

Regional Adipocyte Precursors in the Female Rat

Influence of Ovarian Factors

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Abstract

A flow cytometric immunofluorescence procedure utilizing a specific antibody to rat adipose tissue lipoprotein lipase (LPL) was developed to quantify differentiated and undifferentiated preadipocytes present in the adipose tissue vascular stroma. This method is highly sensitive and specific for cells capable of synthesizing LPL in significant quantities. Pubescence in female rats was associated with an increase in differentiated preadipocytes and in fat cell number with enlargement of the fat depots in the perirenal, parametrial, and the subcutaneous dorsal and femoral regions. A concomitant decline in the percentage of undifferentiated preadipocytes occurred in all but the femoral depot. Ovariectomy reduced pubertal adipose growth in the femoral and parametrial but not the dorsal or perirenal regions. Furthermore, the femoral undifferentiated preadipocyte pool was not preserved in the ovariectomized animals. Thus, ovarian factors influence the pubescence-associated regional preadipocyte differentiation and conversion to adipocytes. The femoral depot contains an ovarian-dependent infinite pool of fat cell precursors. These features could account for the association between ovarian hormones and body fat topography.

Introduction

The well-documented occurrence of intersexual differences in the regional distribution of body fat suggests that the sex steroids may be involved in establishing topographical variations in tissue storage and mobilization of fat (1-4). A number of studies have also demonstrated relationships between sex hormones, particularly estrogen and testosterone, and several forms of obesity (5-9). Since the body fat distribution pattern influences susceptibility to metabolic aberrations such as glucose intolerance, hyperinsulinemia, hyperlipidemia, and their health sequelae (3, 10-12), and since the sex hormone balance correlates closely with the metabolic profile, clarification of events leading to the regional growth of adipose tissue, espe-

cially with regard to the effects of the steroid hormones, has become essential.

The growth potential of an adipose depot is contingent upon the number of cells capable of accumulating fat. Among the lipid-filled cells of adipose tissue exist smaller fibroblastlike cells, some of which are capable of accumulating lipid under the appropriate conditions. Numerous investigations have examined the factors involved in the recruitment of these cells for lipid filling, a problem that underlies the question of how adipose tissue expands. Early attempts have relied primarily upon morphological characteristics such as cell size and/or lipid filling (2, 13, 14) or biochemical markers such as lipogenic enzyme activities (13, 15, 16). These morphological and biochemical events, however, occur after establishment of preadipocyte commitment. More recently, attempts have been made to study these cells through culture cloning (17, 18), to follow the induction of preadipocyte conversion into mature fat cells in vitro (19). Although such analyses are significant in that they are capable of showing which cell types will convert to adipocytes, they are unable to distinguish precursor cells already committed in vivo to becoming adipocytes (differentiated preadipocytes) from those acquiring preadipocyte characteristics only after maximal stimulation in vitro (undifferentiated preadipocytes or adipoblasts).

The major route of lipid acquisition by fat cells in situ is through the uptake of triglycerides from circulating lipoproteins (20). Transfer of these lipids into the cell requires the presence of lipoprotein lipase (LPL),¹ which suggests that the appearance of LPL activity should be a prelude to significant fatty acid accumulation in these cells. LPL activity has been detected in immature fat cells by several groups (17, 21-23), but this finding is subjective since the most sensitive assays for LPL activity cannot detect early synthesis of the enzyme. The availability of a specific antibody to LPL and its application toward immunocytological investigations have expanded the sensitivity of LPL detection (23-25). Indeed systems have been described where immunodetectable LPL was noted in the early stages of preadipocyte differentiation before the appearance of activity changes, other lipogenic enzymes, or lipid deposition (23, 26). Our objectives were to develop a procedure utilizing this antibody and flow cytometric cell sorting to identify and quantify adipocyte precursor cells among the fibroblast-like cells of the adipose tissue stroma, to utilize this procedure to quantify the relative abundance of precursor cells in visceral and subcutaneous adipose tissue regions from the female rat, and to examine the importance of ovarian factors in influencing regional differences in precursor cell development.

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1. Abbreviations used in this paper: GPDH, glycerol-3-phosphate dehydrogenase; LPL, lipoprotein lipase.

Methods

Animals. Female Sprague-Dawley rats (Fasco/King Inc., Madison, WI) were used in all experiments: 3 to 4-wk-old animals (prepubescent rats), 6 to 8-wk-old animals (pubescent rats), and 6 to 8-wk-old animals in which ovariectomy was performed at the age of 3–4 wk (pubescent ovariectomized rats). Rats were maintained at the Animal Resource Center of the Medical College of Wisconsin on a 12-h light/dark cycle and fed normal rat chow ad lib.

Preparation of adipose tissue depots. Four major adipose tissue depots (two visceral and two subcutaneous) were dissected from each animal as described previously (2). The parametrial depot begins at the vagina and continues along the uterus to the ovaries. The perirenal depot consists of a triangular section of fat with its vertex in the inguinal region and the base at the lower pole of the kidney, one side at the midline, and the other extending into the lateral retroperitoneal area. The inguinal (femoral) fat consists of a discrete subcutaneous depot found in the inguinal region. Subcutaneous dorsal (or intrascapular) fat is a diffuse tissue which runs from the midline of the upper back to the shoulders and is carefully dissected away from surrounding brown adipose tissue. The tissues were then washed free of adherent oil droplets with warm Hanks' balanced salt solution (HBSS; Whittaker M.A. Bioproducts Inc., Walkersville, MD), blotted, and weighed.

Preparation and quantification of differentiated and undifferentiated preadipocytes. Stromal-vascular cells from adipose tissues were obtained after collagenase treatment as described by Rodbell (27). Tissues were digested for 45 min at 37°C with shaking. After dissociation cells were filtered through 200- μ m and then 20- μ m nylon mesh. Differentiated preadipocytes were defined as those cells in which LPL surface labeling was detectable after 12 h of monolayer culture. Previous studies have shown that the 12-h period of monolayer culturing allows the adherence of fibroblast-like cells but is not sufficient to induce significant cellular replication (13). In order to demonstrate that the percentage of LPL-labeled cells in the 12-h cultures was not influenced by the occurrence of cell contact, adherence, or replication during this period, parallel studies were performed utilizing a nonadhering, nonreplicating suspension culture system. Cell adherence and replication were prevented by increasing the viscosity of the culture medium with methyl cellulose as described previously (28). This system also allowed evaluation of whether the early detection of LPL-bearing cells represents capacity for lipid accumulation and, consequently, conversion to adipocytes in long-term culture. After a 12-h recovery period, an aliquot of cells was removed for LPL surface labeling. An Intralipid emulsion (10%; Cutter Medical, Berkeley, CA) at a concentration of 0.25 mg/ml, and 16 μ U/ml insulin were then added. After 5–7 d the percentage of cells acquiring adipocyte morphologic characteristics was determined.

Undifferentiated preadipocytes were defined as those cells that lacked surface LPL at confluence but which acquired this antigen after maximal induction with insulin and Intralipid. An aliquot of the fibroblastlike cells was cultured 5–7 d to monolayer confluence and then induced with insulin and Intralipid emulsion for 72 h. Confirmation that these cells were true adipocyte precursors was also demonstrated in long-term suspension culture.

For the time-course studies, aliquots of cells were removed for the simultaneous measurement of LPL immunofluorescence, LPL enzymatic activity, and lipid filling. To evaluate the relationship between LPL surface labeling and adipocyte conversion, harvested cultures with increasing percentages of LPL-positive cells (20–80%) were transferred to suspension cultures enriched with Intralipid and insulin as described above and incubated for an additional 5–7 d. Aliquots were then examined to determine the percentage of cells acquiring the adipocyte morphologic characteristics.

Monolayer culturing system. Fibroblast-like cells were subjected to monolayer culturing (5×10^5 cells/plate) in Dulbecco's minimal Eagle's medium (DMEM; Whittaker M.A. Bioproducts) supplemented with 10% fetal bovine serum (Biologos Inc., Naperville, IL), 20

mM L-glutamine, and penicillin-streptomycin (M.A. Bioproducts) in 60-mm plates and incubated at 37°C. Cells were washed after 12 h to remove nonadherent cells, collagen strands, and other cellular debris. Approximately 60–75% of the cells plated adhered. Cells were either harvested for the quantification of LPL bearing cells (differentiated preadipocytes) or continued in the monolayer cultures until confluency (5–7 d after plating), after which time cells were induced to differentiate by the addition of Intralipid and insulin (undifferentiated preadipocytes or adipoblasts).

Suspension culturing system. Cells that had been plated for 12 h or maximally induced after confluent culturing were removed with 0.5% EDTA, transferred to Leighton tubes (Bellco Biotechnology, Vineland, NJ) containing the same medium plus 2% methyl cellulose, and maintained for up to 12 d in the presence of insulin and Intralipid emulsion. Cells were washed at 3-d intervals by gentle centrifugation.

Immunofluorescent labelling of cells. Cells were removed from the plates by incubating with 0.5% EDTA in HBSS for 10 min at 37°C with shaking, concentrated by centrifugation at 1,000 g for 10 min at 4°C, and washed twice with isotonic PBS. Cells were labeled for 30 min at 37°C with goat anti-rat LPL (primary antibody) diluted 1:2,500 in PBS. After three washes cells were suspended in a 1:200 dilution of fluorescein-labeled rabbit anti-goat IgG (FITC-secondary antibody) purchased from Miles Biochemicals (Elkhart, IN). After 30 min at 37°C, cells were washed twice in PBS, once in PBS containing 0.01% NaN₃, and then suspended in 1.0 ml of the azide-containing buffered saline. Controls were prepared by labeling with normal goat serum in place of the primary antibody (unlabeled cells). In order to demonstrate intracellular LPL, cells sorted out as LPL-positive or negative were recovered, fixed with 2% paraformaldehyde, and made permeable with 0.5% saponin (29). After blocking external sites with the primary antibody and nonfluorescent secondary antibody, cells were labeled internally with primary antibody and a rhodamine-labeled secondary antibody.

The primary antibody was raised against rat adipose tissue LPL using methods described elsewhere (24). Briefly the enzyme was purified to homogeneity with heparin-Sepharose affinity chromatography followed by preparative isoelectric focusing. The purified LPL was then used to immunize goats at a schedule of one injection per month for 5 m. Half-maximal inhibition of 8 mU of adipose tissue LPL was observed with an equal volume of antiserum at a dilution of 1:800. Specificity of the antibody was also demonstrated with double immunodiffusion (24) and by Western blotting (25). The antibody is specific for LPL although it will bind to both the inactive precursor forms of the enzyme and the active surface LPL (25, 30).

Flow cytometric analysis of labeled cells. Appropriately labeled cells (5×10^3 per sample) were analyzed on a flow cytometer (Epics V; Coulter Instruments, Inc. Hialeah, FL). Cells were excited at 488 nm and analyzed at 525 nm, the wavelength for the maximal emission of fluorescein. Small cells (< 2 μ m), cell fragments, or cellular clumps were excluded and accounted for < 5% of the total number of cells examined. The data obtained were displayed in the form of a histogram plotting forward angle light scatter (relative cell size) and orthogonal light scatter (internal cell structure) vs. channel number (fluorescent intensity). Histograms from labeled and unlabeled cells were compared and the corresponding percentiles were subtracted out, giving a new histogram from which the percentage of LPL-positive cells was calculated through internal programming.

Fat cell size and number. Weighed slices of adipose tissue were washed and fixed with OsO₄ before liberation of the cells with 8M urea and subsequent washes with Triton X-100 as described by DeMartinis (31). Cells were sized microscopically using a calibrated ocular (Carl Zeiss, Inc., New York, NY). 300 cells per sample were scored at a magnification of 400, with means being taken from three samples per tissue. Size distribution was determined as the cells were scored, and cell size profiles were thus obtained. This procedure has been shown to correlate well with values obtained by other methods. Cell number from each depot was calculated from triglyceride content and the mean fat cell size.

Other methods. Cells becoming adipocytes were identified by following the increase in cell size upon induction and the conversion of these cells into lipid-filled cells. To stain for lipid accumulation, cells were fixed with 2% paraformaldehyde, stained with Oil Red O, and counterstained with Mayer's hematoxylin for nuclear visualization, using stains and fixatives from Sigma Chemical Co. (St. Louis, MO). A cell was scored as positive if > 50% of the cell appeared stained, clearly demonstrating the presence of lipid droplets. Demonstration of LPL activity in differentiating cells was performed in acetone-ether extracts of cell pellets as described previously (32). Approximately 5×10^6 cells were processed for determination of LPL activity. One unit of LPL activity was defined as the amount of enzyme required to release 1 μmol of free fatty acid from radiolabeled triolein per min at 37°C (33).

Results

Identification and quantification of adipocyte precursor cells in the adipose tissue stroma. A representative set of data illustrating the progression of the LPL-immunofluorescent intensities of fibroblast-like stromal cells during the course of induction of adipocyte conversion in vitro is shown in Fig. 1. Compared to confluent cells (1 B), cells stimulated to differentiate with insulin and Intralipid for 24 and 48 h (1 C and 1 D) demonstrated a progressive shift of fluorescent labeling toward the right, indicating an increasing percentage of cells with surface-bound LPL. An increase in the number of events occurring toward the upper part of the histogram denotes a simultaneous overall increase in cell size. Cells similarly treated but unexposed to the LPL antibody (unlabeled cells) showed only low intensity background fluorescence (1 A). A quantitative estimate of LPL-immunofluorescent intensity was determined via a program comparing the sample with its control. A two-histogram analysis relating the channel number (relative fluorescent intensity) to the number of events (relative number of cells) per channel was thus produced. Fig. 2 A shows a typical

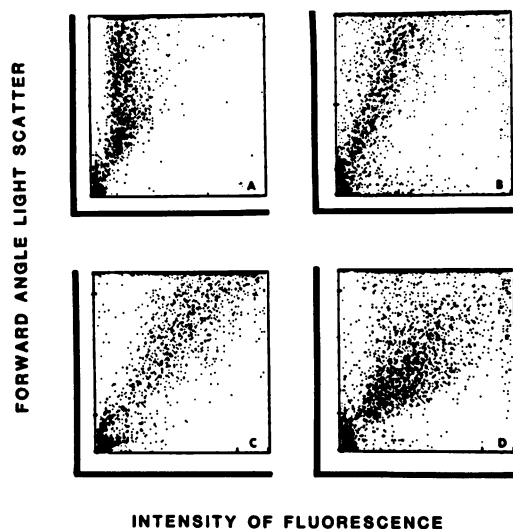


Figure 1. Induction of preadipocyte conversion in vitro: LPL-immunofluorescence intensity progression. Cultured parametrial cells from immature rats were brought to monolayer confluency before the addition of insulin (16 $\mu\text{U}/\text{ml}$) and Intralipid (0.25 mg/ml). 5×10^3 cells per sample were analyzed on a Coulter Epics V flow cytometer. (A) Unlabeled cells. (B) Insulin only. (C) Insulin + Intralipid (24 h). (D) Insulin + Intralipid (48 h). The percentages of LPL-positive cells were determined to be 0, 10, 19, and 58%, respectively.

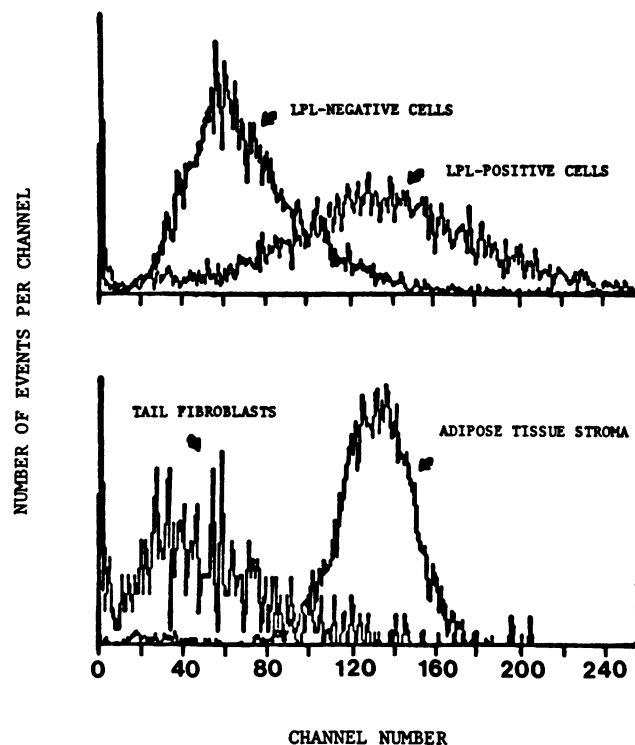


Figure 2. Two histogram immunofluorescence analyses. (A) Comparison of induced preadipocytes vs. unlabeled cells. Fibroblast-like cells from the parametrial depot were cultured to confluency and induced by the addition of insulin and Intralipid for 48 h. Cells from the same pool were similarly treated but were exposed to normal goat serum in place of the LPL antibody (unlabeled cells). (B) Comparison of adipose tissue stromal cells vs. tail fibroblasts. Cells from the parametrial adipose tissue stroma or the tail skin of prepubescent rats were cultured to confluency and induced by insulin and Intralipid as described above.

comparison of stromal cells induced to differentiate for 48 h relative to unlabeled cells from the same source. The two-histogram analysis demonstrates the higher peak fluorescent intensities of the induced cells (a peak fluorescence channel of 152 on an arbitrary scale of 0 to 255) vs. those of the unlabeled cells (peak channel of 47). These cells were then determined to be 57.8% positive for LPL. To demonstrate that the probe is specific for cells that synthesize LPL in significant quantities to become capable of differentiating into preadipocytes, similar studies were performed with rat tail fibroblasts. These cells have been demonstrated to possess LPL activity but are unable to convert to adipocytes in tissue cultures exposed to maximal concentrations of insulin and other inducing agents (34). 3 d of induction barely shifted the peak channel number of these cells from 48 to 57 (Fig. 2 B), comparable to the peak channel number of control cells treated with nonimmunized goat serum (Fig. 2 A). Conversely, induced cells from adipose tissues exhibit peak channel numbers between 120 and 155. Furthermore, adipose tissue stromal cells sorted from the flow cytometer as surface-positive exhibited a comparable degree of LPL immunofluorescence intracellularly. Cells sorted as surface LPL-negative, on the other hand, did not contain detectable quantities of intracellular LPL (data not shown).

The specificity of the immunofluorescent probe for cell surface LPL was demonstrated by pretreating the cells with heparin immediately before labeling. At least 80% of the LPL

label from fully induced parametrial cells was removed after 5 min of heparin treatment at a concentration of 100 U/ml at 37°C. Less than 10% of the fluorescent labeling of tail fibroblasts was removed under these conditions and no further increase in removal of label was demonstrated after longer exposures to heparin.

The temporal relationship between cell surface LPL labeling, LPL enzymatic activity, and lipid filling of cells cultured to confluency and induced to differentiate with insulin and Intralipid is shown in Fig. 3. Increases in surface LPL labeling were observed within 24 h of induction, with maximal labeling attained by 72 h. LPL enzymatic activity assayed in acetone-ether extracts of cells exposed for 1–3 d to insulin and Intralipid was below the level of sensitivity for this assay (32). LPL enzymatic activity, however, was detected after 3–4 d of induction and reached a peak after 6 d. This was followed by the appearance of lipid accumulation with a rapid increase in lipid filling beginning 5–6 d after induction.

Table I shows that the percentages of LPL-positive cells detected in stromal-vascular digests from prepubescent rats determined after 12-h recovery periods in monolayer or suspension cultures were comparable. Furthermore, the percentage of cells acquiring adipocyte characteristics after 5–7 d of exposure to Intralipid in the suspension system was comparable to the percentage of cells bearing the LPL antigen.

During exposure of confluent stromal cells to maximal induction conditions in vitro, acquisition of surface LPL occurred as a continuous process, reaching maxima at ~72 h (Fig. 4), with no further increase in label occurring beyond that time. This stepwise pattern of preadipocyte recruitment was observed with confluent cultures from the four adipose depots of the three animal models examined.

Fig. 5 demonstrates a strong positive relation between the percentage of total LPL-bearing cells measured after 3 d of maximal induction in the monolayer culturing, and the percentage of cells that become filled with lipid after an additional

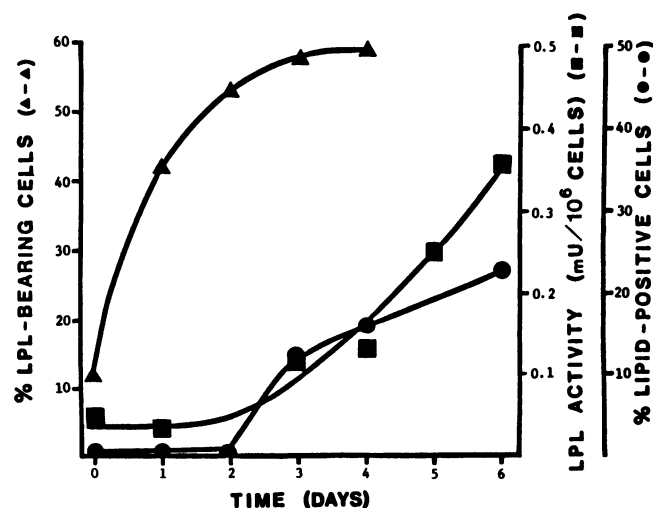


Figure 3. Temporal relationship of LPL immunofluorescence, LPL activity, and cellular lipid filling. Cells taken from the parametrial depot and brought to monolayer confluency were treated with insulin + Intralipid for 6 d. At the indicated times, cells were removed for LPL surface labeling, acetone/ether-extractable LPL enzymatic assay, or lipid staining as described in Methods. Values represent the means from three observations.

Table I. Detection of LPL-bearing Cells in Short-term Cultures of Rat Adipose Tissue Stroma (Differentiated Preadipocytes): Comparison of 12-h Monolayer and Suspension Cultures

Tissue	Percentage of LPL-bearing cells		Percentage of cells with adipocyte morphology
	12-h monolayer	12-h suspension	8–12 d suspension
Parametrial	25.1±4.8	25.5±6.4	32.0±4.8
Dorsal s.c.	27.9±7.5	28.9±5.7	34.5±6.3
Inguinal s.c.	25.5±4.3	26.4±6.4	28.4±5.9

Cells were placed in monolayer or suspension culture 12 h before labeling. Some cells remained in suspension culture for 8–12 d in the presence of insulin and intralipid before cell sizing and lipid droplet scoring. Cells were deemed positive for adipocyte morphology if >50% of the cells stained for lipid. Results are means±SE; *n* = 5–7 samples.

5–7 d of incubation in Intralipid-rich suspension cultures. Regression analysis indicates a strong positive correlation between the increase in lipid accumulation and the increase in cell size in these cells, with the two events occurring simultaneously.

Regional abundance of adipocyte precursor cells: effects of pubescence and ovariectomy. Table II shows that after maximal induction of confluent cells, the total percentages of cells bearing the LPL antigen were not significantly different in the four fat depots of the prepubescent rat. In the pubescent rat, the total number of stromal-vascular cells bearing the LPL antigen were either reduced (dorsal depot) or unchanged (parametrial and perirenal regions) compared to those estimated in the same region of the prepubescent animals. The percentage of LPL-bearing cells in the inguinal (femoral) depot, on the other hand, was significantly increased relative to that from the prepubescent rat and was greater than the percentage of cells from all of the other regions. This pattern was not observed in cells obtained from age-matched ovariectomized rats, where

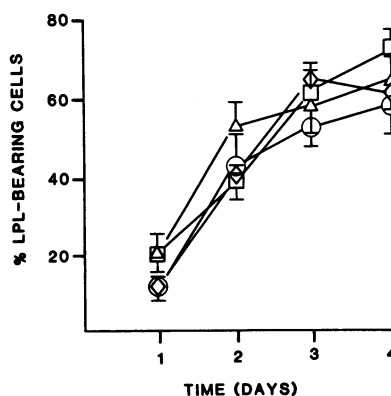


Figure 4. Time course of changes in the expression of immunoreactive LPL during the in vitro induction of confluent precursor cells. Cells from prepubescent rats were brought to monolayer confluency and stimulated with insulin and Intralipid. At indicated time intervals, cells were harvested and labeled with the LPL-immunofluorescent probe. Results represent the mean ± SE from five to seven experiments. □, Dorsal SC; Δ, inguinal SC; ○, parametrial; ◇, perirenal.

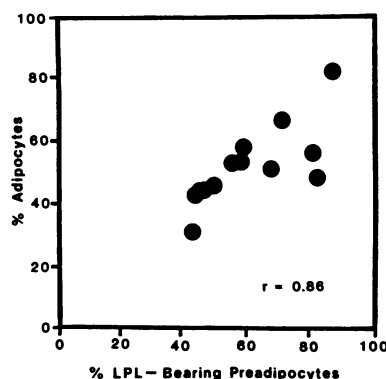


Figure 5. Relationship between the percentage of cells bearing surface LPL and the percentage of cells developing into adipocytes. Cells were harvested after 3 d of maximal stimulation in monolayer cultures, and aliquots analyzed for surface LPL labeling. These cells were transferred to suspension cultures enriched with insulin and Intralipid for an additional 5–7 d. The cells were then sized and lipid content scored to determine the percentage of cells developing adipocyte characteristics. The data points represent single analyses of cells taken from either the parametrial, dorsal, or inguinal depots of prepubescent rats.

the percentages of LPL-bearing cells from the inguinal and dorsal regions were equally decreased.

Fig. 6 shows the percentages of LPL-bearing preadipocytes detected in the 12-h monolayer cultures of fibroblast-like cells from the two subcutaneous and the two visceral fat depots. In the prepubescent rat, the percentage of differentiated preadipocytes was approximately equal in all four depots, ranging from 10 to 30%. In the pubescent animals, the relative size of this preadipocyte pool was significantly increased above that of the prepubescent rats. In the pubescent ovariectomized rats, the percentage of differentiated preadipocytes in the dorsal and perirenal regions was increased to the same extent as in the pubescent animals. Ovariectomy, however, resulted in a significant reduction of the pubescence-associated increase in the percentage of differentiated preadipocytes detectable in the femoral and parametrial depots.

Fig. 6 also shows the regional abundance of LPL-negative stromal cells that acquired the LPL antigen upon maximal induction with insulin and Intralipid in vitro (undifferentiated preadipocytes or adipoblasts). There was no regional difference in the percentage of LPL-bearing cells in the four adipose tissue depots of the prepubescent rat. With pubescence there was a marked decrease in this pool in the parametrial, perirenal, and the dorsal subcutaneous adipose depots, but this pool was preserved in the stromal cells from the inguinal re-

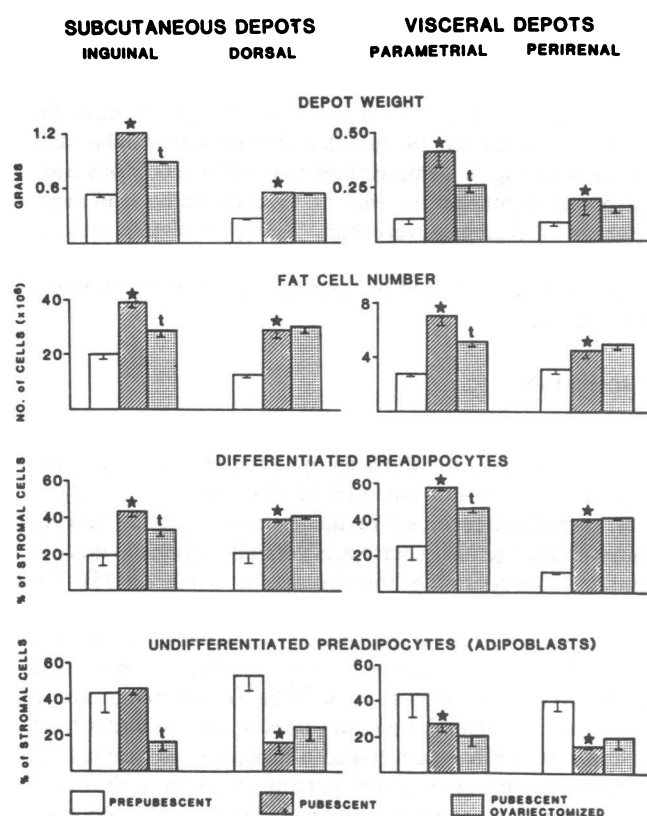


Figure 6. Regional abundance of adipocyte precursor cells in fat depots of prepubescent, pubescent, and pubescent ovariectomized animals: relationship to fat cell number and depot weight. Differentiated preadipocytes represent those cells bearing the LPL surface antigen after 12 h of monolayer culturing. Undifferentiated preadipocytes represent those cells that lacked surface LPL at monolayer confluence and acquired this antigen after maximal induction with insulin and Intralipid in vitro. Fat cell number and depot weight were determined as described in the text. Results represent the mean \pm SE from five to seven animals in each group. * $P < 0.05$ or less, comparing tissues from the same sites of prepubescent rats. † $P < 0.05$ or less, comparing tissues from the same sites of pubescent animals.

gion. The percentage of cells acquiring LPL in cultures from this region was virtually equal to that found in stromal cells from the inguinal region of the prepubertal rat. This protection of the inguinal pool was not observed in ovariectomized rats,

Table II. Percentage of LPL-bearing Cells in Adipose Tissue Stroma after Maximal Induction in Vitro (Total Preadipocytes)

Animals	<i>g</i>	Percentage of LPL-bearing cells			
		Subcutaneous depots		Visceral depots	
		Inguinal	Dorsal	Parametrial	Perirenal
Prepubescent	56 \pm 18	63.6 \pm 7.2	72.7 \pm 6.4	67.2 \pm 7.8	61.4 \pm 5.9
Pubescent	140 \pm 12	86.5 \pm 3.4*	52.3 \pm 4.4†	71.4 \pm 4.2	54.1 \pm 2.1
Ovariectomized pubescent	132 \pm 19	47.9 \pm 4.2†	58.6 \pm 3.4†	65.3 \pm 5.2	59.1 \pm 4.6

Stromal-vascular cells plated in monolayer culture until confluency and maximally induced by the addition of insulin and Intralipid were labeled and analyzed for the percentage of cell surface LPL-bearing cells. Results are means \pm SE of triplicate samples from four to seven experiments. * Significantly increased. † Significantly decreased. $P < 0.01$, compared with the corresponding region in prepubescent rats.

and an equivalent loss from this pool was noted in all four depots.

Mean fat cell size did not differ significantly between different depots in the three animal models tested. Both the means and medians of the fat cell diameters were similar (data not shown). Fig. 6 demonstrates that pubescence was associated with significant increases in fat cell number and depot weight in all regions. Ovariectomy, however, significantly reduced the pubertal increase in adipocyte number and fat depot weight in the femoral and parametrial but not in the dorsal or perirenal regions.

Discussion

We have proposed that both body fat topography and the metabolic profile are influenced by the relative androgenic to estrogenic balance in women (8, 12). Distribution of body fat is an important predictor of the metabolic profile, and the degree of androgenicity correlates with aberrations in glucose, insulin, and lipoprotein homeostasis (3, 8, 11, 12, 35). The differential effects of estrogen and androgen on plasma insulin, adipose tissue LPL, and triglyceride metabolism are generally well known (35–38), but their roles in establishing regional fat deposition and the interrelationships between these hormones, preadipocyte differentiation, and obesity are not understood. To this end, we have developed a procedure to estimate regional fat cell precursor growth potential to examine the role of sex hormones in promoting regional adiposity. We have combined LPL-specific immunofluorescence with flow cytometry to distinguish and quantify fat cell precursors among other cells present in the adipose tissue stroma. Using this procedure, we identified and quantified differentiated and undifferentiated preadipocytes. Identification and quantification of preadipocytes before conversion permitted demonstration of ovarian influence in establishing regional differences in adipose tissue growth potential.

Existing procedures for analyzing adipocyte precursor cells lack the sensitivity or the ability to be quantified. The morphological criteria frequently cited lag behind early events occurring in the developmental process. Indeed, the present study shows that the appearance of assayable LPL activity and the accumulation of lipid droplets occur long after the initial appearance of cell surface LPL. Similarly, analyzing the late-stage enzymes such as glycerol-3-phosphate dehydrogenase (GPDH; 39, 40) or fatty acid synthetase (13, 16), for example, misses the early stages of fat cell conversion and lacks the degree of sensitivity necessary to distinguish these cells from other enzyme-bearing cells. Therefore LPL was chosen as a distinct marker for preadipocyte differentiation since its detection coincides with the early events in cell differentiation (23). Cell surface-bound LPL was induced with insulin and Intralipid in adipocyte precursor cells but not in rat tail fibroblasts. In addition, the percentage of LPL-bearing cells correlated closely with the percentage of cells becoming adipocytes in lipid-rich media.

The heterogeneity of stromal cells underlies the problem of preadipocyte identification and quantification. Induction of LPL activity occurs in differentiating cells before any lipid accumulation (41). Using a differentiating ob17 cell system, Ailhaud has noted that most of the LPL activity was present in cells of higher density than those containing most of the GPDH activity, suggesting that GPDH may play a more criti-

cal role in lipid accumulation after cell commitment (40, 42). This also supports the concept of developmental stages. Our results indicate the presence of at least three distinct cell types in the stromal fractions of rat adipose tissues. First, the differentiated preadipocytes (LPL-bearing cells) are those already committed to differentiating before treatment with insulin and growth factors *in vitro*. Undifferentiated preadipocytes or adipoblasts are those cells that are capable of accumulating cell surface LPL after confluence and induction with insulin and Intralipid. The third class consists of those cells incapable of becoming fat cells even after continued induction. These fibroblast-like cells were treated with insulin and Intralipid in monolayer culture for up to 7 d without a change in their cell size or LPL content. True fibroblasts did not express a sizeable amount of LPL, whereas the preadipocyte pool did.

We have utilized short-term (12 h) monolayer and suspension cultures to demonstrate and quantify the percentage of cells already bearing the surface LPL antigen. When placed in suspension culture and stimulated with insulin and Intralipid, cells committed to differentiation mature into fat cells but do not divide (28). Our results show that the percentages of LPL-bearing cells in both systems were similar. Furthermore, the percentage of LPL-positive cells in the suspension culture was comparable to the percentage of cells becoming adipocytes after 5–7 d of exposure to insulin and Intralipid. Such data strongly support the existence of a pool of differentiated preadipocytes in adipose tissue stroma. With current techniques, it is not possible to determine whether these represent *de novo* differentiated preadipocytes or dedifferentiated fat cells that have lost their lipid content, although the complete lack of any visible lipid before maximal stimulation strongly suggests that these cells have not been lipid-filled previously. Nevertheless, the existence of this pool suggests that there may always be a pool of preadipocytes readily available for recruitment as fat cells, depending upon *in vivo* environmental factors. Understanding the mechanisms regulating the availability of this pool and the factors that influence its recruitment are of particular importance in identifying the role of adipocyte precursors in the pathogenesis of obesity. The regional differences in the relative abundance of these cells and the concomitant changes in fat cell number and depot weight during pubescence and ovariectomy suggest a significant role for this pool in the association between sex hormones and body fat distribution patterns.

In the prepubescent rat, stromal-vascular cells from visceral and subcutaneous adipose depots contained high percentages of undifferentiated preadipocytes. After maximal induction of confluent cells *in vitro*, the percentages of preadipocytes among the fibroblast-like cells reached 60–85%. These findings are consistent with those previously shown using morphologic criteria and cell cloning techniques (18, 28, 43, 44). In the prepubertal rat no significant regional difference in the percentage of undifferentiated preadipocytes was observed. After puberty the degree of preadipocyte recruitment was decreased significantly in the parametrial, perirenal, and the dorsal subcutaneous depots. A similar phenomenon was observed in age-matched rats ovariectomized before pubescence, suggesting that ovarian factors are not involved. Thus, the partial depletion of the undifferentiated preadipocyte pool in these regions represents an age and/or growth-related consequence of the increase in differentiated preadipocytes, their conversion to mature fat cells, and the increase in fat depot

weights. By contrast the percentage of undifferentiated preadipocytes and their recruitment rate in the femoral region of pubescent rats remained significantly higher than those observed in the other regions and were comparable to those seen in the prepubescent animal, in spite of concomitant increases in the percentages of differentiated preadipocytes, fat cell number, and depot weight. This protective effect was contingent upon the presence of ovarian factors. The femoral depot in the female rat thus represents an ovarian-dependent infinite pool of preadipocyte recruitment.

We demonstrated that during induction of the undifferentiated preadipocytes in vitro, the percentage of LPL-bearing cells from each of the depots examined increased progressively. LPL accumulation by these cells is a continuous process, suggesting the presence within this pool of either cell subtypes or cells with differing capacities for LPL acquisition during induction. It is clear from a number of studies that adipose tissue consists of a heterogeneous pool of cells that vary in their rates of replication. When fibroblast-like cells from the stromal-vascular fraction of perirenal adipose tissue of adult rats were grown in culture at clonal density, each confluent clone contained differentiated fat cells maturing at widely different frequencies (18). These results imply that although each fibroblast-like cell might be capable of becoming an adipocyte, each stem cell varies in its susceptibility to become committed to differentiation. Not only may some depots replicate more rapidly, but some may differentiate more extensively. Djian (18) discussed two possible explanations for the different intrinsic capacities for replication and differentiation between cells from different fat depots. The first possibility involved different rates of gene expression and regulation. The existence of a regional or clonal disparity in gene expression may explain quantitative or qualitative differences observed between the femoral and the other regional adipose tissue depots. Recently, Djian et al. showed that in the male rat perirenal tissue does, in fact, contain more clones that differentiate extensively than epididymal fat (46). Whether femoral adipose tissue in the female contains more cells capable of developing into preadipocytes, or cells that are more responsive to hormonal or environmental influences is still uncertain. Alternatively, the greater capacity for recruitment of undifferentiated preadipocytes in the femoral depot compared to other regions may be a consequence of differences in blood flow or innervation that could result in different substrate or hormonal environments in the various tissues (46).

Our results suggest that ovarian factors, presumably sex hormones, promote regional specialization in the growth potential of adipose tissue depots by determining the size and the rate of recruitment of differentiated and undifferentiated preadipocyte pools. Roncari observed that addition of estradiol significantly enhanced the replication of cultured human omental adipocyte precursors (47). Wade and Gray suggested that ovarian steroids act via multiple redundant mechanisms to affect energy balance and adiposity (48). In addition to its central effects on appetite and food intake, estradiol acts peripherally on white adipose tissue to influence LPL and probably hormone-sensitive lipase activities. These responses may occur directly at the fat cell level since high-affinity, hormone-specific cytosolic receptors have been found in rat adipose tissues. Recent studies show that adipocytes from the femoral region exhibit a high degree of metabolic activity in pregnant and lactating women and a considerable increase in LPL activ-

ity with estrogen therapy in postmenopausal women (49). Adipose tissues from pregnant and sex steroid-treated rats also show regional disparities in steroid hormone receptor number (6, 50–52), LPL activity (22, 37, 51, 53), and lipid mobilization during exercise (54), thus providing additional evidence to support our premise that sex steroids may also determine the amount and recruitment rate of preadipocytes and consequently, the regional adipose tissue mass.

In conclusion we have described a method for identifying and quantifying adipocyte precursor cells among other fibroblast-like cells of the adipose tissue stroma and have applied this procedure to the study of regional differences in fat cell recruitment and the possible role of ovarian factors. This will permit future study of the precise involvement of estrogens and androgens in influencing the growth potential of adipose tissue depots and regional adiposity.

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