notes on methodology

Determination of adipose cell size and number in suspensions of isolated rat and human adipose cells

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Summary The osmic acid fixation–Coulter electronic counter method described for determining adipose cell size and number in intact adipose tissue fragments has been modified for use with suspensions of isolated rat and human adipose cells. Mean cell sizes in tissue fragments and isolated cell suspensions prepared from the same tissue are virtually identical in rats of various weights. No statistically significant difference in mean adipose cell size between tissue and isolated cell suspension was observed in human adipose tissue although the variability was much greater than in rat tissue. The distribution of cell sizes among replicate samples is more uniform in the isolated cell preparations, possibly reflecting the considerably larger quantities of tissue used in preparing isolated cells than in determining cell size and number directly from tissue fragments. An example of the utility of the modified method during routine metabolic studies with isolated rat epididymal adipose cells is described; isolated cells of increasing size can be obtained from rats of increasing body weight, or from the separated distal and proximal portions of the fat pads of rats of the same weight.

Supplementary key words osmic acid fixation · Coulter electronic counter · rat weight · obesity · proximal fat pad · distal fat pad

The adipose tissue of an organism increases in mass during normal growth and in obesity through a variable combination of increasing adipose cell size and number (1–4). Recent studies indicate that the in vitro metabolic function of adipose tissue fragments is influenced by the tissue's cellular composition (5–8). Investigations of the relationship between adipose cell structure and function and of the role of altered cellular morphology in the derangement of this cell's metabolic activity in such pathogenic states as obesity and diabetes mellitus have utilized the isolated adipose cell (6, 9–12). A method is, therefore, required for the rapid and routine morphological characterization of this commonly used experimental preparation and for the expression of experimental results on a standardized per cell basis. The present report outlines a modification of the osmic acid fixation–Coulter electronic counter method described by Hirsch and Gallian (13) for determining the size and number of adipose cells comprising fragments of intact adipose tissue, which permits its use with suspensions of isolated rat and human adipose cells.

Procedure

Samples of adipose tissue for cell sizing and counting were obtained from the epididymal fat pads of male albino rats (CD strain, Charles River Laboratories, Wilmington, MA) ranging widely in age and body weight, and from the abdominal subcutaneous fat depots of patient volunteers with different degrees of obesity. The epididymal fat pads of each rat were divided into their proximal and distal halves and the segments were analyzed separately. Human adipose tissue was obtained from the same anatomically defined subcutaneous abdominal fat depot of nonobese and obese patients by surgical biopsy or during elective abdominal surgery. For each rat and human tissue sample, adipose cell size and number were determined both in intact tissue fragments and in isolated adipose cells prepared from these tissues.

The cellularity of intact fragments of adipose tissue was determined by method III described by Hirsch and Gallian (13). Intact shreds of either rat or human adipose tissue were fixed for 48–72 hr at 37°C in 25 ml of 0.05 M collidine buffer, pH 7.4, containing 2 g of osmium tetroxide/100 ml. The osmium-fixed cells were then washed, collected, and counted in isotonic saline in a Coulter electronic counter (Model B, Coulter Electronic, Inc., Hialeah, FL). Mean adipose cell size was calculated by dividing the lipid weight of the fixed tissue sample, determined as described by Hirsch and Gallian (13), by the total number of cells in the sample.

The cellularity of isolated adipose cell suspensions was determined by a modification of method III of Hirsch and Gallian (13). A concentrated, stock suspension of isolated adipose cells was prepared

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TABLE 1. Size of rat epididymal adipose cells in intact tissue fragments or isolated cell suspensions prepared from the same tissue

Rat Weight (n)[a]	Mean Adipose Cell Size	
	Tissue	Isolated Cells
g[b]	*μg lipid/cell*[c]	
208 ± 0 (2)	0.09 ± 0.02	0.09 ± 0.00
212 ± 8 (4)	0.07 ± 0.01	0.09 ± 0.01
212 ± 5 (2)	0.09 ± 0.01	0.10 ± 0.00
301 ± 17 (6)	0.19 ± 0.06	0.21 ± 0.00
307 ± 22 (6)	0.19 ± 0.04	0.19 ± 0.00
342 ± 27 (6)	0.23 ± 0.04	0.23 ± 0.01
354 ± 10 (6)	0.24 ± 0.05	0.23 ± 0.00
654 (1)	0.65 ± 0.10	0.61 ± 0.01
689 (1)	0.72 ± 0.09	0.79 ± 0.02
137 ± 6 (3)[d]	0.07 ± 0.01	0.05 ± 0.00
	0.06 ± 0.01[e]	0.05 ± 0.00[e]
399 ± 7 (3)[d]	0.17 ± 0.02	0.17 ± 0.01
	0.18 ± 0.04[e]	0.16 ± 0.01[e]

[a] Number of rats used in each experiment.
[b] Results are means ± SD of n rats.
[c] Results are means ± SD of at least duplicate, but often triplicate, samples.
[d] Used only distal portion of epididymal fat pads.
[e] Tissue and cells treated 72 hr in osmic acid fixative.

from the remaining tissue by the collagenase method described by Rodbell (14) and modified by Cushman (15). Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 3 g of untreated bovine serum albumin per 100 ml (Bovine Albumin Powder, Fraction V, Armour Pharmaceutical Company, Chicago, IL), with or without glucose, was used in the preparation of cells. Tissue and stock cells were kept at 37°C in 25-ml polyethylene scintillation vials and 50-ml polyethylene sample vials, respectively. In order to minimize cell breakage during the isolation procedure, particularly with larger cells, a maximum of 4 g of adipose tissue was treated in each collagenase incubation containing 3 ml of buffer and 10 mg of collagenase.

Following preparation, the stock isolated adipose cell suspension, housed in a 37°C water jacket designed for this purpose, was gently stirred with a magnetic stirring device in order to maintain its homogeneity. Using an automatic repetitive microliter sampling pipette equipped with a polyethylene tip cut to slightly enlarge its orifice, 0.25–1.0-ml samples of suspension were then transferred into 25-ml polyethylene scintillation vials containing a small volume of osmium tetroxide fixative in 0.05 M collidine-HCl buffer at pH 7.4 and 37°C. The ratio of cell suspension to fixative was always 1:4, volume:volume, for example, 0.5 ml of cell suspension in 2.0 ml of fixative. During preparation of the solution, the concentration of osmic acid was adjusted in increments of 1 g/100 ml such that the ratio of total cell lipid fixed to osmic acid concentration never exceeded 50 mg to 1 g/100 ml, for example, 135 mg cell lipid/sample in 3 g of osmic acid/100 ml. Cell lipid/sample was estimated prior to each experiment on the basis of the number and weights of the rats to be used, the number and size of the adipose cells usually obtained from such rats, and the total volume of cell suspension to be prepared; for example, the 12 whole epididymal fat pads of six rats weighing 200 g each yield roughly 54×10^6 cells at roughly 0.1 μg lipid/cell, or 5.4 g of lipid; the final cell concentration in 20 ml of stock cell suspension is, therefore, roughly 2.7×10^6 cells/ml, or 1.35×10^6 cells and 135 mg lipid/0.5 ml.

After incubation of the cells in the small volume of osmium tetroxide solution for 1 hr at 37°C with or without gentle shaking, sufficient fresh fixative was added to fill each sample vial, and fixation at 37°C was continued for 48–72 hr without shaking. The osmium-fixed cells were then washed, collected, and electronically counted in isotonic saline as described in method III by Hirsch and Gallian (13). At the same time that samples of cells were taken for fixation and counting, total lipid was extracted from identical samples of isolated cells by the method described by Dole (16). The volumes of the components of the extraction system were proportionally reduced, however, such that the final volume of organic phase was 2.3 ml. Lipid weights were then obtained either gravimetrically from dried portions of these extracts or directly by triglyceride assay (17). Mean adipose cell size was calculated as follows:

$$\text{mean cell lipid content } (\mu g/\text{cell}) = \frac{\text{lipid content of cell suspension } (\mu g/\text{ml})}{\text{total number of cells/ml sample.}}$$

Results and discussion

Table 1 summarizes the results of a series of experiments in which adipose cell size was determined either in intact rat epididymal adipose tissue fragments or in isolated adipose cell suspensions prepared from comparable fragments of the same tissue. While the mean adipose cell sizes were not always identical among the different determinations within single experiments, the differences were quite small and no systematic variation in adipose cell size between tissue and cell suspension was observed, independent of rat body weight. In two of the experiments, tissue and cells were fixed with osmium tetroxide for 48 and 72 hr; no significant differences were observed regardless of fixation time. These results suggest a broad applicability, and flexibility, of the osmic acid

fixation–Coulter electronic counter method for determining mean rat adipose cell size and number.

Table 2 summarizes the results of a similar series of experiments carried out with human abdominal subcutaneous adipose tissue and isolated cells. Over the entire series, no statistically significant difference in adipose cell size between tissue and cell suspension was observed. The considerable variation among experiments is, however, reflected in five studies where mean adipose cell size in the cell suspension is up to 25% greater than that in the corresponding tissue fragments, two studies where mean size is roughly 10% greater, two studies where mean size is virtually identical, and three studies where mean size is as much as 28% smaller. While variable numbers of cells are broken during preparation of human adipose cell suspensions, as reflected in the appearance of free lipid above the packed cell float after centrifugation, variations in human adipose cell size between tissue and cell suspension cannot represent the selective breakage of cells of any particular size during preparation of isolated cells or fixation in osmic acid because these variations are observed with both small and large human cells as well as with human cells in the same size range as those from rats. Moreover, the variability is neither consistently positive nor negative.

Species differences in the stability of adipose cells to a variety of experimental manipulations, including collagenase treatment and fixation for electron microscopy, have previously been observed[3] and may be responsible for these variations. In addition, collagenase treatment may be associated with varying degrees of cellular adhesion among isolated cell preparations; indeed, the longer isolated rat adipose cells are exposed to collagenase, the less they tend to stick together.[3] Special care both in keeping the amount of human tissue treated with collagenase below the 4 g upper limit per collagenase incubation vial and in stopping the collagenase incubation at a point just sufficient for complete digestion of the tissue appears to minimize cell breakage and the difference in mean cell size between tissue and isolated cells. When the mean size of human adipose cells from the same subcutaneous fat depot was determined on two different occasions separated by 4 weeks (Table 2), the variation in size of isolated cells was similar to that observed with intact tissue. Perhaps the most likely source of the variations in human adipose cell size between tissue and cell suspension lies in the procedure for selecting samples of tissue to be used either in direct determinations of

TABLE 2. Size of human abdominal subcutaneous adipose cells in intact tissue fragments or isolated cell suspensions prepared from the same tissue

Patient Weight	Adipose Cell Size	
	Tissue	Isolated Cells
Kg	*μg lipid/cell*[a]	
47.5	0.271, 0.383	0.322, 0.326
56.4	0.262, 0.205	0.249, 0.188
65.4	0.258, 0.318	0.386, 0.383
72.7	0.282, 0.300	0.350, 0.352
73.2	0.506, 0.542	0.536, 0.614
75.8[b]	0.663, 0.655	0.484 ± 0.041[c]
75.8[b]	0.856, 0.709	0.668 ± 0.030[c]
87.7	0.580, 0.602	0.671, 0.691
93.6[b]	0.506, 0.557	0.701 ± 0.030[c]
93.6[b]	0.506, 0.549	0.699 ± 0.034[c]
100.2	0.896, 0.911	0.967 ± 0.021[c]
151.9	1.005, 0.928	0.934, 0.865

[a] Results are individual values of duplicate samples, except where noted otherwise.
[b] Two determinations carried out in same patient over a 4 week period.
[c] Results are means ± SD of quadruplicate samples.

cells size or in collagenase digestion. The generally larger variation in cell size among replicate samples of intact tissue than of cell suspension probably represents the true variation of adipose cell size within any given fat depot from which only small samples containing few cells are removed for analysis; adipose cell suspensions are generally prepared from much larger samples or from a greater number of small samples of the same fat depot. The prepared cells are pooled and mixed thoroughly, and only then are virtually identical samples removed for osmic acid fixation and lipid weight analysis. The modification of the osmic acid fixation–Coulter electronic counter method reported here, therefore, provides estimates of human adipose cell size that are reasonably comparable to those obtained from intact tissue shreds in spite of the fact that there is a considerably greater variability in cell size between tissues and cell suspensions from human adipose tissue than from rat adipose tissue. In addition, since the variation in cell size among isolated human cell suspensions prepared from the same individual on separate occasions is entirely comparable to that determined using tissue fragments, the method reported here appears to provide a good estimate of the size of the isolated cells in a given preparation, if not necessarily the size of the cells comprising the fragments of tissue from which they are prepared. The results of metabolic studies with isolated human adipose cells are, therefore, better expressed on the basis of the size and number of cells directly measured in each cell suspension than those determined from separate

[3] Unpublished observations.

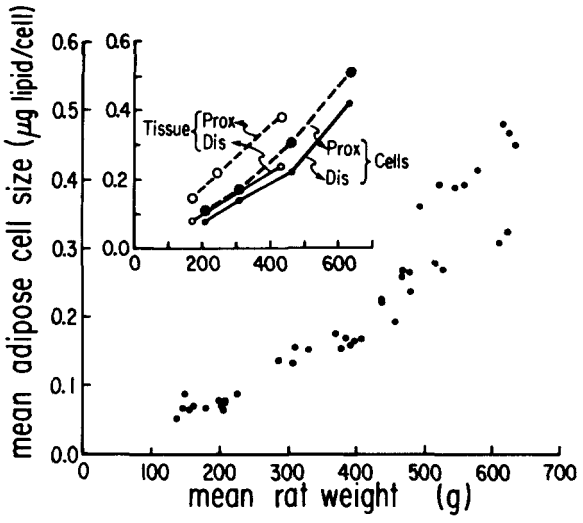

Fig. 1. Relationship between rat epididymal adipose cell size and rat body weight. Mean adipose cell size was determined in suspensions of isolated cells prepared from the pooled distal portions (●), and in tissue fragments (○) (11) or suspensions of isolated cells (○) prepared from the separated distal (——) and proximal (– – –) portions of epididymal fat pads of groups of rats weighing 135–635 g. Results are the means of at least duplicate determinations.

tissue fragments and extrapolated to the isolated cells.

The results of these two series of experiments suggest further that breakage of adipose cells during the isolation procedure is generally independent of cell size and does not affect the distribution of cell sizes around the mean; in fact, an advantage of the isolated cells over intact tissue fragments appears to lie in the increased homogeneity among replicate samples. All of the limitations of the original method, however, such as the lack of identification of very small cells and the need for correction for coincidence counting at high cell concentrations, still apply to the modifications developed here for isolated adipose cell suspensions. Results similar to those above have been obtained using isolated rat adipose cells prepared in an albumin-free medium. Further variations in methodology that have been successfully applied as a means of increasing the flexibility of the technique include gentle shaking during the initial 60-min fixation period, which permits use of the same shaking water bath for fixation and experimental incubation of isolated adipose cells, and storage of fixed cells in the fixative solution at 4°C for several days following the normal 48-hr period at 37°C, which provides the opportunity to collect samples from several experiments prior to the final washing and counting step.

The adipose cell represents a particularly well-studied metabolic unit; the results of a major portion of the studies of adipose cell function have, however, been expressed on the basis of lipid or triglyceride weight and with little regard for the numbers or sizes of the adipose cells under investigation. In order to extrapolate these published results to current studies of adipose cell metabolism as a function of cell size and, in addition, to demonstrate the utility of the present modified osmic acid fixation–Coulter electronic counter method for determining the size and number of cells in isolated cell suspensions prepared for experimental incubation, **Fig. 1** illustrates the relationship between mean adipose cell size and rat body weight when isolated cells were prepared for a series of metabolic studies from the distal and proximal portions of epididymal fat pads. Indeed, routine determinations of cell size and number are now carried out at regular intervals in a typical experiment in this laboratory in order to insure the homogeneity of a given cell suspension and of the individually incubated samples derived therefrom. In addition, Fig. 1 compares the results obtained using isolated cells with those in a previous study using intact epididymal adipose tissue fragments. Increasing rat weight and/or age is accompanied by increasing distal and proximal adipose cell size, but not as a linear function; in fact, if the linear relationship demonstrated by Zinder and Shapiro (9) over a range of rat weights from 80 to 300 g is assumed here, then adipose cell size would appear to increase with increasing rat weight in a biphasic fashion, i.e., a slow increase with weights up to, and a faster increase with weights greater than, roughly 400 g. The isolated adipose cell sizes obtained in the present series of experiments compare well with those obtained by Zinder and Shapiro (9) using an optical method; conversion of published adipose cell activities from a per lipid weight to a per cell basis can be reasonably undertaken using the data presented in Fig. 1.

With rats of a given weight, isolated proximal adipose cells are consistently about 30% larger than the corresponding distal cells (Fig. 1). Salans and Dougherty (Fig. 1) (6) observed greater differences between proximal and distal cells when adipose cell size was determined in intact tissue fragments by the original osmic acid fixation–Coulter electronic counter method; however, only the extreme proximal and distal portions of the fat pads were used. In the present investigation, the distal and proximal halves of the fat pad were compared. Differences in growth rate, physical activity, and body weight may also contribute to this discrepancy between the two studies.

The method reported here for determining the mean size of isolated adipose cells in suspension permits rapid and routine morphological characteri-

zation of this commonly used experimental preparation and the expression of experimental results on a standardized per cell basis. Furthermore, the results obtained with this method indicate that the procedure employed in the isolation of adipose cells from intact tissue does not greatly distort the distribution of cell sizes in a given preparation through a systematically selective destruction of cells of a given size and can, therefore, be usefully employed in the study of relationships between adipose cell size and metabolic function both in experimental animals and in man. Except for the ability to directly characterize the cellular character of a given adipose cell preparation, however, this modification offers no advantages over the original method in which intact fragments of tissue are employed to estimate adipose cell size and number. Until the small, but significant, variations between human isolated cell and tissue adipose cell sizes are entirely resolved, the original tissue method must remain the basis for continuing studies of the growth, development, and cellular character of the adipose tissue mass during the normal aging process and in obesity.

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