

Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression

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ABSTRACT Enlarged adipocytes are associated with insulin resistance and are an independent predictor of type 2 diabetes. To understand the molecular link between these diseases and adipocyte hypertrophy, we developed a technique to separate human adipocytes from an adipose tissue sample into populations of small cells (mean $57.6 \pm 3.54 \mu\text{m}$) and large cells (mean $100.1 \pm 3.94 \mu\text{m}$). Microarray analysis of the cell populations separated from adipose tissue from three subjects identified 14 genes, of which five immune-related, with more than fourfold higher expression in large cells than small cells. Two of these genes were serum amyloid A (SAA) and transmembrane 4 L six family member 1 (TM4SF1). Real-time RT-PCR analysis of SAA and TM4SF1 expression in adipocytes from seven subjects revealed 19-fold and 22-fold higher expression in the large cells, respectively, and a correlation between adipocyte size and both SAA and TM4SF1 expression. The results were verified using immunohistochemistry. In comparison with 17 other human tissues and cell types by microarray, large adipocytes displayed by far the highest SAA and TM4SF1 expression. Thus, we have identified genes with markedly higher expression in large, compared with small, human adipocytes. These genes may link hypertrophic obesity to insulin resistance/type 2 diabetes.—Jernås, M., Palming, J., Sjöholm, K., Jennische, E., Svensson, P.-A., Gabriellson, B. G., Levin, M., Sjögren, A., Rudemo, M., Lystig, T. C., Carlsson, B., Carlsson, L. M. S., Lönn, M. Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression. *FASEB J.* 20, E832–E839 (2006)

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OBESITY-RELATED DISEASE IS the leading cause of death in the industrialized world. Abdominal obesity in particular increases the risk of several metabolic disorders, including type 2 diabetes and cardiovascular disease (1). Obesity and type 2 diabetes both have features of

acute-phase activation and low-grade inflammation (2, 3). Adipocytes produce a number of cytokines and other bioactive molecules, together termed adipokines (4). Some act predominantly in an autocrine or paracrine manner, while others are released into the systemic circulation and act as signaling molecules in other tissues. Therefore, the production and secretion of bioactive molecules by adipocytes may underlie many components of obesity-related disease.

The risk of metabolic complication is increased not only by the amount and location of adipose tissue, but also by the size of the fat cells. Human fat cells can change ~20-fold in diameter and several thousand-fold in volume. Enlargement of subcutaneous (s.c.) abdominal adipocytes is associated with insulin resistance and is an independent predictor of type 2 diabetes (5). Lipid mobilization and glucose metabolism are increased in enlarged adipocytes (6). In contrast, the stimulating effect of insulin on the rate of glucose metabolism is inversely related to the size of the fat cell (7, 8). Cytokine release within adipose tissue also appears to be correlated with adipocyte size (9–12), and hypertrophic adipocytes may contribute to lipotoxicity (13).

In most studies of the impact of adipocyte size, fat cells or biopsies with different mean adipocyte diameters were obtained from different tissue locations or even from different donors (6–10). Therefore, differences in environmental conditions or genetic factors that affect adipocyte gene expression and metabolism could not be excluded. Thus, it is not clear whether the functions of the fat cell vary with adipocyte size *per se*.

In 1972, a procedure was developed to separate human adipocytes of different sizes from the same adipose tissue sample by exploiting differences in the flotation rates of large and small fat cells. Analysis of small and large adipocytes obtained by this rather

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complicated procedure, which required vertical dialysis tubes several meters long, showed that triglyceride turnover increases with cell size (14). In studies of rat adipocytes from a single tissue sample, also separated by size using techniques based on cell buoyancy, various metabolic functions including leptin gene expression were dependent on cell size (15, 16).

The aim of the present study was to detect factors linking human adipocyte hypertrophy to insulin resistance/type 2 diabetes. We developed a simple and accurate procedure for separating human adipocytes from an adipose tissue sample into two populations: large cells and small cells. Using a computerized image-analysis technique that allows the assessment of 10-fold more cells than conventional methods (17), we characterized the size distribution of these populations in detail. Gene expression profiles were analyzed to identify genes differentially expressed in small and large adipocytes. Classification of the genes with markedly higher expression in large compared with small cells revealed that the majority were immune-related, with importance for cell structure, or with unknown function. These genes may connect hypertrophic obesity to metabolic disorders.

MATERIALS AND METHODS

Human adipose tissue samples

Subcutaneous abdominal adipose tissue from 12 subjects, 3 men and 9 women (2 postmenopausal), was obtained after an overnight fast. The subjects were 24–57 yr of age (mean 39.8 ± 3.1 yr) and had BMIs of 23.0–28.7 kg/m² (mean 25.4 ± 0.6 kg/m²). Surgical biopsies were taken from 8 patients undergoing abdominal surgery for nonmalignant conditions and from 1 healthy volunteer. Needle aspirations were obtained from 3 healthy volunteers. One patient had type 2 diabetes. The study protocol was approved by the Regional Ethical Review Board in Göteborg, and all participants gave written informed consent.

Adipocyte isolation and separation

The tissue (4–52 g) was cut into small pieces and treated with 1.05 mg/ml collagenase (Type A, Roche, Mannheim, Germany) in minimum essential medium (Invitrogen, Carlsbad, CA) containing 5.5 mM glucose, 25 mM HEPES, 4% bovine albumin (Fraction V, Sigma, St. Louis, MO), and 0.15 μ M adenosine, pH 7.4, for 60 min at 37°C as described (18). After filtration through a 250 μ m nylon mesh, the adipocytes were washed three times and suspended in fresh medium (cells from ~ 1 g tissue/45 ml medium) in 50-ml Falcon tubes. After gentle agitation of the suspension, cells that resurfaced within 30 s were transferred to new tubes; this procedure was repeated once. These more buoyant cells were then filtered with a 70 μ m nylon mesh and rinsed with fresh medium. Cells not passing through the mesh were resuspended in medium as the final preparation of large adipocytes. The denser cells that did not resurface within 30 s were filtered with a 50 μ m nylon mesh. Cells that passed through the mesh were considered the final preparation of small adipocytes. The medium and the adipocyte suspensions were maintained at 37°C.

Adipocyte size

The mean adipocyte size and the size distribution of the cell populations, before and after separation, were determined by computerized image analysis (KS400 software, Carl Zeiss, Oberkochen, Germany) (17). In brief, the cell suspension was placed between a siliconized glass slide and a coverslip and transferred to the microscope stage. Nine random visual fields were photographed with a CCD camera (Axiocam, Carl Zeiss, Oberkochen, Germany). The surface of the relevant areas was measured automatically, and the diameter of the corresponding circles was calculated. Uniform microspheres 98.00 μ m in diameter (Bangs Laboratories, Fishers, IN) were used as a reference. Because of technical problems, the adipocyte size of the cell populations in one of the 12 separations was determined by a conventional method (18).

RNA preparation

Total RNA was prepared with the phenol-chloroform extraction method of Chomczynski and Sacchi (19) and the RNeasy lipid tissue kit (Qiagen, Chatsworth, CA). After further purification with RNeasy clean-up columns, the RNA concentration was measured spectrophotometrically; the A_{260}/A_{280} ratio was 1.8–2.0. The quality of the RNA was verified by agarose gel electrophoresis before reverse transcription into cDNA.

Microarray analysis

cRNA was prepared and hybridized as recommended in the Affymetrix Gene Chip Expression Analysis manual. In brief, biotin-labeled target cRNA was prepared by *in vitro* transcription (Enzo Diagnostics, Farmingdale, NY) and hybridized to Human Genome U133A arrays (Affymetrix, Santa Clara, CA), composed of 22,283 probe sets representing $\sim 14,000$ expressed genes. Arrays were scanned with a confocal laser scanner (GeneArray scanner G2500A Hewlett Packard, Palo Alto, CA). Gene expression levels were calculated by the Robust Multiarray Average (RMA) method (20). To identify differences in gene expression between small and large adipocytes, Weighted Analysis of paired Microarray Experiments (WAME) was used (21), weighting samples according to precision in calculation of (geometric) signal means and *P* values for differential gene expression.

Tissue expression analysis

Gene expression in different human tissues and cell types was assessed with DNA microarray. Duplicate GeneChip HG-U133A expression profiles from 17 different tissues were downloaded from the SymAtlas dataset (22). The expression profile for each tissue was obtained by calculating the average signal value for each duplicate and gene, respectively. For comparison of gene expression in different tissues, the signal value for each gene was normalized by dividing the signal by the average signal of the entire array for each tissue. In addition, our own expression profiles, originating from small and large adipocytes, were included and normalized as outlined above.

RT-PCR analysis of gene expression

Reagents for real-time RT-PCR analysis of LDL receptor-related protein 10 (LRP10), TM4SF1 and leptin (Assays-on-Demand, TaqMan Reverse Transcriptase reagents, and Taq-

Man Universal PCR Master Mix) were from Applied Biosystems (Foster City, CA) and used according to the manufacturer's protocol. Since SAA1 and SAA2 have highly similar sequences and cannot be studied separately, probe and primer sequences for SAA (Cybergene, Huddinge, Sweden) were designed to span an exon-exon boundary to avoid genomic DNA amplification and to detect both isoforms (23) (sequences available on request). cDNA was synthesized from 500 ng of total RNA in a total reaction vol of 50 μ l. cDNA corresponding to 10 ng of RNA per reaction was used for real-time PCR amplification. Specific products were amplified and detected with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using default cycle parameters. A standard curve was plotted for each primer-probe set with a serial dilution of pooled adipocyte cDNA. Based on our previous report (24) and expression profiles in the present study, human LRP10 was used as reference to normalize the expression levels between samples. All standards and samples were analyzed in triplicate.

Immunohistochemistry

Adipocytes were fixed in 4% buffered formaldehyde, embedded in agar, dehydrated, embedded in paraffin, and cut into 5 μ m-thick sections. The sections were incubated first with monoclonal antibodies against SAA (HyCult Biotechnology) or TM4SF1 (L6 tumor antigen, Chemicon International). As secondary reagent for SAA, alkaline phosphatase antialkaline

phosphatase (Dako Cytomation, Glostrup, Denmark), followed by NBT/BCIP (Roche) as substrate was used. After counterstaining with Nuclear Fast Red, the sections were mounted in glycerol gelatin. For TM4SF1, peroxidase anti-peroxidase (Dako Cytomation, Glostrup, Denmark), followed by diaminobenzidine as substrate was used before mounting the sections in glycerol gelatin.

Statistical analysis

Values are expressed as means \pm SEM. Differences in gene expression between cell populations were analyzed with the Wilcoxon signed-rank test. Differences in adipocyte size distributions were analyzed with the Kolmogorov-Smirnov two-sample test (25). Relationships between gene expression and adipocyte size were analyzed with the Spearman rank correlation test and plotted with a robust regression technique, an M-estimator with Huber's psi-function (26). Bonferroni correction of *P* values from the microarray analysis was used to control for multiple comparisons.

RESULTS

Separation of small and large adipocytes

The mean size and the size distribution of adipocytes were determined by computerized image analysis before and after separation of isolated adipocytes into populations of small and large cells (Fig. 1A, B). In each population, 305–3660 cells (mean 1046 ± 135) were analyzed. The mean diameters of small and large adipocytes from 12 adipose tissue samples were $57.6 \pm 3.54 \mu$ m and $100.1 \pm 3.94 \mu$ m, respectively (Fig. 1C). The mean diameter of the reference microspheres was $97.91 \pm 0.089 \mu$ m (range 97.18–98.22 μ m), $n = 12$. The cell size distributions of the small and large populations differed significantly ($P < 0.001$).

Microarray analysis of gene expression in small and large adipocytes

To identify genes with increased expression in large adipocytes, we performed DNA microarray analyses of small and large cells from three biopsies. Fourteen genes were expressed at > 4 -fold higher levels in the large cells with *P* values that after Bonferroni correction (multiplication with 22,283) were < 0.01 (Table 1). Classification by cellular or organism function based on Gene Ontology definitions (<http://www.geneontology.org/>) revealed that five of those genes were immune-related. The remaining nine genes were referred to structure (four), unknown function (three), growth (one) and transport (one) (Table 1). Differences in sample preparation or hybridization were excluded since there was no difference between small and large adipocytes in the expression of LRP10, CLN3, or COBRA1, previously identified as suitable reference genes for studies of human adipose tissue (24).

One immune-related gene, SAA, and one gene with unknown function, TM4SF1, were selected among the genes with > 4 -fold higher expression in large vs. small

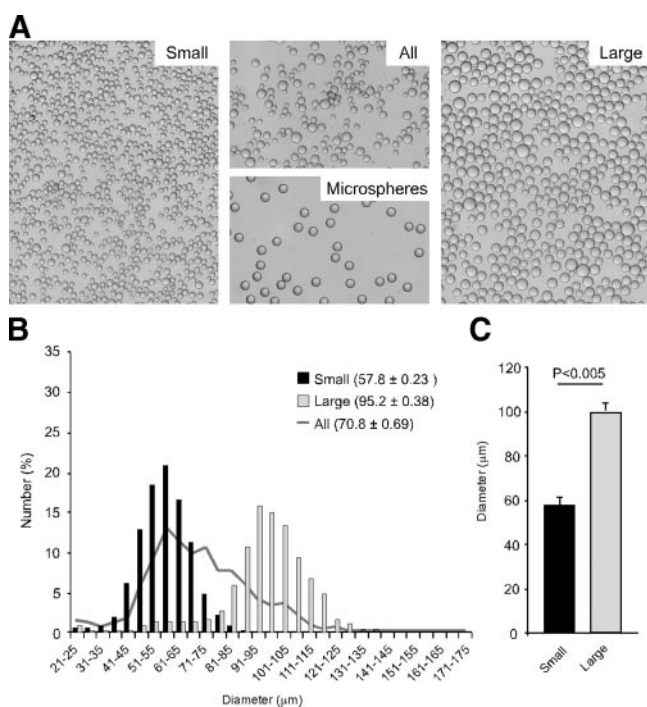


Figure 1. Separation of human adipocytes by size. A) Representative images of human adipocytes from one adipose tissue sample before separation (All) and after separation (Small and Large). Microspheres (98.00 μ m in diameter) were used for reference in the computerized image analysis. B) Size distributions, determined by computerized image analysis, and mean diameters in random samples of all adipocytes ($n=742$), small adipocytes ($n=1965$), and large adipocytes ($n=2382$) from one adipose tissue sample. Patient characteristics; man, age: 41 yr, body mass index (BMI): 26.0 kg/m^2 . C) Mean diameter of small and large adipocytes from 12 adipose tissue biopsies. Error bars indicate SEM.

TABLE 1. Genes detected with more than fourfold higher expression in large adipocytes than small adipocytes as analyzed by DNA microarray

Gene symbol	Gene name	ID	Classification	Fold change	P value	Mean signal small	Mean signal large
SELE	selectin E	206211_at	defense	15.2	1.29E-09	11.5	174.4
SPARCL1	SPARC-like 1	200795_at	unknown	14.9	1.22E-09	33.5	498.8
TM4SF1	transmembrane 4 L six family member 1	209386_at	unknown	11.6	4.46E-11	50.6	586.3
TM4SF1	transmembrane 4 L six family member 1	209387_s_at		9.6	4.19E-09	22.5	216.1
TM4SF1	transmembrane 4 L six family member 1	215034_s_at		9.2	2.29E-10	26.7	245.5
DCN	decorin	211896_s_at	structure	9.4	8.18E-09	59.3	554.7
DCN	decorin	201893_x_at		6.9	3.70E-09	156.8	1076.5
DCN	decorin	211813_x_at		5.6	1.90E-09	118.4	659.1
IL8	interleukin 8	202859_x_at	defense	9.0	4.44E-09	186.8	1682.5
IL8	interleukin 8	211506_s_at		7.5	3.54E-07	55.5	415.2
PALLD	palladin	200897_s_at	structure	8.7	2.53E-09	24.6	213.8
SAA2	serum amyloid A2	208607_s_at	defense	7.8	3.82E-08	245.7	1918.5
SAA2	serum amyloid A2	214456_x_at		7.6	2.24E-07	529.1	4009.6
CLEC3B	C-type lectin domain family 3, member B	205200_at	growth	6.3	8.22E-09	55.8	353.5
CIQR1	complement component 1, q subcomponent, receptor 1	202878_s_at	defense	6.1	9.40E-10	30.0	183.9
COL1A1	collagen, type I, alpha 1	202310_s_at	structure	5.9	2.27E-08	26.2	155.6
CXCL2	chemokine (C-X-C motif) ligand 2	209774_x_at	defense	5.4	8.09E-09	184.4	998.2
COL1A2	collagen, type I, alpha 2	202403_s_at	structure	5.3	2.44E-07	57.1	300.9
FLJ14054	—	219054_at	unknown	4.2	4.65E-09	65.2	273.6
AQP1	aquaporin 1	209047_at	transport	4.2	5.90E-09	43.2	179.7

cells for further analysis (Table 1). SAA, a risk factor for cardiovascular disease and in focus in one of our previous studies (27, 23), was expressed at ~8-fold higher levels in large cells than in small cells. TM4SF1, a highly expressed surface protein of various carcinomas and possibly implicated in signal transduction events mediating cell proliferation and activation (28, 29) was 10-fold higher expressed in the large adipocytes (Table 1). Leptin, being 3-fold higher expressed in the large adipocytes (data not shown), and previously identified with higher expression in large compared with small rat adipocytes (15, 16), was also included in the following studies.

Expression of SAA, TM4SF1 and leptin determined by real-time RT-PCR

The up-regulation of SAA, TM4SF1 and leptin expression in large cells was confirmed by RT-PCR analysis of small (mean $59.3 \pm 4.47 \mu\text{m}$) and large (mean $97.1 \pm 5.69 \mu\text{m}$) adipocytes from seven different adipose tissue samples. In all cases, SAA, TM4SF1 and leptin were expressed at higher levels in large cells ($P=0.018$) (Fig. 2A, C, E). The mean fold increase in expression was 18.7 ± 15.1 for SAA, 22.3 ± 6.4 for TM4SF1, and 3.9 ± 1.4 for leptin. In addition, adipocyte size correlated with the expression of SAA ($P=0.015$), TM4SF1 ($P=0.012$), and leptin ($P=0.0009$) (Fig. 2B, D and F).

Tissue expression analysis

SAA, TM4SF1 and leptin expression levels in large adipocytes were compared to the levels in other human tissues (22) and small adipocytes. The three genes were

expressed at markedly higher levels in large adipocytes than in all other tissues/cell types (Fig. 3A, B C).

Immunoreactivity

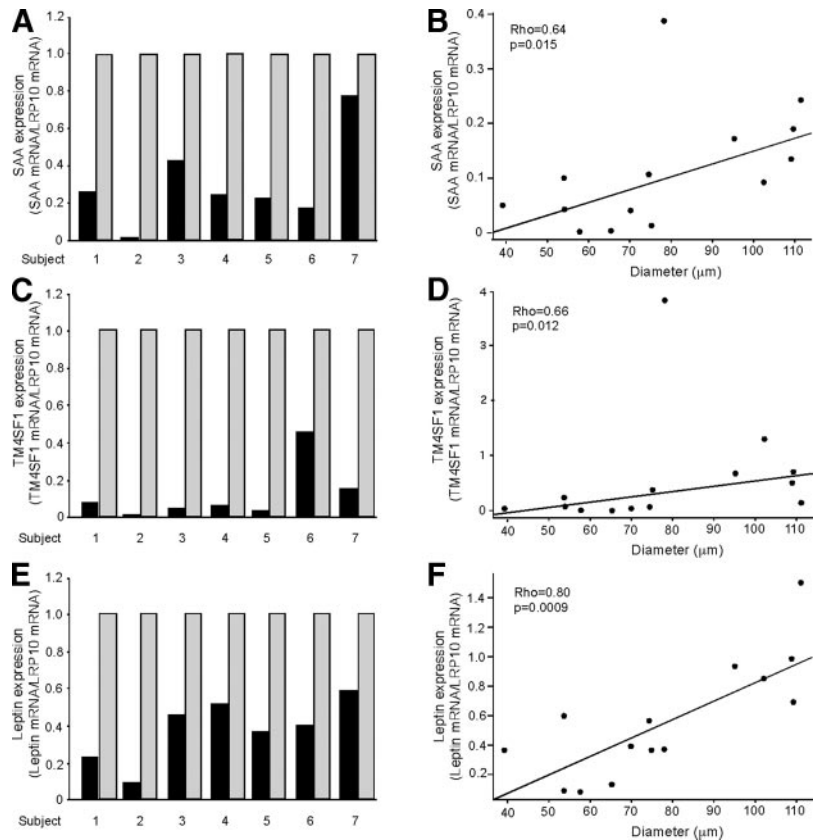
Expression of SAA and TM4SF1 in adipocytes was also demonstrated immunohistochemically. Although SAA immunoreactivity varied between cells of the same size, it was generally greater in large than in small adipocytes in the same sample (Fig. 4, upper). TM4SF1 immunoreactivity was demonstrated mainly in large but to some extent also in medium-sized adipocytes. Positive TM4SF1 signal appeared in a dot-like pattern in the cell membrane. Small adipocytes were completely without TM4SF1 immunoreactivity (Fig. 4, lower).

DISCUSSION

In this study, we developed a new technique to separate populations of small and large human adipocytes from a single adipose tissue sample. The two populations of cells obtained with our technique differed significantly in size, as determined with a computer-based image-analysis method that allows rapid analysis of 10-fold more cells than conventional methods (17). DNA microarray analysis of the two populations showed that several genes were expressed at markedly higher levels in the large cells, demonstrating that hypertrophy *per se* can significantly alter gene expression and thereby presumably adipocyte function.

Previous studies of the metabolic activity of small and large adipocytes have indicated that adipocyte size influences several metabolic functions (6–10). How-

Figure 2. Expression of serum amyloid A (SAA), transmembrane 4 L six family member 1 (TM4SF1), and leptin in small adipocytes (solid bars) and large adipocytes (shaded bars) isolated from adipose tissue samples from seven subjects, measured by RT-PCR. The expression of SAA, TM4SF1, and leptin in the large cells from each sample was set to 1.0. In all cases, SAA (A), TM4SF1 (C), and leptin (E) were expressed at higher levels in the large cells. Robust regression and rank correlation for SAA expression and adipocyte size (B), for TM4SF1 expression and adipocyte size (D), and for leptin expression and adipocyte size (F). Rho; Spearman's correlation coefficient.



ever, the cells of different sizes were obtained from different adipose tissue locations or from different donors. Thus, it was not possible to exclude environmental influences, such as nutritional/hormonal conditions, or genetic factors that might have affected gene expression and thus adipocyte metabolism. Our technique avoids these problems and will facilitate metabolic studies of fat cells of different sizes. For example, a positive correlation between human adipocyte size and leptin expression/secretion has previously been suggested after analysis of adipocytes from two depots: omental fat cells and twice as large s.c. fat cells (9). In the present study of human adipocytes from a single adipose tissue sample, the previous findings were confirmed since leptin was indeed expressed at higher levels in the large cells in all cases. Moreover, we identified several genes that, compared with leptin, showed a more pronounced differential expression in large vs. small adipocytes.

Among the fourteen genes with markedly higher expression in large compared with small adipocytes, five were classified as immune-related; E-selectin, interleukin (IL)-8, SAA, C1q receptor 1, and CXCL2 also known as MIP-2 or macrophage inflammatory protein-2. Components of the metabolic syndrome, such as obesity and type 2 diabetes, are associated with a systemic increase in inflammatory markers (30–32). The acute-phase proteins SAA and C-reactive protein have attracted particular attention because they are independent risk factors for coronary artery disease (27, 33, 34). We (23), and others (35), have shown that adipose tissue is a major site of SAA production and is

likely to be a major source of circulating SAA in obese patients. Moreover, serum SAA concentrations were correlated to fasting insulin levels indicating a link to insulin resistance (23). The current study extends previous findings by demonstrating that SAA is expressed at the highest concentration by the large adipocytes. SAA has been implicated in inflammation, insulin resistance and impairment of reverse cholesterol transport. Our data may therefore suggest that adipocyte-derived SAA, likely having both local effects and endocrine functions, is a potential mediator of the link between hypertrophic adipocytes and type 2 diabetes.

Further support for an association between obesity and inflammation comes from studies showing macrophage infiltration of adipose tissue (36). Moreover, the proportion of cells expressing CD68, a macrophage marker, increases with increasing average adipocyte area in human s.c. adipose tissue (37). This relationship between adipocyte size and macrophage accumulation suggests that hypertrophic adipocytes secrete factors that attract monocytes. Again, SAA derived from hypertrophic adipocytes may be involved in this process because SAA activates the chemotactic formyl peptide receptor like-1, which results in migration of blood monocytes and neutrophils (38). IL-8, also found to be expressed at high levels in hypertrophic adipocytes, may act as an other potential monocyte recruiting factor in adipose tissue (39). Accumulation of macrophages in adipose tissue is likely to further increase the levels of inflammatory cytokines in adipose tissue and thereby increase insulin resistance.

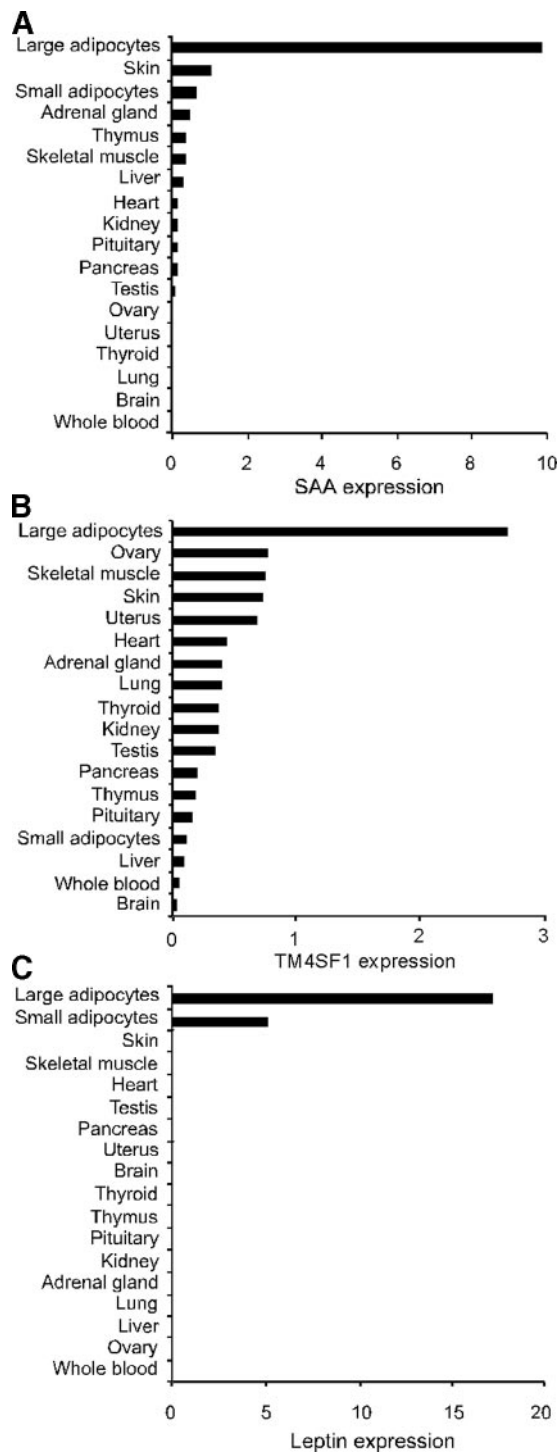


Figure 3. Expression of SAA (A), TM4SF1 (B), and leptin (C) in large adipocytes and other human tissues/cell types, measured by microarray analysis. The relative expression levels of SAA, TM4SF1, and leptin in each tissue were determined as described in Materials and Methods.

SAA in the circulation is primarily associated with HDL and may modulate its role in cholesterol transport (40). The presence of SAA on HDL has been reported both to promote (41), and to reduce (42), cholesterol efflux to HDL suggesting that SAA may alter cholesterol removal from cells. Moreover, SAA reduces cellular uptake of cholesterol from HDL by inhibiting the

HDL receptor, SR-BI (scavenger receptor class B type I) (43). Such an effect would be expected to impair reverse cholesterol transport—the transfer of cholesterol from peripheral tissues (vascular wall) to the liver for excretion. Non-HDL-bound SAA has been reported to promote cholesterol efflux from cells (44).

Recent studies suggest an intricate relationship between adipocyte size, cholesterol concentration and insulin sensitivity (45). Cholesterol accumulates within the adipocyte lipid droplet proportionally to the triglyceride content. However, within an adipose tissue sample, large adipocytes have reduced membrane cholesterol concentrations compared with small fat cells, demonstrating that a changed cholesterol distribution is characteristic of adipocyte hypertrophy. By reducing adipocyte plasma membrane cholesterol it is possible to reproduce part of the defects seen in hypertrophic adipocytes such as insulin resistance (45). SAA may impair adipocyte uptake of HDL cholesterol by the same mechanisms described above for reverse cholesterol transport. Furthermore, locally produced lipid-

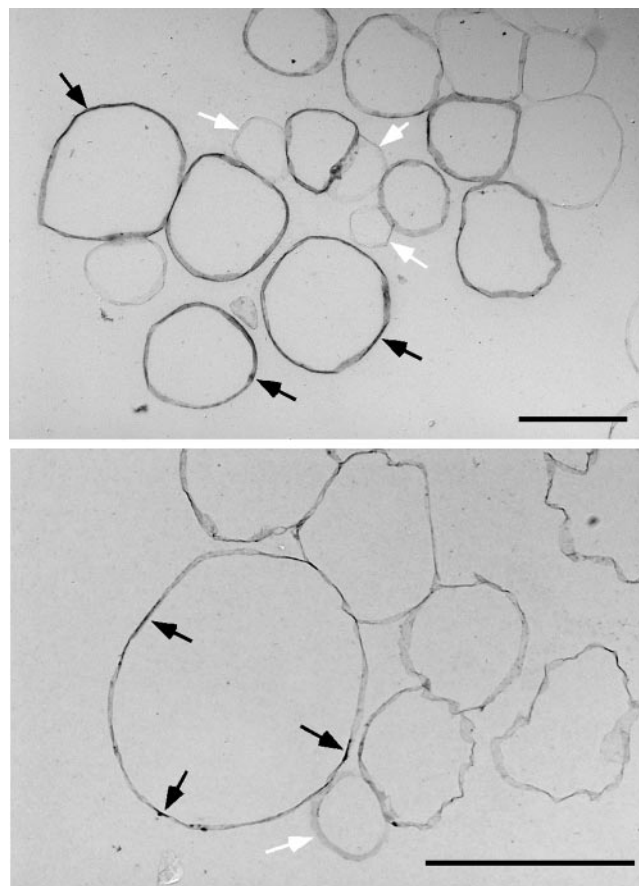


Figure 4. SAA immunoreactivity of human adipocytes in a paraffin section. Positive signal appears dark gray. Black arrows indicate large adipocytes with strong SAA signal; white arrows indicate small adipocytes with weak SAA immunoreactivity that are barely visible. Scale Bar = 100 μ m. (top). TM4SF1 immunoreactivity of human adipocytes in a paraffin section. Positive signal appears dark gray and in a dotlike pattern as indicated by black arrows. White arrow indicates small adipocyte completely without immunoreactivity. Scale Bar = 100 μ m. (bottom).

free SAA may reduce adipocyte levels of cholesterol (44). Our findings may therefore suggest that the insulin resistance in hypertrophic adipocytes could be related to the high expression of SAA in these cells. SAA has also been shown to bind to cell-surface receptors, including Tanis/Sels, that have been implicated in regulation of insulin sensitivity (46). Thus, SAA may influence insulin sensitivity in adipose tissue by several mechanisms, including interactions with receptors, recruitment of inflammatory cells and local impairment of cholesterol metabolism.

The significance of the increased expression of TM4SF1 in large adipocytes remains to be elucidated. TM4SF1 shares topological features with members of the tetraspanin superfamily (29). However, recent analysis suggests that TM4SF1 belongs to a new transmembrane-4 superfamily comprising TM4SF1, TM4SF5, IL-TMP and L6D (28). The biological properties of this superfamily remain largely unknown while the activities of tetraspanin proteins often are attributed to their association with integrins and specific surface proteins (29). We have previously identified TM4SF1 as one of the genes with higher expression in visceral compared to s.c. adipose tissue in obese men (47). Moreover, genomic scans have identified loci linked to fasting plasma insulin levels in Pima Indians (48) and BMI in white, black, Asian and Mexican-American ethnic groups (49) in the chromosomal region harboring the TM4SF1 gene. We have analyzed a publicly available DNA microarray dataset originating from isolated abdominal s.c. adipocytes from obese and nonobese Pima Indians (50). In comparison with the lean group, the obese group had higher fasting glucose and fasting insulin levels and higher 2-h insulin levels after an oral glucose tolerance test. Interestingly, the TM4SF1 expression was significantly higher in adipocytes from the obese subjects compared with adipocytes from the nonobese subjects (50). Furthermore, in a recent study, TM4SF1, together with several genes involved in inflammatory processes in the brain, was found to be up-regulated in C/EBP β -overexpressing neuronal cells (51). Thus, a potential role of TM4SF1 in immunological processes in human adipose tissue, and in subsequent development of metabolic disturbances, may be speculated.

To summarize, we have developed a technique to separate human adipocytes, from a single adipose tissue sample, by size. The resulting populations of small and large adipocytes have significantly different cell size distributions. Gene expression profiling of the small and large adipocytes identified genes, many of them immune-related, with markedly higher mRNA expression in the large cells. Two of those genes, SAA (an acute-phase protein implicated in inflammation, insulin resistance and impairment of reverse cholesterol transport) and TM4SF1 (a membrane protein with unknown function), were \sim 20-fold higher expressed in the large cells, a difference reflected also at the protein level. Moreover, in comparison with several other human tissues, large adipocytes displayed by far the high-

est SAA and TM4SF1 expression. In the light of previous studies reporting that adipocyte hypertrophy is associated with insulin resistance and is an independent predictor of type 2 diabetes, the findings in the current work provide novel insights into the molecular connection between hypertrophic obesity and insulin resistance/type 2 diabetes. FJ

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Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression

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SPECIFIC AIMS

Enlargement of subcutaneous (s.c.) abdominal adipocytes is associated with insulin resistance and is an independent predictor of type 2 diabetes. The aim of the present study was to detect factors linking human adipocyte hypertrophy to insulin resistance/type 2 diabetes.

PRINCIPAL FINDINGS

1. Isolated human adipocytes, from a single adipose tissue sample, can be separated into populations of small cells and large cells

Most previous studies of the impact of adipocyte size, have studied fat cells or biopsies with different mean adipocyte diameters obtained from different tissue locations or even from different donors. Therefore, differences in environmental conditions or genetic factors that affect adipocyte gene expression and metabolism could not be excluded. Thus, it is not clear whether the functions of the fat cell vary with adipocyte size *per se*.

In the present study, a technique for separation of human adipocytes by size was developed. The technique, based on cell buoyancy and mesh filtration, separated isolated adipocytes from an adipose tissue sample into populations of small cells (mean 57.6 ± 3.54 μm) and large cells (mean 100.1 ± 3.94 μm). The mean size and the size distribution of the small and large populations, determined by computerized image analysis, differed significantly ($P < 0.005$ and $P < 0.001$, respectively) (Fig. 1).

2. Microarray analysis of the cell populations identified genes with markedly higher mRNA expression in large cells than small cells

Gene expression profiling (Affymetrix GeneChip HG-U133A arrays composed of 22,283 probe sets) of the

cell populations separated from adipose tissue from three subjects identified 14 genes with more than 4-fold higher expression in large cells than small cells ($P < 0.01$) (Table 1). Classification by cellular or organism function based on Gene Ontology definitions revealed that five of those genes were immune-related. The remaining nine genes were referred to structure (four), unknown function (three), growth (one) and transport (one) (Table 1). Differences in sample preparation or hybridization were excluded since there was no difference between small and large adipocytes in the expression of LRP10, CLN3, or COBRA1, suitable reference genes for studies of human adipose tissue.

3. SAA and TM4SF1 were ~20-fold higher expressed in the large adipocytes as determined by real-time RT-PCR, and adipocyte size correlated with the expression of both SAA and TM4SF1

One immune-related gene, serum amyloid A (SAA), and one gene with unknown function, transmembrane 4 L six family member 1 (TM4SF1), were selected among the genes with >4-fold higher expression in large vs. small cells for further analysis (Table 1). Leptin, being 3-fold higher expressed in the large adipocytes (data not shown), and previously suggested to be higher expressed in large compared with small adipocytes, was also included in the following studies.

The up-regulation of SAA, TM4SF1 and leptin expression in large cells was confirmed by real-time RT-PCR chain reaction analysis of small (mean 59.3 ± 4.47 μm) and large (mean 97.1 ± 5.69 μm) adipocytes from seven different adipose tissue samples. In all cases, SAA, TM4SF1 and leptin were expressed at

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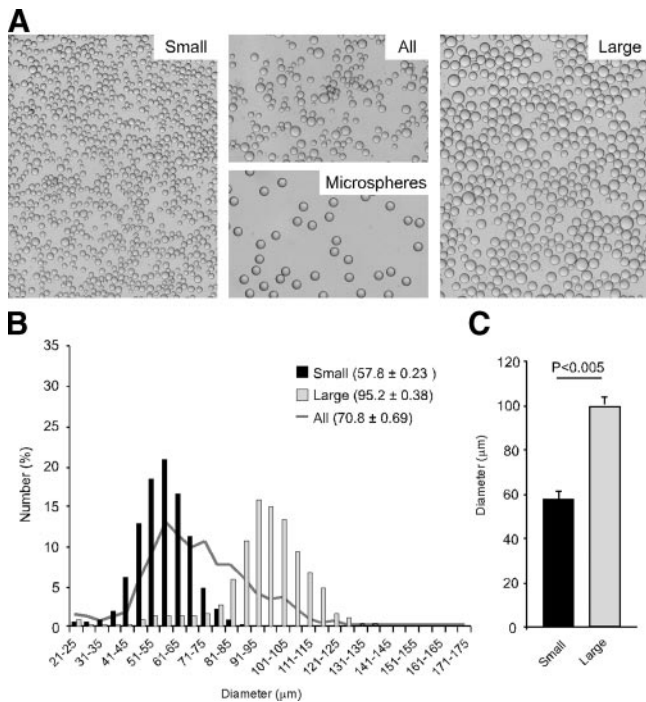


Figure 1. Separation of human adipocytes by size. *A*) Representative images of human adipocytes from one adipose tissue sample before separation (All) and after separation (Small and Large). Microspheres (98.00 μm in diameter) were used for reference in the computerized image analysis. *B*) Size distributions, determined by computerized image analysis, and mean diameters in random samples of all adipocytes ($n=742$), small adipocytes ($n=1965$), and large adipocytes ($n=2382$) from one adipose tissue sample. Patient characteristics; man, age: 41 yr, body mass index (BMI): 26.0 kg/m^2 . *C*) Mean diameter of small and large adipocytes from 12 adipose tissue biopsies. Error bars indicate SEM.

higher levels in large cells ($P=0.018$). The mean fold increase in expression was 18.7 ± 15.1 for SAA, 22.3 ± 6.4 for TM4SF1, and 3.9 ± 1.4 for leptin. In addition, adipocyte size correlated with the expression of SAA ($P=0.015$), TM4SF1 ($P=0.012$), and leptin ($P=0.0009$).

4. In comparison with 17 other human tissues/cell types by microarray, large adipocytes displayed by far the highest SAA and TM4SF1 expression

GeneChip HG-U133A expression profiles from 17 different tissues were downloaded from the SymAtlas dataset (<http://symatlas.gnf.org/Symatlas/>). For comparison of gene expression in different tissues, the signal value for each gene was normalized by dividing the signal by the average signal of the entire array for each tissue. In addition, our own expression profiles, originating from small and large adipocytes, were included and normalized as outlined above. SAA, TM4SF1 and leptin expression levels in large adipocytes were compared to the levels in other human tissues and small adipocytes. The three genes were expressed at markedly higher levels in large adipocytes than in all other tissues/cell types.

5. The higher mRNA expression of SAA and TM4SF1 in large compared to small adipocytes was reflected also at the protein level

Expression of SAA and TM4SF1 in adipocytes was also demonstrated immunohistochemically. Although SAA immunoreactivity varied between cells of the same size, it was generally greater in large than in small adipocytes in the same sample. TM4SF1 immunoreactivity was demonstrated mainly in large but to some extent also in medium-sized adipocytes. Positive TM4SF1 signal appeared in a dot-like pattern in the cell membrane. Small adipocytes were completely without TM4SF1 immunoreactivity.

CONCLUSIONS AND SIGNIFICANCE

In this study, we developed a new technique to separate populations of small and large human adipocytes from a single adipose tissue sample. The two populations of cells obtained with our technique differed significantly in size, as determined with a computer-based image-analysis method that allows rapid analysis of 10-fold more cells than conventional methods. DNA microarray analysis of the two populations showed that several genes were expressed at markedly higher levels in the large cells, demonstrating that hypertrophy per se can significantly alter gene expression and thereby presumably adipocyte function (Fig. 2).

Previous studies of the metabolic activity of small and large adipocytes have indicated that adipocyte size influences various adipocyte metabolic functions. However, the cells of different sizes were obtained from different adipose tissue locations or from different donors. Thus, it was not possible to exclude environmental influences, such as nutritional/hormonal conditions, or genetic factors that might have affected gene expression and thus adipocyte metabolism. Our technique avoids these problems and will facilitate metabolic studies of fat cells of different sizes. For example, a positive correlation between human adipocyte size and leptin expression/secretion has previously been suggested. In the present study of human adipocytes from a single adipose tissue sample, the previous findings were confirmed since leptin was indeed expressed at higher levels in the large cells in all cases. Moreover, we identified several genes that, compared with leptin, showed a more pronounced differential expression in large vs. small adipocytes (Fig. 2).

Among the fourteen genes with markedly higher expression in large compared with small adipocytes, five were classified as immune-related; E-selectin, interleukin-8, SAA, C1q receptor 1, and CXCL2 also known as MIP-2 or macrophage inflammatory protein-2. Components of the metabolic syndrome, such as obesity and type 2 diabetes, are associated with a systemic increase in inflammatory markers. The acute-phase proteins SAA and C-reactive protein have attracted particular attention because they are independent risk factors for

TABLE 1. Genes detected with more than fourfold higher expression in large adipocytes than small adipocytes as analyzed by DNA microarray

Gene symbol	Gene name	ID	Classification	Fold change	P value	Mean signal small	Mean signal large
SELE	selectin E	206211_at	defense	15.2	1.29E-09	11.5	174.4
SPARCL1	SPARC-like 1	200795_at	unknown	14.9	1.22E-09	33.5	498.8
TM4SF1	transmembrane 4 L six family member 1	209386_at	unknown	11.6	4.46E-11	50.6	586.3
TM4SF1	transmembrane 4 L six family member 1	209387_s_at		9.6	4.19E-09	22.5	216.1
TM4SF1	transmembrane 4 L six family member 1	215034_s_at		9.2	2.29E-10	26.7	245.5
DCN	decorin	211896_s_at	structure	9.4	8.18E-09	59.3	554.7
DCN	decorin	201893_x_at		6.9	3.70E-09	156.8	1076.5
DCN	decorin	211813_x_at		5.6	1.90E-09	118.4	659.1
IL8	interleukin 8	202859_x_at	defense	9.0	4.44E-09	186.8	1682.5
IL8	interleukin 8	211506_s_at		7.5	3.54E-07	55.5	415.2
PALLD	palladin	200897_s_at	structure	8.7	2.53E-09	24.6	213.8
SAA2	serum amyloid A2	208607_s_at	defense	7.8	3.82E-08	245.7	1918.5
SAA2	serum amyloid A2	214456_x_at		7.6	2.24E-07	529.1	4009.6
CLEC3B	C-type lectin domain family 3, member B	205200_at	growth	6.3	8.22E-09	55.8	353.5
CIQR1	complement component 1, q subcomponent, receptor 1	202878_s_at	defense	6.1	9.40E-10	30.0	183.9
COL1A1	collagen, type I, alpha 1	202310_s_at	structure	5.9	2.27E-08	26.2	155.6
CXCL2	chemokine (C-X-C motif) ligand 2	209774_x_at	defense	5.4	8.09E-09	184.4	998.2
COL1A2	collagen, type I, alpha 2	202403_s_at	structure	5.3	2.44E-07	57.1	300.9
FLJ14054	—	219054_at	unknown	4.2	4.65E-09	65.2	273.6
AQP1	aquaporin 1	209047_at	transport	4.2	5.90E-09	43.2	179.7

coronary artery disease. We, and others, have previously shown that adipose tissue is a major site of SAA production and is likely to be a major source of circulating SAA in obese patients. The current study extends these findings by demonstrating that SAA is expressed at the highest concentration by the large

adipocytes. SAA has been implicated in inflammation, insulin resistance and impairment of reverse cholesterol transport. Our data may therefore suggest that adipocyte-derived SAA, likely having both local effects and endocrine functions, is a potential mediator of the link between hypertrophic adipocytes and type 2 diabetes (Fig. 2).

To summarize, we have developed a technique to separate human adipocytes, from a single adipose tissue sample, by size. The resulting populations of small and large adipocytes have significantly different cell size distributions. Gene expression profiling of the small and large adipocytes identified genes, many of them immune-related, with markedly higher mRNA expression in the large cells. Two of those genes, SAA (an acute-phase protein implicated in inflammation, insulin resistance and impairment of reverse cholesterol transport) and TM4SF1 (a membrane protein with unknown function), were ~20-fold higher expressed in the large cells, a difference reflected also at the protein level. Moreover, in comparison with several other human tissues, large adipocytes displayed by far the highest SAA and TM4SF1 expression. In the light of previous studies reporting that adipocyte hypertrophy is associated with insulin resistance and is an independent predictor of type 2 diabetes, the findings in the current work provide novel insights into the molecular connection between hypertrophic obesity and insulin resistance/type 2 diabetes. [E]

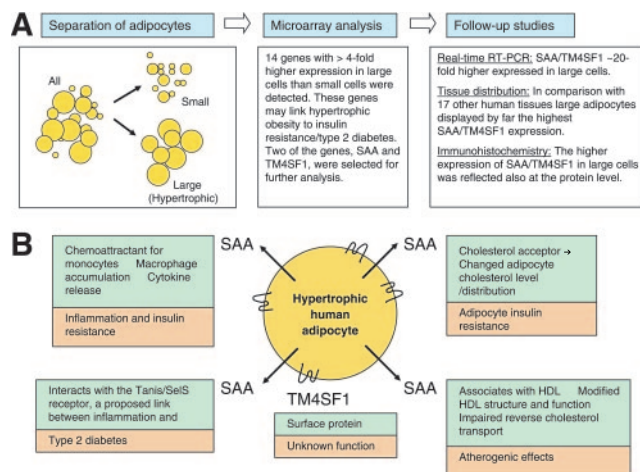


Figure 2. Schematic diagram. Flow chart and summary of the principal findings (A). Potential mechanisms by which serum amyloid A (SAA) and transmembrane 4 L six family member 1 (TM4SF1), expressed at the highest levels by large adipocytes, may mediate a link between hypertrophic obesity and insulin resistance/type 2 diabetes (B).