly predominate in secondary culture, the findings to be described in secondary culture must have been derived from cell populations composed predominantly of adipocyte precursors.

Effect of anatomic site and animal age on the replication rates of adipocyte precursors. In these studies, populations of perirenal and epididymal precursors were obtained and their doubling times were determined. The observations were made in secondary culture to ensure the absence of any persisting in vivo influence. The data are shown in Table I. The cells from each anatomic site were obtained from three sets of rats, with average weights of 73, 178, and 398 g. In animals of all ages studied, the precursors from perirenal tissue replicated at significantly more rapid rates than cells from the epididymal depot. It is important to note that the differences observed in population-doubling times between perirenal and epididymal precursors represent large differences in cell number. For example, in one experiment with 180-g animals (with the population-doubling time of perirenal precursors at 19.6 h and that of epididymal cells at 24 h), after 3 d of growth of similarly sized inocula, 6.1×10^5 perirenal cells were present, compared with 3.1×10^5 epididymal cells.

In addition to anatomic site, the age of the rats had a significant influence on replicative rates. Aging reduced the rate of replication of precursors from both depots and had a particularly marked effect on perirenal cells. Hence, the difference in the population-doubling times between perirenal and epididymal precursors was much greater in 73-g than in 398-g animals.

Size of colonies derived from perirenal and epididymal adipocyte precursors. The data in Table I were derived from studies of cell populations obtained from the two anatomic sites. It was considered very likely that each population was composed of primordial fat cells that were heterogeneous in capacity for replication. It was therefore of interest to compare perirenal and epididymal precursors using methods that allowed individual cells to be studied. This was accomplished by plating precursors from 180-g rats in primary culture under clonal conditions. After 2 wk of incubation, the number of cells in each colony was determined, as was the colony size distribution of perirenal and epididymal precursors expressed as a percent of colonies attaining at least a specified size. Pooled data from four experiments are shown in Fig. 2.

Perirenal and epididymal precursors did not differ in the fraction of inoculated cells that formed colonies. From the shape of the colony size distribution curves it was apparent that, as expected, both precursor pools were composed of cells heterogeneous in their capacity for replication. In all studies, perirenal precursors exhibited a greater proliferative ability than did epididymal cells. For example, 50% of perirenal cells were able to achieve a colony size of at least nine $\log_2$ cells/colony, while only 35% of epididymal cells produced colonies of that size or larger. These data, derived from individual cells, thus confirm the cell population studies in establishing that perirenal precursors have a greater proliferative capacity than epididymal elements.

Anatomic site and differentiation of adipocyte precursors. Having established that perirenal and epi-

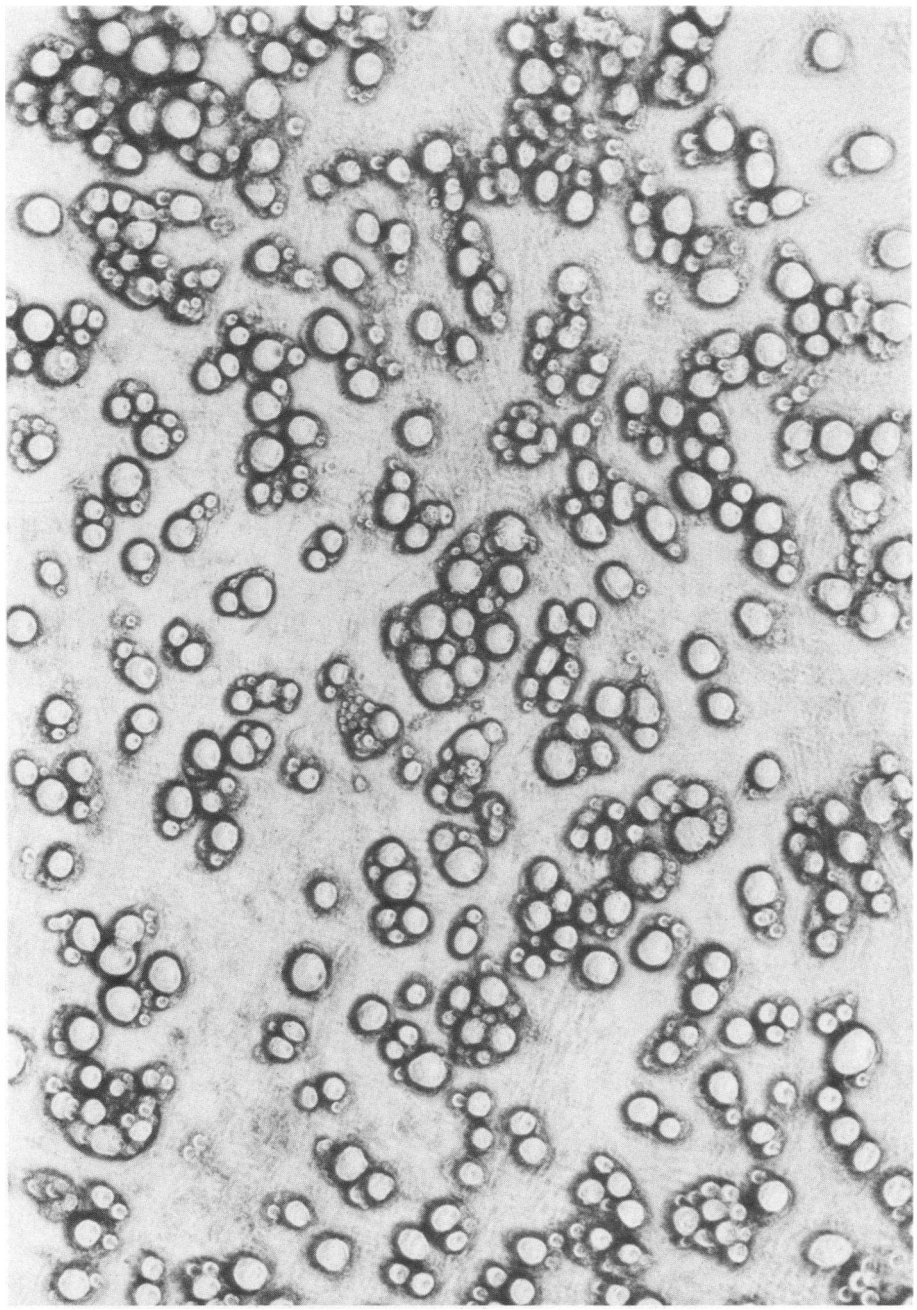

FIGURE 1 Differentiation of a perirenal adipocyte precursor clone in primary culture. Cells were obtained from the stromal fraction of perirenal adipose tissue of a 170-g rat. After 21 d in primary culture, differentiation was promoted with the collagen gelation mixture, insulin, and plasma-derived serum, and observations were made after an additional 21 d. × 125.

didymal precursors differ in replicative behavior, it was of interest to determine whether polyclonal populations from the two sites also displayed differences in capacity for differentiation. In these studies, populations of perirenal and epididymal precursors were grown to confluence in secondary culture and then differentiation was induced by addition of the collagen gelation mixture. Capacity for differentiation in the two cell populations was determined by the activity of glycerophosphate dehydrogenase and was followed

TABLE I
Effect of Site and Age on the Replication Rate of Adipocyte Precursors

No. of animals	Mean wt	Population-doubling times*	
		Perirenal cells	Epididymal cells
	g	h	
6	74±2	15.8±0.9	22.2±0.7
7	179±1	19.3±0.7	25.5±1.2
8	397±4	23.2±0.7	25.8±0.8

* Means±SEM. Perirenal vs. epididymal, all pairs significant by two-tailed t test at $P < 0.03$; perirenal vs. perirenal, all pairs $P < 0.005$; epididymal vs. epididymal, 179- vs. 397-g NS, other pairs $P < 0.02$. The ages of 74-, 179-, and 397-g rats were 28, 48, and 93 d, respectively.

as a function of time after confluence. The results from three experiments using 150-g rats were similar; one experiment is shown in Fig. 3.

Enzyme activity was undetectable in subcellular preparations from both cell types just before achieving confluence. With induction of differentiation, enzyme activity rose progressively up to 6–8 d postconfluence. At all times, enzyme activity in perirenal cells was greater than in epididymal elements. During the 6–8-d period, microscopic examination revealed rapid accumulation of lipid in cells from both sites. This accumulation was always more extensive amongst perirenal precursors. Fig. 4 illustrates the morphological change in perirenal cells over a 6-d period of differentiation. In one experiment derived from a 400-g rat, the same pattern of results was obtained. In this experiment, during preconfluence no enzyme activity was detected. At 6 and 8 d postconfluence, glycerophosphate dehydrogenase activity of epididymal cells was 44 and 68 U/10^6 cells, respectively, while the corresponding figures for perirenal precursors were 58 and 83 U/10^6 cells.

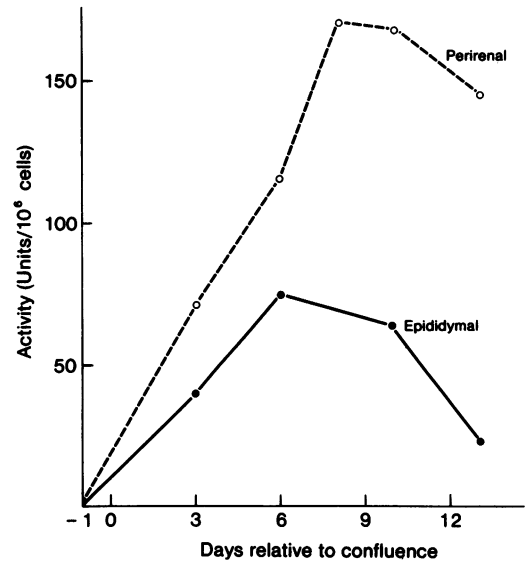

FIGURE 3 Glycerophosphate dehydrogenase activity during differentiation of perirenal and epididymal adipocyte precursors. Differentiation was promoted as described in Fig. 1 legend and the text. One unit of enzyme activity was defined as the oxidation of 1 nmol of NADH/min.

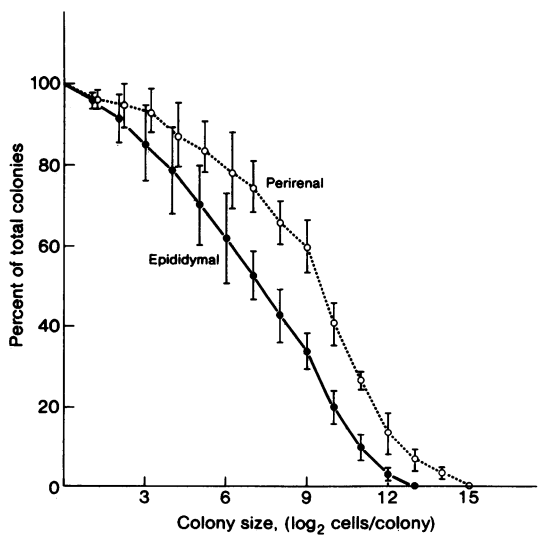

FIGURE 2 Colony size distribution of adipocyte precursors. Precursors were obtained from perirenal and epididymal adipose tissue of 170-g rats. After cell isolation, precursors were allowed to adhere for 12 h during which time no replication occurred (Methods). Clones were then allowed to replicate for 14 d, after which time colony size was determined. The means and SD of four experiments, each involving one animal, are shown.

These data indicate that perirenal and epididymal cells differ not only in rate of replication but also in their capacity for differentiation. Although differentiation was noted in animals of both 150 and 400 g, additional studies would be required to determine whether aging influences not only the rate of replication but also the differentiative process.

Weight gain and replication of adipocyte precursors. Previous studies have indicated that although rapid weight gain induced by high-fat feeding is associated with an increase in DNA synthesis and in fat cell number in both perirenal and epididymal regions, the number of mature adipocytes in perirenal fat is particularly increased. The cell culture techniques of this study were used to explore the possibility that these findings may reflect an effect of a diet that in-

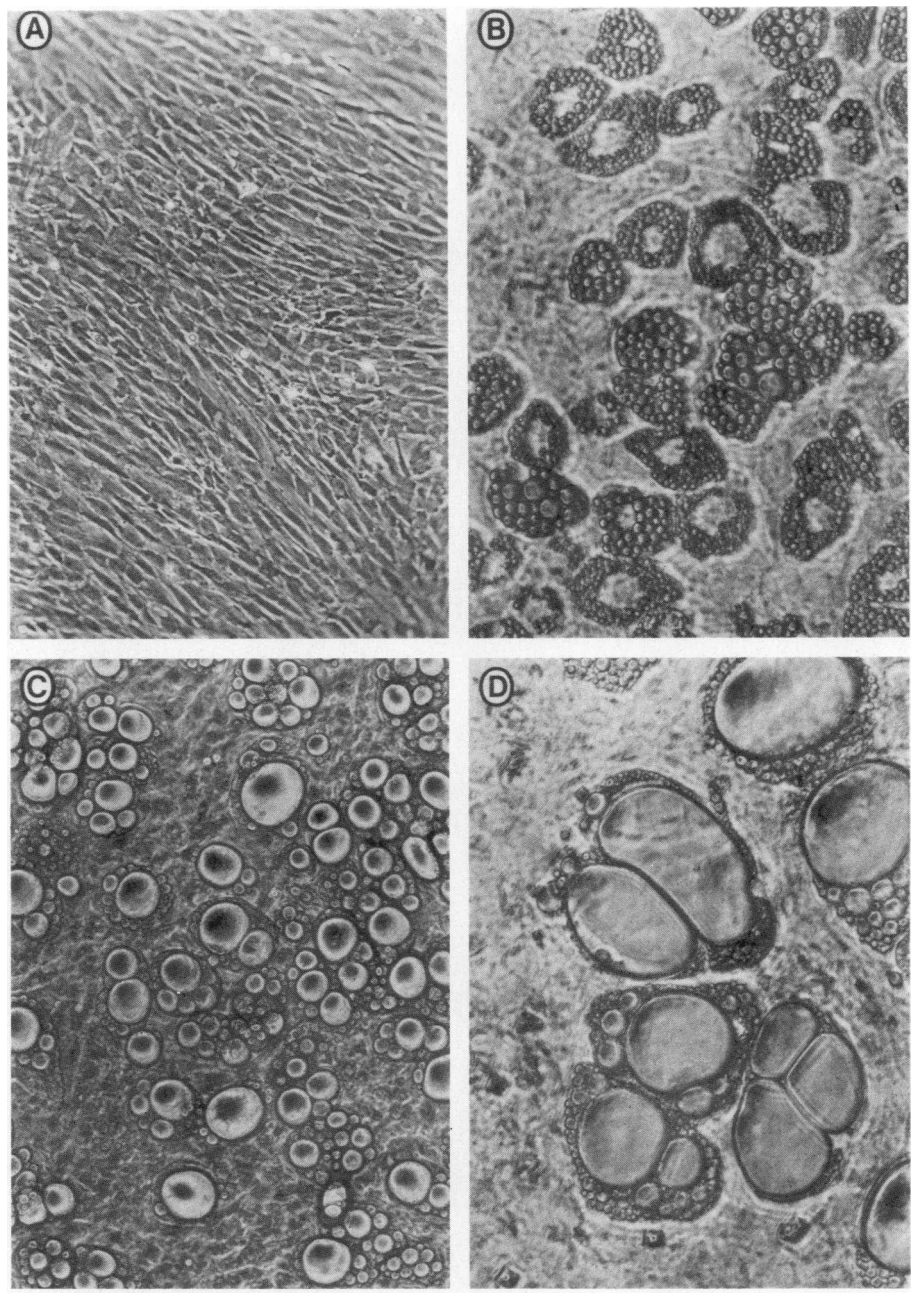

FIGURE 4 Differentiation of perirenal adipocyte precursors in secondary culture. Cells were obtained from pooled stromal fractions from perirenal adipose tissue of 170-g rats. After 21 d in first subculture, differentiation was promoted as indicated in Fig. 1 and as detailed in the text. *A* shows cells before addition of the promoters of differentiation. *B* and *C* were obtained at 6 and 21 d of differentiation, respectively. The magnification for *A*, *B*, and *C* is 125. *D* is a higher magnification of *C* (× 450).

duces rapid weight gain on the replication rate of adipocyte precursors in secondary culture.

As expected, high-fat feeding resulted in greater weight gain than low-fat diets (Table II). However, the population-doubling times of perirenal precursors in secondary culture were not affected by the diet; indeed, replicative rates were similar to those of cells from rats of the same age maintained on standard chow

TABLE II
Effect of High-Fat Diet on the Replication Rate of Perirenal Adipocyte Precursors

	Low-fat diet	High-fat diet
Mean weight (*g*)	429±27	512±23
Population doubling times (*h*)	23.8±1.0	24.8±1.9

Cells from five rats were studied in each group. Doubling times were not significantly different. Means±SEM.

(Table I). Hence, rapid weight gain was not associated with an enduring effect on adipocyte precursors.

DISCUSSION

The present study provides new information in the following aspects of adipose tissue biology: nature of the fibroblastlike cells derived from adipose tissue, influence of anatomic site, age, and diet on replicative rates of adipocyte precursors, and influence of site on adipocyte differentiation.

The techniques that have been used indicate the feasibility of obtaining from the stromal fraction of adipose tissue clones consisting of adipocyte precursors and also cell populations in secondary culture composed predominantly of adipocyte precursors. Indeed, the present studies of differentiation indicate that, in contrast with other observations (10), precursors can be obtained from 400-g rats as well as from younger animals.

The more extensive differentiation of perirenal rather than epididymal cells was evident not only morphologically, but also when a biochemical marker of adipocyte differentiation was followed, i.e., glycerophosphate dehydrogenase activity. Hence, perirenal precursors not only replicate more rapidly than epididymal cells, but also differentiate more extensively. The regional variations in differentiative capacity of adipocyte precursors are reminiscent of the locational specialization of skin fibroblasts reported by Griffin et al. (11). Genital skin fibroblasts are unique in their expression of steroid 5 α-reductase, the enzyme catalyzing the formation of dihydrotestosterone.

There are several possible explanations for the greater capacity for replication and differentiation of perirenal as compared with epididymal adipocyte precursors. Perhaps the most obvious explanation relates to the possibility that gene expression may be regulated differently in precursors from various fat tissue regions. Such differential control in perirenal and epididymal adipocyte precursors could explain the observed disparities in replication and differentiation. Regional differences in gene expression could have many possible consequences. For example, differential responsiveness to hormones and growth factors might result from dissimilarities in receptor and postreceptor events. These differences, e.g., regional disparities in alpha adrenergic receptor number (1, 2), might in turn influence the time of appearance and activity of enzymes and regulators involved in replication and differentiation. Another consequence might relate to quantitative or qualitative differences in the elaboration of trophic paracrine and autocrine factors in diverse adipose depots.

There is, however, another explanation for differences between perirenal and epididymal precursors that does not involve the postulation of variations in gene control. From the colony size distribution data, it is clear that both precursor pools contain cells that are heterogeneous with respect to capacity for replication, or the number of cell doublings that can be attained over a period of time, and that the perirenal pool contains more cells with greater capacity. Studies with other cell systems have established that under these experimental conditions the size of colonies is inversely related to the number of previous in vivo doublings. Because of this relationship, cells from younger animals produce larger colonies (12). Application of this principle to the present study suggests that at a given animal age, perirenal precursors may have undergone fewer in vivo replications than epididymal cells and that perirenal fat may thus be an embryologically "younger" tissue than epididymal. This concept is consistent with observations in our cell system as well as others (13); indicating that cell populations from younger animals replicate more rapidly in culture than populations from older animals. This concept also explains the present observation that aging has a greater effect on the replication rate of perirenal than epididymal cells. If perirenal cells had undergone fewer in vivo divisions than epididymal precursors it might be expected that decrements in replication rate with aging would be more marked in perirenal elements.

There is, as yet, no clear understanding of why the age of a depot or of an animal influences the replication rate of adipocyte precursors. It is possible that this phenomenon is dependent upon differentiation of precursors with redistribution of differentiated cells into the mature fat cell compartment. It is well-established in other cell systems that the frequency of differentiation increases with an increasing number of cell generations (14). Hence, it would be predicted that in an adipocyte precursor pool containing cells that replicate at different rates, there would be more frequent differentiation in those clones that replicate most rapidly. With time, the precursor pool would be depleted of the rapidly dividing elements, which would become

part of the differentiated mature adipocyte compartment, leaving behind an increasing proportion of more slowly replicating precursor clones. Thus, if two adipose depots differed in time of development, it would be expected that the older would display a lower average rate of precursor replication. It should be noted that this concept also explains the differences in extent of differentiation that were noted in this study between perirenal and epididymal cells. If differentiation were more frequent amongst rapidly dividing cells, it would be expected that markers of differentiation would be more prominent in a pool enriched with these cells. It is also possible, although unproven, that amongst these cells differentiation proceeds not only with greater frequency but also with greater rapidity.

Having found that anatomic site and aging influence the capacity of replication of cells in primary culture and of their progeny in secondary culture, it was of interest to explore any possible perpetuating influence of an environmental factor such as a high-fat diet. Previous data from other laboratories have established that induction of obesity by high-fat feeding leads to a preferential increase in fat cell number in perirenal tissue (15, 16). The results of the fat feeding experiments in the present study indicate that this effect is not because of any persisting influence of this diet on the replication rate of a population of precursors. In other words, the effect is not transmitted to the generations of cells that were not exposed to the stimulus in vivo. It is very likely that the preferential effect of fat feeding on perirenal fat cell number noted by others reflects the more rapid replication and more frequent differentiation displayed by perirenal as opposed to epididymal precursors.

Exposure of adipocyte precursors to an abundance of circulating lipoprotein-triglyceride could result in more extensive maturation of fat cells in the pool that is relatively enriched in rapidly dividing and extensively differentiating cells. Hence, it is conceivable that in a developing adipose depot, the rate of lipid accumulation may be a function of not only the mass of nutrient ingested, but also of the composition of the adipocyte precursor pool to which the circulating nutrient is exposed. This composition will determine the frequency and the rate of new mature fat cell formation. Further, it is possible that the extent of assimilation of circulating triglyceride by the mature fat-cell pool is influenced by the age of the differentiated adipocytes, and that the assimilative process is greater in the younger differentiated cells. This concept bears some analogy to the varying functions of immature and older erythrocytes. It suggests that the extent of lipid accumulation in an adipose depot, in addition to being dependent on factors external to the cells such as the supporting vascular net as well as the availability of nutrients, hormones, and growth factors, would also be a function of the composition of the precursor pool and its direct derivative, the young differentiated adipocyte. Differences in composition between depots could lead to disparities between fat tissue regions in lipid accumulation, despite exposure of the depots to the same amount of circulating nutrient.

It will be of great interest to determine the processes that control the clonal composition of an adipocyte precursor pool, and to note the effect of alterations in this composition on the subsequent growth of adipose tissue. Influences such as genes, hormones, and nutrition, which may partly mediate the effect of aging, could affect not only the replicative rate but also the frequency and extent of differentiation of adipocyte precursors. Indeed, the resulting differences in the composition of adipocyte precursor pools might explain not only regional, but also interindividual variations in adipose mass in response to a nutrient load.

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