Needle and surgical biopsy techniques differentially affect adipose tissue gene expression profiles1–3

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ABSTRACT

Background: Adipose tissue gene expression analysis in humans now provides a tremendous means to discover the physiopathologic gene targets critical for our understanding and treatment of obesity. Clinical studies are emerging in which adipose gene expression has been examined in hundreds of subjects, and it will be fundamentally important that these studies can be compared so that a common consensus can be reached and new therapeutic targets for obesity proposed.

Objective: We studied the effect of the biopsy sampling methods (needle-aspirated and surgical) used in clinical investigation programs on the functional interpretation of adipose tissue gene expression profiles.

Design: A comparative microarray analysis of the different subcutaneous adipose tissue sampling methods was performed in age-matched lean (n = 19) and obese (n = 18) female subjects. Appropriate statistical (principal components analysis) and bioinformatic (FunNet) functional enrichment software were used to evaluate data. The morphology of adipose tissue samples obtained by needle-aspiration and surgical methods was examined by immunohistochemistry.

Results: Biopsy techniques influence the gene expression underlying the biological themes currently discussed in obesity (eg, inflammation, extracellular matrix, and metabolism). Immunohistochemistry experiments showed that the easier to obtain needle-aspirated biopsies poorly aspirate the fibrotic fraction of subcutaneous adipose tissue, resulting in an underrepresentation of the stroma-vascular fraction.

Conclusions: The adipose tissue biopsy technique is an important caveat to consider when designing, interpreting, and, most important, comparing microarray experiments. These results will have crucial implications for the clinical and physiopathologic understanding of human obesity and therapeutic approaches. Am J Clin Nutr 2009;89:51–7.

INTRODUCTION

White adipose tissue (AT) is a critical player in obesity-related metabolic functions (1). Indeed, AT is no longer considered to be simply a passive lipid reservoir but rather an endocrine organ capable of secreting factors that profoundly influence processes such as feeding behavior, energy flux, and immunoinflammation. As such, obtaining AT samples is paramount to the understanding of the pathophysiology of human obesity (2, 3). Much of our recent understanding about the role of AT in human obesity is a consequence of using modern molecular biology tools, such as the microarray (4, 5). More specifically, evidence indicates that various inflammatory pathways are activated in obese subcutaneous AT (scAT) and that these same pathways are downstream-regulated after weight loss (1, 6–9). The described transcriptional profiles in AT are concomitant with variations in immune cell types whose phenotypes and roles are not yet fully elucidated (7, 10–13). Recently, it was postulated that the significant up-regulation of constituents of the extracellular matrix, most notably the enhanced presence of interstitial regions of fibrosis infiltrated with different types of inflammatory cells, may serve to mediate the interactions between local inflammatory events and perturbed AT metabolic functions (7).

Although this “fibrotic link” between inflammation and metabolism requires further exploration, evidence that metabolic pathways in obesity are unbalanced was recently reinforced. Pietiläinen et al. (1) studied rare monozygotic twin pairs discordant for body weight and provided further evidence of mitochondrial dysfunction in obesity, as shown by the down-regulation of pathways related to branch chain amino acid catabolism, fatty acid synthesis, and β-oxidation in scAT. Taken together, the global analysis of gene expression has shown the pathologic perturbations of AT biology in obese states and the important contribution of AT to obesity-related complications. A rapidly growing number of studies are now designed to identify new candidate genes and related gene products based on

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AT gene expression signatures from humans varying in corpulence or whose weight fluctuates in response to environmental stimuli (ie, by modifying diet, physical activity, medical treatment, etc). The accessibility of human AT samples for gene expression analyses has been notably facilitated by the development of obesity surgery programs, thereby enabling laboratories to establish AT bio-banks for large-scale studies. Progress in the analytic platforms, experimental protocols, and analysis of microarrays has seen this technology take significant steps from the bench toward the bedside (5, 14). Multiple examples now exist in which researchers have used this tool to classify and predict diseases and to assess the interindividual variations in response to environmental stresses (15–19). Although many biological and technical limitations still exist (eg, interindividual differences, the quality and quantity of tissue biopsy samples, and analytic sensitivity), steps are being taken to overcome these obstacles to obtain the most relevant and physiologically “accurate” information possible. Indeed, this accuracy can only be achieved if the quality and authenticity of the biological material studied is assured. It is currently unknown whether the different biopsy methods used to obtain AT samples in humans may affect microarray interpretation.

The present study was designed to explore the effect of different scAT biopsy methods on the functional interpretation of gene expression data. The knowledge revealed by this comparison of sampling methods will serve as a caveat for the experimental design of future microarray work in human obesity and will lead to a dramatic improvement in the reproducibility of results and conclusions.

SUBJECTS AND METHODS

Subjects and AT samples

The clinical investigations were performed according to the Declaration of Helsinki and were approved by the ethics committees of Hôtel-Dieu Hospital (Paris, France). Informed consent was obtained from all subjects. All tissue biopsies were obtained in the morning from subjects after an overnight fast. Eighteen obese and 19 lean women were prospectively recruited in 2007 for the present study (Table 1). Abdominal subcutaneous fat specimens were obtained by needle aspiration in the periumbilical area under local anesthesia (1% xylocaine), from a comparable site of the abdomen. A region 5 cm lateral from the umbilicus (either to the left or right side of the abdomen) was sterilized. A needle (BD Microlance Hypodermic Needle, 16 G, 40 mm, regular bevel, part no. 300637; Becton Dickinson France SAS, Le Pont-De-Clai, France) was then adapted to a 20-mL syringe and the piston compressed. Approximately one-third of the length of the needle was inserted into the subcutaneous fat, and the needle piston was released maximally until it was locked by a stopper, thereby creating a vacuum. Tissue resistance was created by the surgeon gripping the abdominal wall with one hand while the other hand rotated the needle throughout the tissue in an up-down motion. Once the tissue was aspirated by the syringe, the needle was withdrawn, and the piston was removed; AT samples were washed in physiologic serum, placed immediately in liquid nitrogen, and stored at −80°C until analysis. Needle-aspirated biopsies that appeared bloody were not used for the present comparative analysis.

Surgical biopsy

This technique has been standardized in accordance with previous work performed within the European Framework 5 project NUGENOB (http://www.nugenob.com/). Superficial subcutaneous AT samples of ~1–2 cm³ (corresponding to ~1–2 g) were obtained from the periumbilical area, under local anesthesia (1% xylocaine), from a comparable site of the abdomen. A region 5 cm lateral from the umbilicus (either to the left or right side of the abdomen) was sterilized. A needle (BD Microlance Hypodermic Needle, 16 G, 40 mm, regular bevel, part no. 300637; Becton Dickinson France SAS, Le Pont-De-Clai, France) was then adapted to a 20-mL syringe and the piston compressed. Approximately one-third of the length of the needle was inserted into the subcutaneous fat, and the needle piston was released maximally until it was locked by a stopper, thereby creating a vacuum. Tissue resistance was created by the surgeon gripping the abdominal wall with one hand while the other hand rotated the needle throughout the tissue in an up-down motion. Once the tissue was aspirated by the syringe, the needle was withdrawn, and the piston was removed; AT samples were washed in physiologic serum, placed immediately in liquid nitrogen, and stored at −80°C until analysis. Needle-aspirated biopsies that appeared bloody were not used for the present comparative analysis.

Sample preparation and microarray analysis

Total RNA was extracted from biopsies with the use of the RNeasy total RNA Mini kit (Qiagen, Courtaboef, France). Both needle and surgical biopsy samples were directly homogenized in RLT buffer with the use of a VDI 25 adaptable homogenizer (VWR, Fontenay-sous-Bois, France). Total RNA concentration and quality were confirmed with the use of the Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France). Total RNA (200 ng) from each sample was amplified and transcribed into
fluorescent cRNA with the use of Agilent’s Low RNA Input Linear Amplification kit (Agilent Technologies). Cyanine-5 dye was incorporated into all samples, and an in-house obese reference pool was labeled with cyanine-3 dye. In brief, the in-house reference pool was created by mixing equal amounts of total RNA extracted from AT samples of subjects undergoing plastic surgery, as previously described (20). All samples were hybridized to Agilent whole human genome microarrays. These microarrays comprise >41,000 unique 60-mer oligonucleotide human sequences and transcripts. Sample preparation, hybridization, and microarray washing were performed according to the manufacturer’s recommendations (Agilent Technologies). Arrays were scanned with the use of a GenePix 4000A Scanner (Axon Instruments-Molecular Devices, Sunnyvale, CA).

AT morphology

Both needle-aspirated and surgical subcutaneous AT biopsies were obtained from the periumbilical area of subjects undergoing gastric surgery. Samples were fixed overnight at 4°C in 4% paraformaldehyde and then embedded in paraffin. Thin sections (~5 μm thickness) were stained with hematoxylin-eosin and examined with a Zeiss 20 Axiosstar Plus microscope (Zeiss, Jena, Germany). Digital images were captured with a Sony triCCD camera (Sony, Paris, France). Fibrotic regions were identified by incubating tissue sections in 0.2% phosphomolybdic acid, 0.1% picrosirius red (direct red 80 in saturated picric acid), and 0.01 N HCl and then dried and mounted in Permount (21).

Immunohistochemistry

Immunohistochemical detection of HAM56 (Dako Cytomation, Trappes, France) was performed with the use of the avidin-biotin peroxidase method (22), as previously described (10).

Statistical analysis

For all microarrays, background signal was not subtracted before the Loess normalization of log-transformed microarray data with the use of the Goulphar Version 1.1.3 package (http://transcriptome.ens.fr/goulphar/index.php) (23). Gene lists were filtered to identify genes common to all microarrays with the use of Microsoft Excel (Microsoft, Redmond, WA). Differential gene expression, using a 0.5% false discovery rate (FDR), was assessed with the use of the Significance Analysis of Microarrays (version 3.02) procedure (available at http://www-stat.stanford.edu/tibs/SAM/). Multivariate analysis [hierarchical clustering and principal components analysis (PCA)] was performed with the use of Umetrics SIMCA-P (version 11.0.0.0; Umetrics AB, Umeå, Sweden) and TM4 version 4.0 software (http://www.tm4.org/) (24). The functional profiling of gene expression data was performed with the use of FunNet (http://www.funnet.info), which was extensively described elsewhere (7). The complete data set is publicly available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) through the series accession number GSE12050.

RESULTS

Subjects for this study were placed into 4 groups according to BMI and biopsy method: Lean-Needle (n = 10), Lean-Surgery (n = 9), Obese-Needle (n = 9), and Obese-Surgery (n = 9). The subjects were matched for age and BMI (Table 1). Microarray data were filtered so that only probe-sets targeting genes identified in the NCBI EntrezGene database were used (ie, known GeneIDs). This represented ≈32,000 probes of the ≈45,000 probes present on the microarray platform. Next, we averaged the logarithmic ratios of GeneIDs represented by multiple probe-sets on the microarray so as to have a single value per GeneID per subject. Finally, only probe-sets identified across all 37 microarrays were considered for analyses. This resulted in a final list of 9689 unique GeneIDs. With the use of these 9689 GeneIDs, all subjects were simultaneously analyzed to identify potential outlying data. PCA identified one outlier in the Lean-Surgery group; therefore this subject was removed from all further analyses (Figure 1). Both PCA and hierarchical clustering (by Pearson correlation) showed that subjects were organized into distinct groups, based on BMI and biopsy technique (Figure 1). Despite obtaining the various scAT samples from independent subjects, the tight clustering of the 4 groups illustrated in Figure 1 shows that group differences are considerably more important than interindividual differences. Interindividual differences in gene expression in scAT were previously shown to be low (25), thereby reinforcing the dominant effect of the sampling method on gene expression profiles.

Differential gene expression for all pairwise comparisons was determined with a standard analysis of microarray analysis with the use of an FDR of ≈0.5%. Although the use of an FDR of 0.5% can be considered stringent, we made the choice to adhere to the highest degree of statistical stringency possible so as to achieve the greatest confidence in our biological interpretations. Despite the use of this stringent FDR, the comparisons showed the anticipated highly significant differences between obese and lean subjects, but more astonishingly between needle and surgical biopsy techniques as well. The Obese-Needle compared with Lean-Needle analysis identified 3141 differentially expressed genes and the Obese-Surgery compared with Lean-Surgery analysis identified 3910 differentially expressed genes; however, the Lean-Needle compared with the Lean-Surgery identified 2358 differentially expressed genes and the Obese-Needle compared with the Obese-Surgery identified 3946 differentially expressed genes. Variability in gene expression data sets was equivalent between all 4 groups (see Figure S1 under “Supplemental Data” in the online issue).

Although thousands of genes were identified as differentially regulated between the 2 biopsy techniques, this did not necessarily imply that these gene lists were enriched in biologically meaningful data that could affect data interpretation. Therefore, we performed a functional analysis of these gene lists with the use of the FunNet tool (7) and confirmed results with DAVID Bioinformatics Resources (confirmatory data not shown) (26). KEGG pathways and Gene Ontology Cellular Component annotations were examined to identify those functional annotations that were significantly enriched in the different gene lists. This analysis showed that biological themes commonly discussed in obesity (eg, inflammation, extracellular matrix, and metabolism) are influenced by the biopsy sampling technique; a crucial caveat to consider when designing experiments in humans.

Indeed, the 2 tissue sampling methods had a different effect on the functional interpretation of gene expression data in both lean and obese subjects, indicating that these differences occur
independently to changes in body corpulence. The comparison between the Surgery and Needle groups showed that genes involved in KEGG inflammatory pathways (cytokine-cytokine receptor interaction, FcεRI signaling pathway) and in the metabolism of lipids (biosynthesis of unsaturated fatty acids, glycerolipid metabolism, glycerophospholipid metabolism, etc), carbohydrates (glycolysis-gluconeogenesis, starch and sucrose metabolism), and amino acids (valine, leucine, and isoleucine degradation) are not equally represented in the 2 biopsy techniques (Figure 2, A and B). These differences coincided with changes in the Gene Ontology cellular component analysis, in which the membrane and mitochondrial fractions were not equally represented in the 2 biopsy methods (Figure S2, A and B). Therefore, the different biopsy methods will influence the biological interpretation of global gene expression data sets and suggest that it is not appropriate to combine samples obtained by different biopsy techniques within a study.

We next determined whether similar biological conclusions concerning the obese state could be reached with the use of both needle-aspirated and surgical biopsies. The Obese-Needle and Lean-Needle comparison showed that elements related to inflammation were both up- (eg, natural killer cell–mediated cytotoxicity, and antigen processing and presentation) and down-regulated (eg, cytokine-cytokine receptor interaction, and complement and coagulation cascades) in obese subjects (Figure 2C), whereas the extracellular component was up-regulated (Figure S2C). The only mitochondrial-related KEGG functional pathways identified was oxidative phosphorylation, and the cellular component analysis showed that the mitochondrial fraction was decreased in obese subjects. In contrast, the surgical biopsies provided a more complete overview of the complex biological changes in AT associated with obesity. Indeed, inflammatory (B cell signaling, T cell receptor signaling, Toll-like receptor signaling, etc), cytoskeleton (regulation of actin cytoskeleton), and cellular interaction pathways (cell adhesion molecules) were all up-regulated in obese subjects, whereas metabolic pathways related to lipid (eg, biosynthesis of unsaturated fatty acids and glycerophospholipid metabolism, etc), carbohydrate (starch and sucrose metabolism), and amino acid (eg, valine, leucine, and isoleucine degradation) were down-regulated (Figure 2D), as was the oxidative phosphorylation pathway. As expected, extracellular and membrane-related fractions were up-regulated in obese subjects, and the mitochondrial fraction was down-regulated (Figure S2D).

We therefore hypothesized that the structure and morphology of the tissue samples obtained with the use of these 2 biopsy techniques may provide an explanation for the aforementioned differences in gene expression (Figure 3). With the use of biopsies obtained from the same obese subjects, both biopsy methods maintained the structure of adipocytes (Figure 3, A and B) and were comparable for the study of macrophage infiltration, as shown by the visualization of crownlike structures (Figure 3, C and D) previously observed in scAT (27). However, needle-aspirated scAT biopsies were more fragmented and contained more sanguineous regions on the periphery of
FIGURE 2. KEGG functional analysis. Up-regulated and down-regulated genes in the different pairwise comparisons were functionally studied to identify pathways enriched in one list compared with the other. The list of 9689 GeneIDs identified in all microarrays was used as the common reference list for all comparative analyses. These comparative analyses used only differentially expressed genes identified within the use of a 0.5% false discovery rate with the Significance Analysis of Microarrays (SAM) analysis. A: Pathways up-regulated (Lean-S, Lean-N) and down-regulated (Lean-S, Lean-N) in surgical biopsies compared with needle-aspirated biopsies from lean subjects. B: Pathways up-regulated (Obese-S, Obese-N) and down-regulated (Obese-S, Obese-N) in surgical biopsies compared with needle-aspirated biopsies from obese subjects. C: Pathways up-regulated (Obese-N, Lean-N) and down-regulated (Obese-N, Lean-N) in obese subjects compared with lean subjects, whereby both groups have had needle-aspirated biopsies. D: Pathways up-regulated (Obese-S > Lean-S) and down-regulated (Obese-S > Lean-S) in obese subjects compared with lean subjects, whereby both groups have had surgical biopsies. Transcriptional domain coverage indicates the most significant biological functions represented among annotated genes, ranked by percentage. Lean-N, lean subjects having needle biopsies; Lean-S, lean subjects having surgical biopsies; Obese-N, obese subjects having needle biopsies; Obese-S, obese subjects having surgical biopsies.
DISCUSSION

Research on human AT is rapidly advancing, and obtaining tissue samples has been greatly facilitated by gastric surgery procedures, which are performed most often in severely obese subjects. However, AT samples are also relatively easy to obtain from lean and obese subjects during elective or programmed surgery (including plastic surgery), thereby permitting comparative AT analyses to be performed in subjects varying in body weight.

Although clinic-based programs have facilitated the access to surgical AT samples, this biopsy method is considerably more laborious and limited in number compared with the “easier to obtain” needle-aspirated biopsy samples. Furthermore, an additional advantage of needle-aspirated biopsies is that they are significantly easier to repeat in the same person throughout the course of a medical or nutritional intervention, thus enabling the measurement of a dynamic individual response to a given intervention (30, 31).

Studies are emerging in which adipose gene expression has been examined in hundreds of subjects simultaneously (32), and it is just a matter of time before such large-scale population studies become routine for the study of human obesity thanks to the rapidly growing number of AT bio-banks around the world. It will be fundamentally important that these studies can be compared so that a common consensus can be reached and potential therapies for human obesity can be postulated. However, there is currently a paucity of information on the comparability of AT microarray data when tissue samples are obtained with the use of different biopsy sampling methods.

Our findings indicate that, although the rapid and more easily obtainable needle-aspirated biopsies can generate data sets that show perturbations in inflammatory and extracellular pathways to a certain degree, they might not capture a comparable overview of the biological changes seen with the more laborious surgical biopsies, especially for the stroma-vascular fraction of AT. The stroma-vascular fraction, which includes components of the extracellular matrix, is now considered important for the study of AT because of its influence on adipocyte cell biology (33–35). In this regard, our immunohistochemistry experiments confirmed that needle-aspirated biopsies are not the optimal method to study the extracellular matrix modifications or the development of fibrosis that can occur in AT and in obesity.

This study shows that it is inappropriate to combine samples obtained by different biopsy techniques for the analysis of AT gene expression within a study. Moreover, we consider that it is absolutely imperative that researchers of future work studying adipose gene expression clearly indicate the sampling methods used, because this will serve to improve the capacity to independently reproduce and compare microarray data. As such, highly authentic and reproducible data from scAT can be generated, ensuring the physiologic relevance and applicability of all findings and ameliorating our ability to successfully move research in obesity from the bench to the bedside.

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The authors’ responsibilities were as follows—DMM and KC: designed the study and wrote the paper; DMM, JT, BH, and VP: performed all experimental and statistical work; CH and J-DZ: implemented the FunNet tool and assisted with the analysis; NV and CP: coordinated obtaining subcutaneous adipose tissue samples with the various biopsy techniques; DMM, JT, VP, BH, CH, CP, NV, J-DZ, and KC: read and approved the manuscript. None of the authors had a personal or financial conflict of interest.

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