Association of fat cell size and paracrine growth factors in development of hyperplastic obesity

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Marques, Brenda G., Dorothy B. Hausman, and Roy J. Martin. Association of fat cell size and paracrine growth factors in development of hyperplastic obesity. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1898–R1908, 1998.—Inguinal, epididymal, and retroperitoneal adipose tissue from lean and obese Zucker rats, 3–15 wk of age, was used to determine the association among adipocyte size distribution, the presence of paracrine growth factors in adipose tissue, and subsequent changes in adipocyte number. For each specific depot and time point, obese rats had a greater percentage of large adipocytes than did lean rats. A positive correlation (P < 0.02) was found in obese rats between the percentage of inguinal and epididymal adipocytes in the 140- to 180-µm size range and the ability of conditioned medium prepared from these depots to stimulate cellular proliferation in a bioassay system utilizing preadipocytes from inguinal fat pads of normal rats. Proliferative activity of the conditioned medium from all depots in obese rats was positively correlated (P < 0.01) to subsequent changes in fat cell number. The data presented here for the inguinal and epididymal depot of obese Zucker rats are consistent with the hypothesis that enlarged adipocytes secrete growth factors that induce preadipocyte proliferation.

Determination of a fat cell size distribution profile more accurately represents the number and size of large fat cells present in the tissue (18, 29) and may help delineate whether a critical size precedes the development of adipocyte hyperplasia.

Regional differences in adipocyte hypertrophy and hyperplasia suggest that locally produced growth factors may be involved in the regulation of adipogenesis (12). Adipocyte counting and sizing can determine changes in cellularity, but other methods are needed to determine if mitogenic factors that may regulate the development of hyperplasia are present in adipose tissue when fat cells reach a critical size. Primary culture of stromal vascular cells from adipose tissue is an effective in vitro model to study factors that alter the proliferation of preadipocytes (26, 28). Media exposed to cells isolated from adipose tissue or fragments of mature fat are known to contain adipogenic factors that alter the growth of preadipocytes in culture (7, 21, 22, 30), but these effects have not been linked to specific changes in adipocyte size distribution.

This project was designed to study the possible association among changes in characteristics of the fat cell distribution profile, the presence of local mitogenic factor(s) derived from adipose tissue, and the development of fat cell hyperplasia. The experimental protocol was selected to help determine if enlarged adipocytes are responsible for the secretion of growth factors that regulate fat cell proliferation. Zucker rats provide a genetic model suitable for examining the role that a critical fat cell size may play in the development of obesity because adipocyte hypertrophy precedes hyperplasia in a regional and developmental pattern (6, 16, 17). Adipose tissue cellularity parameters for the inguinal, epididymal, and retroperitoneal depots were determined in lean and obese rats at five ages. Primary preadipocyte cultures were used as a bioassay system to demonstrate the presence of mitogenic factors in adipose tissue-conditioned medium prepared from the above depots. Correlation coefficients were calculated to determine if a specific fat cell size was associated with the in vitro proliferative activity of the conditioned medium and the in vivo development of hyperplasia.

MATERIALS AND METHODS

Animals. Lean (Fa/?) and obese (Fa/fa) male Zucker rats, ages 3, 6, 9, 12, and 15 wk, were obtained from the University of Georgia colony. Lean and obese 3-wk-old Zucker rats cannot be distinguished through visual examination. Phenotypic identification of this age group was accomplished through comparisons of serum insulin levels (ICN Pharmaceuticals, Irvine, CA), inguinal pad weight vs. body weight, and adipose tissue cellularity data (20, 23). For the remainder of the age groups, identification of phenotype was performed through visual inspection. Postweanling rats were housed in hanging wire cages in a room environmentally regulated for temperature (23 ± 3°C) and humidity (40–50%), with a 12:12-h light-dark cycle. Rats had free access to pelleted diet (Ralston...
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Purina, St. Louis, MO) and tap water throughout the duration of the study. All procedures for the care of the animals used in this study were approved by the University of Georgia Institutional Animal Care and Use Committee.

On the day of sample collection, rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body wt). The inguinal, epididymal, and retroperitoneal fat pads were immediately removed and weighed. Because of limited amounts of adipose tissue, pads from two lean rats were pooled to provide an adequate quantity. Aliquots from all three depots were taken for adipocyte cellularity studies. Adipose tissue-conditioned medium was prepared from the inguinal depot for all age groups, from the epididymal pads for rats aged 6, 9, 12, and 15 wk, and from the retroperitoneal depot for 9-, 12-, and 15-wk-old rats.

Adipose tissue cellularity. Fat cell size and number were determined through electronic quantification using the method of Hirsch and Gallian (15) as modified by Cartwright (5). Triplicate adipose tissue samples (50–70 mg) were fixed in a solution containing 0.12 M osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA) in 50 mM collidine (2,4,6-trimethylpyridine) buffer. Samples were fixed for at least 1 wk at room temperature, rinsed with 0.9% NaCl, and then placed in 8 M urea for several days to facilitate separation of cells from the tissue. Fixed adipocytes were rinsed with 0.9% NaCl through a 240-µm nylon screen and then collected on a 20-µm nylon screen (Tetko, Briarcliff Manor, NY). Samples of cells that met quality standards during the fixation process were analyzed on a Coulter (model ZM, Coulter Electronics, Hialeah, FL) electronic particle counter. Sizing was accomplished by dual threshold analysis at nine defined size ranges (30–240 µm) calibrated with microsphere standards (Coulter Electronics). Counts in all size ranges were performed in triplicate and are reported as percent distribution. Number of adipocytes per pad was calculated by multiplying the mean cells per milligram of sample by the total mass of the corresponding depot.

Conditioned medium preparation. Adipose tissue not used for cellularity studies was micro-dissected to remove visible blood vessels, finely minced, rinsed three times in fresh 37°C Hanks' balanced salt solution (HBSS; Sigma Chemical, St. Louis, MO), blotted dry, and weighed. Tissue aliquots were incubated in DMEM-nutrient mixture F-12 Ham (DMEM-F-12, Sigma) containing 72 mM gentamicin sulfate, 120 mM cefazolin, and 27 µM amphotericin B for 4 h at 37°C in a humidified 5% CO₂ atmosphere. Because the number of adipocytes per milligram of tissue is known to differ with age and phenotype, the ratio of tissue-to-medium was adjusted (1 g tissue to 5–10 ml medium) to more closely approximate an equal number of fat cells per milliliter of medium. After incubation, the conditioned medium was separated from the tissue fragments by filtration through Whatman qualitative paper, sterile filtered into aseptic vials, and stored frozen at −20°C.

Bioassay system: primary cell culture. Stromal-vascular cells, including preadipocytes, were obtained from the inguinal adipose tissue of specific pathogen-free male Sprague-Dawley rats (80–100 g; Harlan Sprague Dawley, Indianapolis, IN) by methods previously described (27). Briefly, animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg), and inguinal adipose tissue was removed aseptically and pooled. Each gram of minced adipose tissue was incubated with 5 ml of a digestion buffer consisting of 0.1 M HEPES and 6.21 × 10⁻⁵ U/ml collagenase type 1 (Worthington Biomedical, Freehold, NJ) for 90 min in a 37°C shaking water bath. Digests were filtered through nylon mesh screens with 240 and 20 µm openings, resuspended in DMEM-F-12, and centrifuged for 10 min at 600 g. Floating fat-filled cells were discarded. Cells in the stromal-vascular pellet were washed with plating medium (DMEM-F-12 with 10% fetal bovine serum), centrifuged, and then resuspended in plating medium. An aliquot of the suspension was mixed with Rappaport's stain, and cells were counted on a hemocytometer. Cells were diluted with plating medium and seeded on 12.5 or 25 cm² tissue culture flasks at a density of 4.8 × 10³ cells/cm². Cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere.

Bioassay system: proliferation assay. Day 1 after seeding the plating medium was removed and replaced with DMEM-F-12 until treatment medium was applied on day 2. Proliferation of preadipocytes and stromal-vascular cells in response to test medium was determined through the use of [³H]thymidine incorporation during the exponential growth phase (25). Cultures were treated with basal control medium (DMEM-F-12) or a test medium [25%, (vol/vol) adipose tissue-conditioned medium; 75% (vol/vol) DMEM-F-12] containing a final overall concentration of 0.5% porcine serum and 0.2 µCi [³H]thymidine (ICN Pharmaceuticals, Irvine, CA) per flask for days 2–5 of culture. On day 5 the flasks were rinsed and refed with lipid-filling medium (10% porcine serum, 1.0 mM porcine insulin, and 10,000 U/l heparin in DMEM-F-12) to promote fat accretion in the preadipocytes. Lipid-filling medium was changed every other day through day 13. On day 15 the cells were enzymatically harvested using HBSS containing 0.5% bovine serum albumin, 4,185 U/l trypsin, and 1.05 × 10⁵ U/l collagenase. The lipid-filled cells (preadipocytes now fully differentiated) and the nonlipid-filled stromal-vascular cells were separated by density gradient centrifugation through Percoll (Sigma) as described by Novakofski (25). Both cell fractions were assayed for incorporation of [³H]thymidine by scintillation counting. Data were corrected to represent activity per 100,000 cells in the adipose tissue used for preparation of the conditioned medium and are expressed as the difference in uptake of [³H]thymidine compared with cultures treated with the basal control medium.

Statistical analysis. Data were subjected to analysis of variance using SuperAnova (version 1.11, Abacus Concepts, Berkeley, CA) to determine the effect of phenotype, age, and depot on adipose tissue cellularity parameters and the proliferative response of preadipocytes in culture to the presence of adipose tissue-conditioned medium. Least-squares contrasts were used for comparisons between means. Simple linear regression and sample correlation coefficients were calculated to determine the association between the percent of cells in specific size ranges, the in vitro proliferative activity of conditioned medium, and in vivo fat cell number changes during the subsequent 3-wk period. Differences were accepted as significant at the P < 0.05 level.

RESULTS

Adipose depot development. The mass of the inguinal, epididymal, and retroperitoneal depots was greater (P < 0.002) in obese rats compared with lean rats at all ages (Table 1). Over time a progressive and significant (P < 0.01) increase in weight for each depot was noted in obese rats with the greatest percent change occurring between 3 and 6 wk. A similar pattern of develop-
ment was also noted in the epididymal depot of lean rats. However, the inguinal and retroperitoneal depots of lean rats had progressive increases in pad weight (P < 0.01) only until 12 wk, with no further significant increase noted at week 15. The number of fat cells increased with advancing age in all depots, for both phenotypes (Table 1). At 3 wk, despite increased pad weights, there was no significant difference in fat cell number between obese and lean rats in the inguinal, epididymal, or retroperitoneal depots (Table 1). However, at all subsequent ages (6, 9, 12, and 15 wk), obese rats had a significantly greater (P < 0.01) number of fat cells for each of the three depots compared with lean rats. These age-related changes in fat cell number do not necessarily represent absolute increases in the quantity of adipocytes because lipid filling of existing very small fat cells would allow them to reach the size detectable by the electronic counter (8).

Adipocytes size distribution profiles. As shown in Figs. 1–3, major differences in cell size distribution were readily apparent at 3 wk in adipose tissue from lean compared with obese Zucker rats. In the inguinal (Fig. 1), epididymal (Fig. 2), and retroperitoneal (Fig. 3) fat pads of the lean rats the majority (70–75%) of the cells were <50 µm in diameter. In contrast, fat cell size of the 3-wk-old obese rats was distributed over a larger range with ~45–55% of the cells being >50 µm. A further shift in the overall pattern of fat cell distribution between the two phenotypes was detected at 6 wk. In the lean rats an enlargement in fat cell size was evidenced with the majority of the cells in the three fat depots now detected in the 30- to 70-µm size ranges. However, whereas obese rats continued to have a significantly (P < 0.001) greater percentage of large adipocytes (80–240 µm) than did the lean rats, a biphasic distribution profile emerged at this time with marked increases in the proportion of cells in the 80- to 140-µm size ranges (approximate increase from 3 to 6 wk of 2.7-, 3.9-, and 3.6-fold for inguinal, epididymal, and retroperitoneal fat pads, respectively). The biphasic cell size distribution pattern for obese rats is consistent with previous observations (18, 29) and was even more apparent at 9 wk. Compared with lean rats, the 9-wk-old obese rats had not only a greater (P < 0.05) percentage of cells in the 100- to 240-µm size ranges (approximate increase from 3 to 6 wk of 2.7-, 3.9-, and 3.6-fold for inguinal, epididymal, and retroperitoneal fat pads, respectively). The biphasic distribution profile persisted in the obese rats at 12 wk. At this point the obese rats had a significantly (P < 0.05) greater proportion of cells from all three fat depots in both the smallest (30–40 µm) and larger (100–240 µm) size ranges compared with the lean rats.

Table 1. Adipose depot characteristics in male Zucker rats

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<tr>
<th></th>
<th>Pad wt, g</th>
<th>Fat cell number, ×10⁶</th>
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<tr>
<td></td>
<td>3 Wk</td>
<td>6 Wk</td>
</tr>
<tr>
<td><strong>Inguinal</strong></td>
<td></td>
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<tr>
<td>Lean</td>
<td>0.29 ± 0.05a</td>
<td>1.27 ± 0.36b</td>
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<tr>
<td>(5)</td>
<td>(6)</td>
<td>(9)</td>
</tr>
<tr>
<td>Obese</td>
<td>0.71 ± 0.05a,b,f</td>
<td>8.15 ± 0.31b,f</td>
</tr>
<tr>
<td>(5)</td>
<td>(8)</td>
<td>(9)</td>
</tr>
<tr>
<td><strong>Epididymal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lean</td>
<td>0.04 ± 0.01a</td>
<td>0.46 ± 0.15b</td>
</tr>
<tr>
<td>(6)</td>
<td>(6)</td>
<td>(10)</td>
</tr>
<tr>
<td>Obese</td>
<td>0.09 ± 0.01a,b,f</td>
<td>2.48 ± 0.13b,f</td>
</tr>
<tr>
<td>(5)</td>
<td>(8)</td>
<td>(10)</td>
</tr>
<tr>
<td><strong>Retroperitoneal</strong></td>
<td></td>
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<tr>
<td>Lean</td>
<td>0.03 ± 0.01a</td>
<td>0.17 ± 0.05b</td>
</tr>
<tr>
<td>(6)</td>
<td>(5)</td>
<td>(8)</td>
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<tr>
<td>Obese</td>
<td>0.08 ± 0.01a,b,f</td>
<td>1.53 ± 0.05b,f</td>
</tr>
<tr>
<td>(5)</td>
<td>(5)</td>
<td>(8)</td>
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Values are least-square means ± SE; nro. rats/group in parentheses. Values in a row not sharing a letter are significantly different (P < 0.05).

fP < 0.05 between phenotypes for given age and depot.
In contrast, adipocytes from 12-wk-old lean rats had a bell-shaped distribution, with the majority of the cells from all fat pads in the 50- to 100-µm size ranges. The emergence of a more biphasic distribution profile was noted in lean rats at 15 wk. In both the epididymal and retroperitoneal fat pads, ~60% of the cells from the lean rats were in the 80- to 140-µm size ranges and an additional 10–15% were in the smallest (30–40 µm) size range. This pattern of cell size distribution is reminiscent of what had been observed in the obese rats at 6 wk, suggesting that developmental changes in adipose tissue cellularity occur in a much more compressed time frame in the obese compared with the lean rats.

In vitro preadipocyte proliferation. Conditioned medium was used to investigate the potential of paracrine substances secreted from adipose tissue to influence proliferation of preadipocytes in a primary cell culture system. This bioassay system utilized cultured stromal-vascular cells (including preadipocytes) derived from inguinal fat pads of normal rats for testing the proliferative influence of conditioned medium prepared from inguinal, epididymal, and retroperitoneal fat depots of the lean and obese Zucker rats. Data have been corrected to represent the proliferative activity of medium conditioned by exposure to 100,000 fat cells and are expressed as the difference in uptake of [3H]thymidine from cultures treated with a DMEM-F-12 basal control medium.

As shown in Fig. 4A, preadipocytes exposed to conditioned medium prepared from inguinal adipose tissue of 3-, 6-, and 12-wk-old obese rats demonstrated significantly (P < 0.01) greater proliferative stimulation compared with cultures treated with comparable adipose tissue-conditioned medium from age-matched lean rats. Although not significant there was a trend for greater proliferative activity in the medium prepared from 9-wk-old obese rats compared with lean rats (P =...
There was no significant difference in the ability of inguinal adipose tissue-conditioned medium from 15-wk-old lean or obese rats to stimulate preadipocyte proliferation. Stromal-vascular cells in primary culture did not differ significantly in their response to treatment with inguinal adipose tissue-conditioned medium from 3-, 6-, 9-, or 12-wk-old lean and obese rats (data not shown). However, conditioned medium from inguinal adipose tissue of 15-wk-old obese rats significantly \( (P < 0.05) \) stimulated the proliferation of stromal-vascular cells (7.1 pM [3H]thymidine) compared with cultures exposed to medium prepared from 15-wk-old lean rats where the proliferative activity was less than the basal control (2.77 pM [3H]thymidine).

Conditioned medium prepared from epididymal adipose tissue of 6- and 12-wk-old obese rats had a significantly \( (P < 0.01) \) greater ability to stimulate preadipocyte proliferation than the medium prepared from age-matched lean rats (Fig. 4B). However, the proliferative activity of epididymal-conditioned medium from 9-wk-old lean rats was significantly \( (P < 0.05) \) greater compared with that of the obese rats. The proliferation of preadipocytes was not stimulated in cultures treated with epididymal adipose-conditioned medium from 15-wk-old rats of either phenotype. The stimulated response of stromal-vascular cells was significantly \( (P < 0.05) \) greater in cultures treated with conditioned medium from 9-wk-old lean rats (20.1 pM [3H]thymidine) compared with obese rats (2.6 pM [3H]thymidine). In the other age groups (6, 12, and 15 wk) there was no significant difference between phenotypes on the replicative response of stromal-vascular cells exposed to conditioned medium from epididymal adipose tissue (data not shown).

There was no significant difference in the proliferation of preadipocytes in cell cultures treated with

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**Fig. 2.** Cell size distribution profile for adipocytes from the epididymal fat pad of lean and obese Zucker rats of various ages. Data shown are least-square means ± SE for 5–10 animals per phenotype at a given age. For each age group values within a size range not sharing a letter are significantly different \( (P < 0.05) \) between phenotypes.
conditioned medium from retroperitoneal adipose tissue of 9- or 15-wk-old lean and obese rats (Fig. 4C). However, when exposed to conditioned medium prepared from retroperitoneal adipose tissue of 12-wk-old obese rats, proliferation of preadipocytes was significantly (P < 0.05) stimulated compared with cultures treated with similar medium prepared from lean rats. No significant differences in the proliferation of stromal-vascular cells were noted in cultures treated with conditioned medium prepared from retroperitoneal tissue of lean or obese rats (data not shown).

Association of fat cell size, proliferative activity of conditioned medium, and changes in fat cell number. Depot specific correlation coefficients (Table 2) were calculated to ascertain if there was any significant relationship between the proportion of fat cells in defined size ranges, the capacity of adipose tissue-conditioned medium to promote preadipocyte proliferation in vitro, and the change in fat cell number in vivo over the subsequent 3-wk period (i.e., from 9 to 12 wk). Data analysis was performed with the original nine size ranges, as well as with the nine individual adipocyte size distributions ranges equally collapsed to form three general size categories: small (30–60 µm), medium (60–100 µm), and large (100–240 µm). Limited sample size did not allow for statistical interpretation of the data by phenotype at specific time points.

Initial analysis was performed by using data from all age groups, all pads, and both phenotypes. A significant (P < 0.001) negative association between the percentage of small fat cells and proliferative activity of the conditioned medium was noted. There was no significant correlation between the proportion of medium-size adipocytes and the ability of the conditioned medium to stimulate preadipocyte replication. There was a significant (P < 0.001) positive correlation between the...
percentage of large fat cells and proliferation of preadipocytes in vitro. A significant ($P < 0.001$) association was also noted for the proliferative activity of the conditioned medium and the change of fat cell number in vivo at the next age group.

However, adipose tissue is not a unitary organ, and discrete analyses by pad and phenotype were also performed (Table 2). When data from the inguinal depot of both phenotypes were pooled, the percentage of large adipocytes was significantly ($P < 0.001$) and positively correlated with the stimulated replication of preadipocytes exposed to adipose tissue-conditioned medium. Furthermore, the proliferative activity of the medium also had a significant ($P < 0.001$) positive correlation with the change in the number of inguinal adipocytes observed in the next age group. In contrast, there was a significant ($P < 0.001$) negative correlation between the percentage of small adipocytes and the ability of the conditioned medium to induce proliferation of preadipocytes in culture. Further analysis of the inguinal depot by phenotype revealed a positive association ($r = 0.494, P = 0.02$) between the percentage of large adipocytes in the obese rats and the ability of the conditioned medium to stimulate preadipocyte proliferation. Specific size range analysis indicated that the proportion of fat cells with diameters 140–180 µm was the only size to be significantly related ($r = 0.870, P < 0.001$) to the proliferative capacity of the conditioned medium. As with the combined inguinal data, the proliferative activity of the conditioned medium prepared from obese rats was positively associated ($P < 0.001$) with subsequent changes of fat cell number in vivo. In lean rats, only the percentage of medium-size inguinal fat cells was positively correlated ($P < 0.01$) with proliferative activity of the conditioned medium, the proportion of 60–70 µm ($r = 0.685, P < 0.01$) and 70–80 µm ($r = 0.656, P = 0.01$) cells contributing to this association. However, the proliferative activity of conditioned medium from lean rats was not significantly correlated to subsequent changes in fat cell number in the inguinal depot.

In the epididymal depot, neither data from lean rats nor data from the combined phenotypes revealed any significant correlation between the percentage of small, medium, or large adipocytes and the ability of epididymal-conditioned medium to affect preadipocyte proliferation in vitro (Table 2). However, in obese rats there was a significant ($P < 0.01$) positive association between the proportion of large epididymal cells and proliferative activity in the conditioned medium. Adipocytes in both the 140- to 180-µm ($r = 0.829, P < 0.001$) and 180- to 240-µm ($r = 0.552, P < 0.05$) size ranges contributed to this effect. For data from the lean, obese, and combined phenotypes, proliferative activity of the conditioned medium from epididymal fat depots was positively ($P < 0.05$) associated with the in vivo fat cell number changes in the next age group.

Neither data from lean rats, obese rats, nor combined data for both phenotypes were found to have a significant correlation between the percentage of small, medium, or large fat cells and the ability of retroperitoneal depot-conditioned medium to stimulate in vitro preadipocyte proliferation. However, a strong positive correlation ($P < 0.005$) was observed between the proliferative activity of the conditioned medium from both the obese
and combined phenotypes and subsequent in vivo change in fat cell number of the retroperitoneal fat pad.

**DISCUSSION**

The hypothesis that a critical fat cell size precedes adipocyte hyperplasia is not a new one. Increases in mean fat cell size have been noted in mice (24) and several strains of rats (1, 11, 13, 19) fed high-fat diets. Studies of adipose tissue cellularity in the genetically obese Zucker rat also indicated that hypertrophy develops before hyperplasia (16, 17, 18). Based on these observations Faust (10) proposed that attainment of a critical fat cell size initiated the events that lead to proliferation of adipocytes. Because regional differences in the development of cellular hypertrophy and hyperplasia were observed when several fat pads were studied, it is reasonable to assume that locally produced growth factors may in part account for this differential development. However, these previous studies have typically focused on adipocyte cellularity parameters and did not investigate possible associations between fat cell size and paracrine growth factors in adipose tissue.

In the present study, examination of adipose tissue cellularity parameters and the demonstration of stimulated preadipocyte proliferation in response to exposure to adipose tissue-conditioned medium has allowed us to link specific aspects of changes in fat cell size, fat cell number, and the presence of locally derived growth factors in adipose tissue. This paradigm allowed us to examine the hypothesis that enlarged adipocytes secrete growth factors that are involved in the regulation of fat cell proliferation and to evaluate if a “critical size” range is a significant factor. From the fat cell distribution profiles (Fig. 1–3) it is apparent that obese rats consistently have a higher percentage of large adipocytes compared with depot- and age-matched lean rats. Despite the greater presence of large fat cells in the adipose tissue of obese rats used in preparation of conditioned medium, there was not a consistent pattern of enhanced preadipocyte proliferation compared with cultures treated with conditioned medium prepared from lean rats (Fig. 4). However, even though consistent in vitro evidence of augmented proliferative activity in obese rats is lacking, the in vivo data on fat cell number (Table 1) demonstrate the progressive development of hyperplasia in these rats. Thus the hypothesis that fat cells of a critical size secrete growth factors that stimulate adipocyte proliferation may not be applicable to all depots or both phenotypes.

Regional differences were observed in this study with regard to the strength of association between adipose tissue cellularity characteristics and proliferative response of the conditioned medium. The strongest associations between the percentage of fat cells in small, medium, and large size ranges; the proliferative capacity of the adipose tissue-conditioned medium; and subsequent changes in fat cell number were found in the inguinal depot. In this depot there was a high correlation between the percentage of large fat cells and the proliferative activity of the conditioned medium. More strikingly, it was found that the percentage of 140- to 180-µm fat cells contributed to 76% of the variability in proliferative activity in the inguinal adipose tissue-conditioned medium from the obese animals. In addition, the proliferative activity of the conditioned medium in the obese animals was significantly correlated with subsequent changes in fat cell number. Thus in the inguinal depot it appears that the percentage of large fat cells contributes significantly to the presence of locally derived adipogenic growth factors and that these growth factors regulate in part the increase in fat cell number of the depot. In the epididymal depot of the obese rats the percentage of large fat cells was highly correlated with proliferative activity in adipose tissue-conditioned medium. A similar correlation was not observed for the lean rats; however, the proliferative capacity of epididymal-conditioned medium for both lean and obese rats was significantly associated with subsequent increases in fat cell number. This would imply that for the epididymal depot something other than the proportion of large fat cells

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<th>Table 2. Association of cellularity parameters and proliferative activity</th>
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<tr>
<td>All data</td>
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<td>Ingual</td>
</tr>
<tr>
<td>Lean</td>
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<td>Obese</td>
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<td>Retroperitoneal</td>
</tr>
<tr>
<td>Lean</td>
</tr>
<tr>
<td>Obese</td>
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Correlation coefficients of relationship between proportion of small (30–60 µM), medium (60–100 µM), and large (100–240 µM) adipocytes, ability of adipose tissue-conditioned medium to promote preadipocyte proliferation in vitro, and in vivo change in fat cell number over a subsequent 3-wk period (i.e., from 9 to 12 wk).
contributes to the factors present in conditioned medium that are associated with increases in fat cell number. In the retroperitoneal fat depot the activity of the conditioned medium from the obese rats was highly correlated with subsequent changes in fat cell number. However, there was no association between the proportion of retroperitoneal fat cells in different size ranges and the proliferative capacity of the conditioned medium. Thus unlike the other depots, the data from this depot do not support the hypothesis that large fat cells are contributing substantially to the presence of local adipogenic factors involved in regulating the development of increased fat cell number.

In this study, conditioned medium prepared from all three fat depots was tested in a primary culture bioassay that utilized cells derived from inguinal fat pads of normal rats. Coincidentally, the strongest correlations between proliferative activity of the conditioned medium and fat cell size and number parameters were observed with conditioned medium from the inguinal fat pad of the obese Zucker rats. However, we do not believe that the higher degree of correlation observed for inguinal fat pad compared with the other two depots can be accounted for on the basis that for this depot alone the conditioned medium was tested on a similar cell type from which it was produced. First of all, it is anticipated that regional differences in the putative proliferative factor(s) would be quantitative rather than qualitative in nature. Furthermore, even on the slight chance that fat pad specific differences in the putative proliferative factor(s) did exist, these would undoubtedly be minor in comparison to known species differences in growth factor composition. In this regard the cell system used for testing the activity of the conditioned medium has been shown to be quite capable of responding to growth factors from various species (D. Hausman, unpublished data). Finally, the finding of the strongest correlations between proliferative activity and cellularity parameters in the inguinal fat pad is not unexpected in light of a recent longitudinal study by DiGirolamo et al. (9). They observed that the cumulative growth of both the inguinal and retroperitoneal fat pads of Wistar rats was due primarily to hyperplasia, whereas the growth of the mesenteric and epididymal fat pads was due predominately to hypertrophy. In our study we observed the strongest correlations between proliferative activity and subsequent change in fat cell number for the inguinal and retroperitoneal fat depots. However, for the inguinal depot there was an additional association between cell size and proliferative activity, which was not observed with the conditioned medium collected from the retroperitoneal depot.

It should be noted that interpreting measures of association are complicated in that multiple comparisons for the correlation increase the possibility of errors in inference. Collapsing the data obtained from the nine original cell size ranges into three equal overall classifications reduced the number of comparisons and reduced risk of error. Furthermore, most of the inferences of positive association in this data set were significant at $P \leq 0.01$, a level that reduces the risk of erroneous interpretations. However, as with all inferences of association it should be noted that a positive correlation is not absolute proof of cause, and yet correlation is an essential characteristic of causality.

Several other recent investigations have also focused on the presence of locally derived growth factors in adipose tissue and their effect on the development of preadipocytes in vitro. Medium conditioned by exposure to preadipocytes derived from omental tissue of massively obese human has been demonstrated to stimulate the replication of rat preadipocytes in primary culture (21). Exposure of preadipocytes to either mature fat or isolated adipocytes enhances the process of differentiation (30). Of greater interest to the question of fat cell proliferation, Lau et al. (22) have found that medium conditioned by exposure to endothelial cells, but not mature fat or isolated adipocytes, stimulates preadipocyte replication. Coculture of preadipocytes with isolated adipocytes from obese humans demonstrated that these cells significantly enhanced proliferation compared with cultures treated with adipocytes from lean controls (7). Unfortunately, none of these studies investigated the association between large adipose cells and alterations of preadipocyte development and thus cannot directly advance our understanding of the critical fat cell size hypothesis.

In general, this study provides evidence that factors present in medium conditioned by exposure to adipose tissue from lean and obese Zucker rats are positively associated with changes in fat cell number. However, the relative contribution of the proportion of small, medium, and large adipocytes to proliferative activity in the conditioned medium is dependent on both depot and phenotype. It should be noted that adipose tissue contains not just mature adipocytes, but a variety of cell types including, fibroblasts, endothelial cells, preadipocytes at various stages of development, and other cells that may be involved singly or together in contributing to the local milieu that regulates adipogenesis. The use of adipose tissue-conditioned medium has allowed us to evaluate the contribution of not just adipocytes but other cells as well and increases the possibility that growth factors secreted as a result of interactions between cell types will be present in the medium. In depots where no significant correlation was found between the percentage of large fat cells and enhanced proliferative activity of conditioned medium, it may be assumed that smaller adipocytes and nonadipose cells influence the overall activity.

In conclusion, the data presented here for the inguinal and epididymal depots of obese Zucker rats are consistent with the concept that enlarged adipocytes, especially those in the 140- to 180-µm diameter size range, secrete growth factors that induce the proliferation of preadipocytes. The stimulation of adipogenesis in different depots may, however, be modulated by factors released from other cells present in the tissue. Preparation of conditioned medium with discrete populations of isolated adipocytes that have been separated into specific size ranges and characterization of the
substances present in the conditioned medium will help to further elucidate the role fat cells play in the paracrine regulation of adipogenesis.

Perspectives

The incidence, health implications, and economic cost of obesity make it one of the most serious public health issues in the United States. Obesity is a well-known risk factor for the development of several costly chronic diseases, including diabetes, hypertension, cardiovascular disease, and some cancers. Recent data from the National Center for Health Statistics have indicated that >30% of the adults in the United States have developed excess body fat to a point that increases their risk of developing these chronic disorders. Furthermore, the incidence of obesity in children and adolescents has increased dramatically in the past decade with almost 25% of this population classified as overweight or obese. Early onset and the most severe forms of adult onset obesity are often characterized by the hyperplastic growth of adipose tissue. These cases also represent the strongest correlation with the risk of chronic disease, present the poorest prognosis, and are the hardest to treat. Conventional forms of obesity treatment may bring about a reduction in fat cell size but cannot decrease fat cell number. This persistence in elevated numbers of adipocytes may contribute to the difficulty in achieving and maintaining optimal body weight. Understanding the cellular mechanisms that underlie the process of adipocyte proliferation is essential before appropriate intervention strategies to prevent the development of hyperplastic obesity can be designed. It had been previously hypothesized, although never conclusively demonstrated, that reaching a critical fat cell size may be the triggering event that results in an increase in fat cell number. The present study has demonstrated a link between specific aspects of changes in fat cell size, fat cell number, and the presence of locally derived growth factors in adipose tissue. The identification and characterization of these paracrine and/or autocrine factors may eventually lead to more effective treatment strategies for hyperplastic obesity.

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