Scanning electron microscopy of very small fat cells and mature fat cells in human obesity

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Summary To determine the effect of obesity on the size distribution of fat cell populations in human adipose tissue, omental fat tissue biopsies were obtained from lean, moderately obese, and massively obese patients. The size distributions of adipocytes from lean and obese fat tissues examined by the scanning electron microscopic method were bimodal, consisting of populations of very small fat cells and mature fat cells, in contrast to collagenase-derived isolated cells that showed only the large mature fat cells. The very small fat cell population represented 21 to 26% of the total fat cell number in the lean and in both obese groups. In contrast, preparations of human fat cells isolated by the collagenase method systematically excluded the very small fat cells. In massive obesity, both cell populations participated in the hyperplastic growth but only the larger mature fat cells increased in size, implying that these two cell populations differ in their physiological role.—**Julien, P., J-P. Despres, and A. Angel.** Scanning electron microscopy of very small fat cells and mature fat cells in human obesity. *J. Lipid Res.* 1989. **30:** 293–299.

Supplementary key words adipocyte cellularity • adipose tissue hyperplasia • collagenase • fat cell sizing • omentum • osmic acid fixation

Adipose cell size has a modulating effect on adipocyte metabolism and hormone action (1). In exploring the relation of cell size and function, a variety of methods have been developed to study the cellularity of adipose tissue. The histologic methods used to evaluate cell diameters of sectioned slices of fixed or frozen adipose tissue are only valid if an even distribution of fat cells is assumed and if they are cut through their equators (2). Diameter measurements of adipocytes isolated after collagenase treatment of tissue assume that no cells are systematically lost or destroyed during the isolation procedure (2). The validity of these assumptions is justifiably questioned.

Evidence for a bimodal distribution of adipocyte diameters exists in isolated cell preparations of several mammalian species (3) but not in humans. In the experimental rat, the smaller cells, called very small fat cells (VSFC), contribute 30–60% of the total cell population in adipose tissue, and the proportion of these VSFC is greater in fat from obese animals compared to lean (4). In view of the unknown function of VSFC and the hypercellularity of obesity in the massive form, it was of interest to determine whether human fat tissue displays cellular heterogeneity. In the present study we examined the cellularity of human omental fat tissue in lean and obese human subjects comparing two methods: light microscopic examination of fat cells isolated by the classical collagenase method (5) and scanning electron microscopic visualization of intact fragments of fat tissues. Our findings demonstrate a biphasic distribution of adipocytes in intact fat tissue from both lean and obese subjects indicating cellular diversity in human fat. In collagenase-derived isolated fat cells, a single size population was found consistent with a systematic loss of small cells. Only the volume of the large mature fat cells (MFC) was altered by obesity; the VSFC remained constant in size and proportion.

METHODS

Omental adipose tissue biopsies were obtained from the major omentum of a total of 20 patients: 10 massively obese patients (9 females, 1 male, age 42 ± 3 yr, weight 114 ± 8 kg, BMI 43 ± 1 kg/m^2, mean ± SEM) undergoing gastric stapling, and 3 moderately obese subjects (2 females, 1 male, age 49 ± 6 yr, weight 70 ± 4 kg, BMI 29 ± 1 kg/m^2) and 7 lean subjects (3 females, 4 males, age 51 ± 5 yr, weight 62 ± 4 kg, BMI 24 ± 1 kg/m^2) undergoing elective surgical procedures. The research protocol was approved by the Research Ethics Committee of the Toronto General Hospital, and the patients gave informed consent.

Adipose tissue samples were freshly washed in Krebs bicarbonate buffer (KRB) and processed immediately for light microscopic examination of isolated cells, and for the preparation of intact fat tissue fragments for scanning electron microscopy. Adipocytes were isolated by collagenase digestion as described by Rodbell (5) with minor modifications (6). The liberated cells were separated from undigested tissue by filtration through a fine nylon mesh and washed three times with KRB containing 2% bovine serum albumin. Fat tissue fragments (approximately 3-mm^3 blocks) were fixed for 48 hr at 4°C with 5% glutaraldehyde–4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) and post-fixed for 24 hr in 0.1 M phosphate buffer containing 1% osmium. The osmium fixation procedure was repeated three times. The blocks were dehydrated in graded ethanol (50 to 99%) and dried in a critical point dryer using liquid CO_2. Fixed intact adipose tissue blocks were then coated with 200 Å of gold using a sputter coater, and examined with a JEOL scanning electron microscope. Adipocyte diameters (250 cells) were determined from photomicrographs (magnification 100 ×) obtained by scanning electron microscopy of intact fat pads using a com-

Abbreviations: BMI, body mass index; MFC, mature fat cell; KRB, Krebs bicarbonate buffer; VSFC, very small fat cell.

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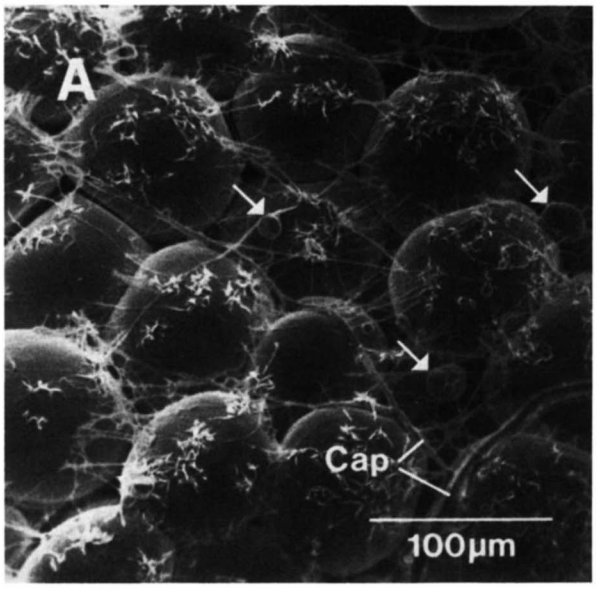
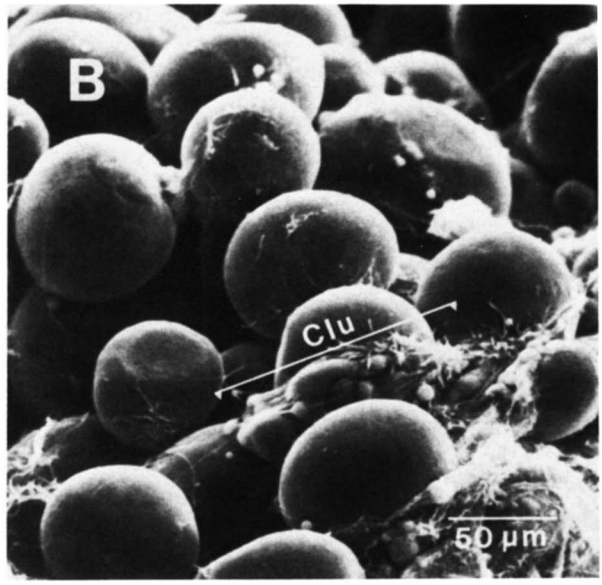
puterized image analysis system (7) and grouped in 5-μm class intervals. Isolated fat cell diameters (200 cells) were determined using a microscope equipped with a graduated ocular as previously described (8). An aliquot of the cell suspension was taken and transferred to 1 ml of 0.4% trypan blue. Fat cell diameters were measured with a precision of 1 μm and recorded in 5-μm classes from 0 to 250 μm.

Omental adipose tissue biopsies were obtained from four additional control female patients and subcutaneous adipose tissue biopsies from a female patient undergoing cesarian section and from another undergoing hysterectomy. Intact adipose tissue fragments from these subjects were immediately washed in KRB and processed for both light microscopic examination and transmission electron microscopy. The fat tissue fragments were fixed in glutaraldehyde–formaldehyde and osmium, and dehydrated in graded ethanol as described above. Fixed adipose tissue blocks were then embedded in standard low-viscosity Spurr resin (J. B. EM Services Inc., Montreal, Canada). Thin sections stained with toluidine blue were examined by light microscopy using a Zeiss photomicroscope. Adipocyte diameters (100 cells) were determined from photomicrographs. Ultrathin sections were stained using standard procedures (lead citrate and uranyl acetate) and viewed with a Hitachi H-7000 electron microscope.

Values are expressed as mean ± SEM. Isolated fat cells and fixed intact fat tissue from the same fat pad were considered to be paired samples. The mean values of their fat cell volumes were compared using the Student's *t* test. The





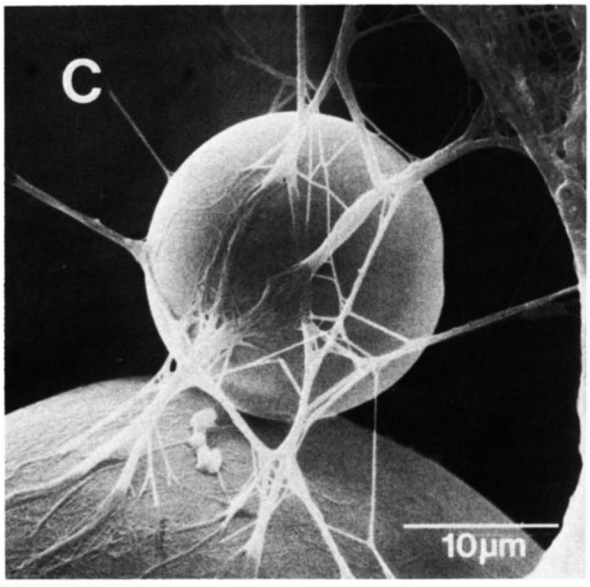
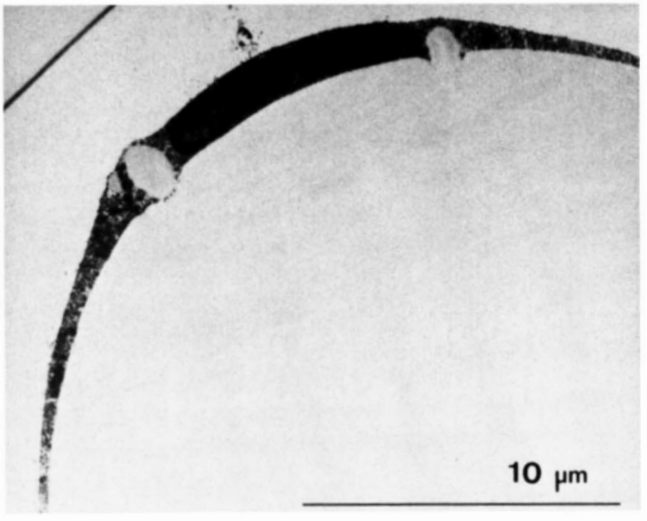


Fig. 1. Scanning electron photomicrographs of omental fat tissue obtained from a lean subject showing (A) mature and very small fat cells (arrows) supported by a stroma of collagen fibers, and (B) a cluster or pocket of VSFC surrounded by a dense network of collagen fibers. (C) A VSFC is retained to a larger MFC (> 100 μm) by collagen fibers. The surface of these adipose cells is surrounded by a thin layer of delicate reticular fibrils. Cap, blood capillary; Clu, cluster of VSFC.

MFC



VSFC

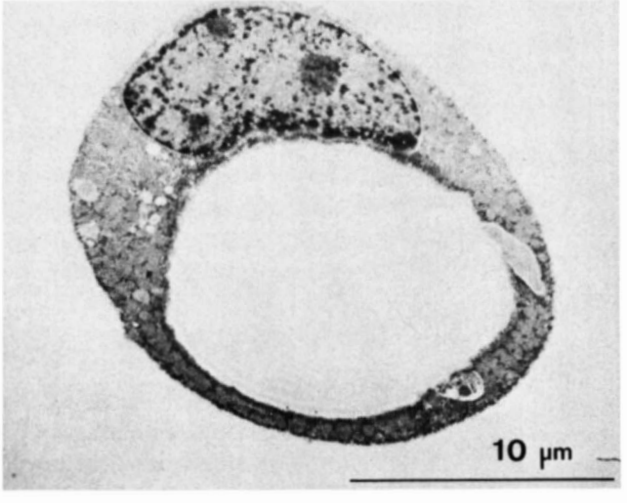


Fig. 2. Transmission electron photomicrographs of omental fat tissue obtained from a control female subject showing mature and very small fat cells. The nucleus is flattened, as commonly observed in mature fat cells, and denser than VSFC nucleus.

mean values of cell diameters and volumes and the mean percentage of VSFC and MFC in cell populations were compared between lean and obese patients using the unpaired *t* test. The reproducibility of the scanning method and its reliability compared to the isolated-cell method were determined by analysis of variance using ANOVA-randomized block factorial design (9). Analysis of the average histograms was carried out by the χ^2 test for comparison of two distributions. Relationships between variables were analyzed using the Pearson's interclass correlation coefficient.

RESULTS AND DISCUSSION

Adipose tissues obtained from the three groups of patients consisted of lobules of fat cells separated by septae of connective tissue fibers. VSFC (< 53 μm in diameter) were visible and found randomly distributed through the tissue (**Fig. 1A**). Occasional clusters or "pockets" of VSFC occurred in both lean and obese subjects (Fig. 1B). The lobular structure composed of MFC and VSFC was maintained by a stroma of collagen fibers (Fig. 1A). Fig. 1 also shows that, at the exposed surface of the tissue fragments, in situ adipocytes were round and separated artifactually by large interstitial spaces. A distinct network of reticular fibers was closely associated with the cell surface of both MFC and VSFC (Fig. 1C).

Transmission electron microscopy of intact fat tissue shows ultrastructural characteristics of MFC and VSFC (**Fig. 2**). The typical flattened nucleus, as shown in MFC, was not seen in VSFC which contained relatively less lipid inclusion than MFC as previously reported in rat fat tissue (10). This ultrastructural study of adipocytes confirms that VSFC are nucleated cells and not fat cell protrusions or lipid droplets. Intact fat tissues embedded in Spurr resin (**Fig. 3**) were also examined by light microscopy. The histogram of adipocyte diameters determined from sections of intact fat tissue embedded in resin shows a skewed distribution of diameters toward small cells. It is evident that this method does not allow equatorial sectioning of every adipocyte; however, the presence of a peak in the diameter distribution between 7.5 and 47.5 μm cannot be explained by simple methodological inaccuracy and is consistent with a bimodal distribution of fat cells. The mean diameter of the small fat cells (7.5 to 47.5 μm) was 28.7 ± 0.9 (mean ± SD) compared to the diameter of the larger fat cells (> 47.5 μm: 86.2 ± 2.7 μm).

In paired experiments, three sets of fat tissue blocks were prepared from each biopsy obtained from eight patients. These blocks were examined in duplicate by scanning of intact fat tissues and by light microscopy of iso-

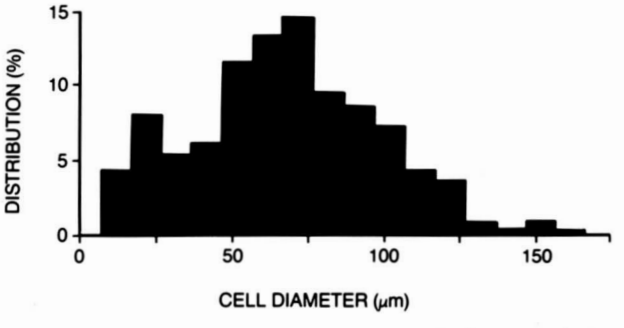


Fig. 3. Combined distribution of diameters of adipocytes obtained from five control female subjects. Adipocyte diameters (n = 500) were measured by light microscopy from intact tissue embedded in Spurr resin. Each class interval is 10 μm wide.

lated adipocytes (**Table 1**). Comparison of first and second size scanning determinations of VSFC and MFC mean volumes showed that there was no significant variation between these two independent measurements from the same fat pads. Moreover, the mean MFC volumes determined by the isolated fat cell method were also not statistically different compared to those measured by scanning. **Table 2** indicates that the reliability for mean MFC diameter, as determined by scanning of tissue or light microscopy of isolated fat cells, was high (a reliability coefficient of r_i = 0.87 was obtained when analysis of variance was performed on MFC volumes as measured by scanning electron microscopy in duplicate and by light microscopy), thus most of the variance in MFC volume was between subjects and not between methods. VSFC were apparent by scanning microscopy but were not evident in freshly isolated cell preparations. Statistical analysis of the mean VSFC volumes obtained in duplicate shows high repeatability (Table 1), validating the scanning method to determine fat cell size in a wide range of diameters from 7.5 μm to more than 200 μm.

Histograms of the diameters of omental adipocytes obtained from lean, moderately obese, and massively obese patients are shown in **Fig. 4**. The histograms of intact fat tissues processed by scanning (Fig. 4A,B,C) clearly show a biphasic distribution of adipocyte diameters. By contrast, no VSFC were evident in the samples processed by the collagenase-derived isolated fat cell method (Fig. 4D,E,F). The mean diameter of VSFC measured by scanning was 21.7 ± 1.2 μm (mean ± SEM) for the lean subjects and was not statistically different compared to moderately and massively obese patients (28.3 ± 1.1 and 23.7 ± 1.2 μm, respectively). The mean diameter of lean MFC was 84.7 ± 5.0 μm which was smaller ($P < 0.025$) than moderately obese MFC mean diameter (107.6 ± 3.7 μm). No significant differences were found between MFC diameters of moderately and massively obese patients. Within each weight group, χ^2 analysis of MFC distributions showed no significant difference between distributions of MFC analyzed by the scanning method and the isolated cell method indicating that they represent the same population of cells.

Our findings represent the first evidence of a bimodal size distribution of fat cells in human omental fat tissues from lean and obese patients. Similar results were obtained with subcutaneous as well as mediastinal fat cells (data not shown) indicating the widespread characteristic of this feature. Previous reports based on collagenase-derived isolated cells have shown a bimodal size distribution in animal fat, but this was not always noted and on occasion VSFC were considered contaminants (11). A variety of instruments and methods have been used to size fat cells in order to meet every particular experimental circumstance, as reviewed by Gurr and Kirtland (2). Methods were introduced to preserve the integrity of isolated adipocytes by prefixing them (3). However, VSFC were not found, probably because their higher density (10) precluded separation from the stromal fraction which was washed away from the large fat cell suspension (12). Fat cells have also been isolated from osmium-fixed fat tissues using urea to solubilize the connective tissue matrix (13). This method, combined with automatic particle counting, improved the cell sizing methodology leading to the demonstration of a population of smaller size cells in bovine subcutaneous adipose tissue (2). However, several factors, in particular sample storage, limited the reproducibility and precision of isolated cell sizing by cell counter (11). Small fat cells were noted in cut sections of fat tissue obtained from human (Fig. 3), guinea pig, mouse, and swine (3, 14, 15). However, this histological method did not provide equatorial measurements of all cells (2), necessitating a correction factor to derive a calculated mean diameter representative of the mean diameter obtained from isolated cell measurements (16).

Although VSFC contribute less than 1% of the total fat tissue mass, they represent 21 to 26% of the total fat cell

TABLE 1. Comparisons of VSFC and MFC mean volumes measured from lean and obese subjects in intact fat tissues by scanning electron microscopy in duplicate experiments (I and II) and in isolated fat cell preparations by light microscopy

Methods of Analysis	VSFC	MFC
	$\mu m^3 \times 10^4$	
Electron microscopy		
I	1.4 ± 0.2	51.8 ± 8.1
II	1.6 ± 0.3	53.2 ± 10.1
Light microscopy		51.3 ± 8.2

Diameters of 250 (electron microscopy) or 200 (light microscopy) randomly selected cells were calculated from each subject. Fat cell volume was calculated from each cell diameter using the equation of a sphere. The mean fat cell volumes were compared using the Student's t test for paired measurements. Differences between methods were not statistically significant; mean ± SEM, n = 8.

TABLE 2. Reproducibility of the scanning method and its reliability compared to isolated-cell method were determined by analysis of variance using Anova-randomized block factorial design for repeated measurements on MFC volumes by scanning electron microscopy in duplicate and light microscopy

Source of Variation	SS	DF	MS	F
Between subjects	0.89	7		
Within subjects	0.27	16		
Between methods	0.00	2	0.00	0.04[a]
Residual	0.26	14	0.02	
Total	1.16	23		

Mean volumes are reported in Table 1. SS = Sum of Squares; DF, degrees of freedom; MS, mean square reliability as determined by intraclass correlation coefficient was 0.87 ($P < 0.01$).
[a] P = NS (for $P < 0.05$; 2,14; F > 3.74).

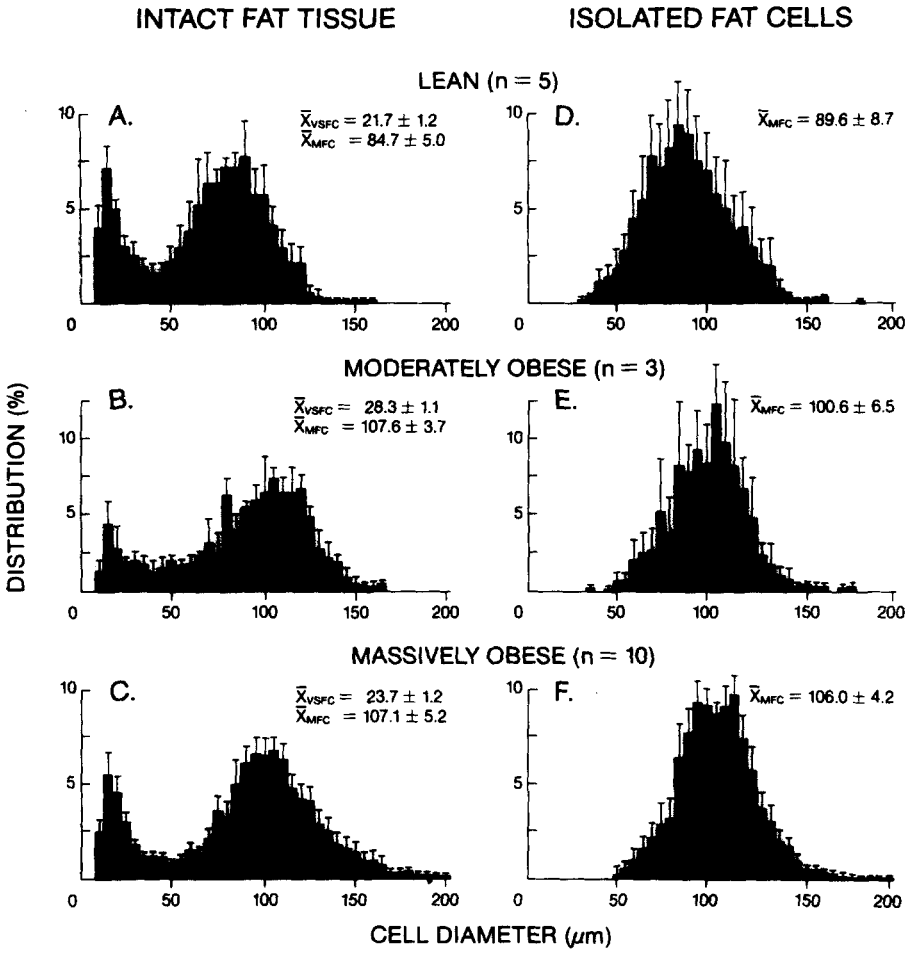


Fig. 4. Comparisons of average distributions of adipocyte diameters measured from intact tissue by scanning electron microscopy (A,B,C) and from fresh isolated cells by light microscopy (D,E,F). Samples were obtained from omental tissue of lean, moderately obese, and massively obese patients. Each class interval represents mean ± SD of cells in that interval obtained from a number (n) of patients. Each interval is 5 μm wide.

population in human omental fat tissue (**Table 3**). Thus, the smaller mean diameters found in cut sections (14, 15) compared to the isolated-cell method is partly explained by the presence of a population of VSFC. In lean mature animals, both VSFC and MFC populations have been reported, and in a number of studies in obese animals, VSFC were present in greater proportion than in the lean animals (4, 17, 18). In contrast, in the present work on human omental fat tissue, obesity did not alter the relative proportion of VSFC and MFC (Table 3) nor did it affect VSFC volume. The only apparent change was an increase in MFC volume. Further, a significant correlation ($P < 0.001$) was found between MFC volume and body mass index for all three groups of subjects taken together (**Fig. 5**) whereas VSFC volume remained constant. These findings demonstrate a differential effect of obesity on these two cell populations suggesting that VSFC could have a physiological role distinct from MFC. It was also noted that in moderately obese subjects MFC reached a maximum cell volume without a further increase in massive obesity. These findings confirm previous reports that massive obesity is associated with adipocyte hypertrophy as well as an increase in total fat cell number (18, 19), but goes on to show that this hyperplasia affects both VSFC and MFC populations similarly.

Many metabolic reactions, including lipolysis (20), triglyceride synthesis (21), lipoprotein metabolism (22), and lipoprotein lipase activity (23), are routinely correlated with fat cell size (1, 19). Thus, studies using intact tissue fragments which contain a mixture of both cell types, small and large, will give information reflecting combined activity. Because VSFC and MFC differ in metabolic reactivity (10), cellular heterogeneity should be taken into account in studies involving fragments of adipose tissue or isolation of total tissue membranes and organelles when compared to collagenase-isolated adipocytes from which VSFC have been systematically excluded.

TABLE 3. Effect of obesity on VSFC and MFC distribution and size measured by scanning electron microscopy

Parameter	Lean (n = 7)	Moderately Obese (n = 3)	Massively Obese (n = 10)
Cellular distribution (%)			
VSFC	25.9 ± 3.4	21.3 ± 3.8	22.0 ± 3.1
MFC	74.1 ± 3.4	78.7 ± 3.8	78.0 ± 3.2
Mass distribution (%)			
VSFC	0.9 ± 0.2	0.7 ± 0.2	0.5 ± 0.1*
MFC	99.1 ± 0.2	99.3 ± 0.2	99.5 ± 0.1*
Cellular volume ($\mu m^3 \times 10^4$)			
VSFC	0.9 ± 0.1	2.0 ± 0.2	1.2 ± 0.2
MFC	37.2 ± 5.7	74.0 ± 6.6**	76.8 ± 12.0**

The inflexion points between VSFC and MFC populations were determined by the polynomial least square method and were not significantly different between moderately (53.3 ± 2.0 μm) and massively (52.5 ± 2.9 μm) obese patients, but were higher than lean (43.8 ± 4.1 μm, $P < 0.025$). VSFC maximum diameter was determined using these inflexion points which had a very high reliability with an intraclass correlation coefficient of 0.82 ($P < 0.01$). The mean distributions and volumes of VSFC and MFC were compared between lean and obese subjects using Student's t test. Mean ± SEM;* $P < 0.025$ and ** $P < 0.05$ compared to lean.

This report describes a simple scanning microscopic method that minimizes cell loss or disruption and emphasizes the importance of analyzing both VSFC and MFC populations separately, so that each is accounted for in studies where fat cell size is a determinant of function. Because VSFC appear to be a separate population of cells without a known unique function, it is premature to regard them simply as immature cells. In view of the paucity of cell lipid compared to MFC, they may support adipose tissue functions other than glyceride storage. While VSFC may represent precursors of MFC, their constancy in both lean and obese fat tissues, as shown here, supports the suggestion that they could also be involved in the development and growth of adipose tissue or some other, yet to be established, function (3).

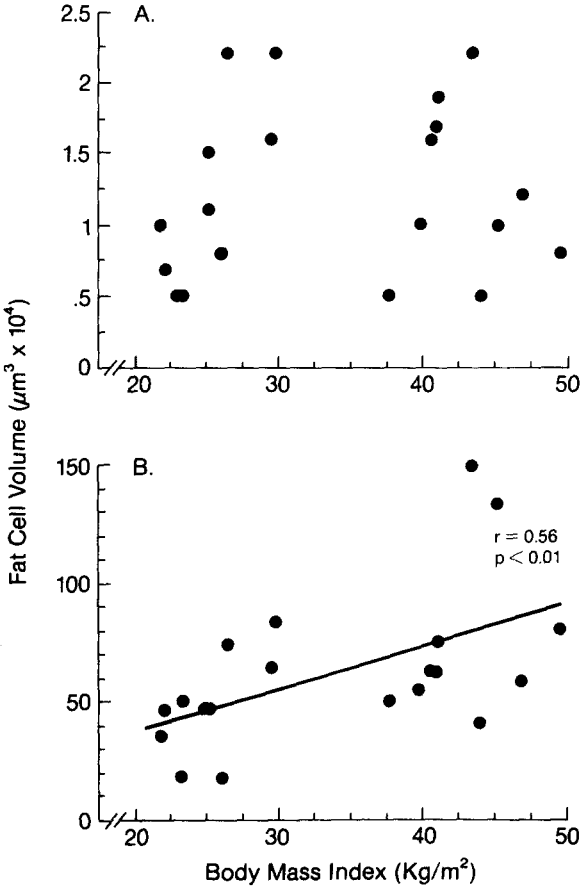


Fig. 5. Correlations between VSFC (A) and MFC (B) volume and body mass index for all the subjects studied; n = 20 (lean, moderately obese, and massively obese patients).

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