International Journal of Obesity (2008) 32, 112–120
 0307-0565/08 $30.00
www.nature.com/ijo

ORIGINAL ARTICLE

Contribution of mesothelial cells in the expression of inflammatory-related factors in omental adipose tissue of obese subjects

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Objective: The objective of this study was to determine the contribution of mesothelial cells, present in human omental adipose tissue (OAT) but not in the subcutaneous depot (SAT), on the expression of inflammation-related factors.
Design: Comparison of the expression profiles of inflammation-related genes in mesothelial cells with those in the adipocyte-enriched (AEF) and stromal vascular fractions (SVF) and localization of interleukin-18 (IL-18) expression in adipose depots.
Subjects: Eleven obese Caucasian female subjects undergoing gastric bypass surgery (body mass index: $43.6 \pm 1.3\,kg/m^2$; age: 41.6 ± 2.3 years).
Measurements: The expression profiles of cytokine and chemokine-related genes in mesothelial cells and in cell fractions prepared from OAT were assessed by the microarray technique. The differential expression of IL-18 was confirmed by real-time PCR and the protein was localized in adipose depots by immunohistochemistry.
Results: Microarray data analysis demonstrated that, of the 16 cytokine and chemokine-related genes that were upregulated in mesothelial cells compared with the AEF, IL-18 was the cytokine with the highest differential expression. IL-18 expression was similar in mesothelial cells and the SVF. In both SAT and OAT, IL-18 was immunolocalized in neutrophils and mast cells, but not in macrophages nor adipocytes. This cytokine was also detected in mesothelial cells in OAT. This additional source of expression may explain the higher IL-18 expression levels in OAT than SAT ($+5.9$-fold).
Conclusion: By their capacity to express inflammatory-related factors, and in particular the proinflammatory cytokine IL-18 in OAT, mesothelial cells appear as a new player in the process of low-grade inflammation associated with obesity.
International Journal of Obesity (2008) **32,** 112–120; doi:10.1038/sj.ijo.0803688; published online 17 July 2007

Keywords: cytokines; mast cells; neutrophils; transcriptomics; adipose tissue

Introduction

Obesity is associated with increased systemic inflammation that has been proposed to participate in the development of type 2 diabetes and cardiovascular risk. Adipose tissue is involved in this inflammatory process mainly by its capacity to produce inflammatory mediators such as tumor necrosis factor-α (TNF-α), IL-6 and monocyte chemoattractant protein-1 (MCP-1).[1] The proinflammatory interleukin-18 (IL-18), which has also recently been associated with the development of obesity and related metabolic diseases, has been reported to be increased in the plasma of obese and type 2 diabetic patients.[2–4] Its precise involvement in the development of metabolic disorders is still unclear, since it has also been shown mice deficient in IL-18 are obese and hyperglycemic.[5]

Adipose tissue is composed of various cell types, which include adipocytes and many other cells identified in the stromal vascular fraction (SVF) such as preadipocytes, blood cells, endothelial cells and macrophages. Adipocytes are able to secrete proinflammatory cytokines such as IL-6 and IL-1β but also a wide variety of chemokines.[6] Although the proinflammatory cytokine, IL-18, has also been reported to be produced by adipocytes,[7,8] a more recent study has demonstrated that it was secreted more by the tissue matrix and the SVF than the adipocytes themselves.[9] Macrophages, which have been shown to infiltrate the adipose tissue of obese subjects,[10,11] are an important source of inflammatory

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Received 5 March 2007; revised 29 May 2007; accepted 4 June 2007; published online 17 July 2007

mediators in fat. Indeed, these cells have been reported to produce almost all of the TNF-α in the adipose tissue of obese subjects, whereas IL-6 was equally expressed in adipocytes and macrophages.[10] A greater macrophage infiltration was recently observed in visceral fat when compared with subcutaneous adipose tissue (SAT).[11,12] This difference in the population of resident macrophages may underlie differences in the proinflammatory state previously observed between these two fat depots.[11,13]

The omentum consists of a network of peritoneum sheets, rich in mesothelial cells and filled with fat.[14] Therefore, in contrast with SAT, omental adipose tissue (OAT) is characterized by the presence of mesothelial cells which surround the fat lobules.[14–16] Mesothelial cells, which are also present in the lung and in the peritoneal cavity, participate in the coagulation, fibrinolysis and inflammatory processes, via their endocrine/paracrine capacities (for a review see[17]). Peritoneal mesothelial cells have also been shown to secrete chemokines that promote the migration of neutrophils and monocytes from the vascular compartment to the serosal space.[18] The function of mesothelial cells in adipose tissue has yet to be elucidated.

The objective of the present study was to determine the contribution of mesothelial cells in the expression of inflammatory-related factors in human adipose tissue. Using a DNA array approach, we compared the expression profile of cytokines and chemokines in mesothelial cells, the adipocyte-enriched fraction (AEF) and SVF. We found that IL-18 was preferentially expressed in mesothelial cells as compared with adipocytes. We confirmed by immunochemistry that, in addition to neutrophils and mast cells, mesothelial cells also express IL-18. This additional source of IL-18 was associated with a higher expression of this interleukin in OAT compared with SAT. Our results demonstrate, for the first time, that mesothelial cells are a potential key player in the development of the inflammation process in OAT and may contribute to the specificity of this fat depot.

Methods

Tissue collection and mesothelial cell preparation

The protocol of the present study was submitted to and approved by the 'Ethical committee of the faculty of biology and medicine, Lausanne University' and all patients provided their informed consent to participate. Those using oral glucose or lipid-lowering agents, weight-reduction therapies or antihypertensive drugs were excluded from the study. OAT and SAT were collected in 11 obese Caucasian female patients undergoing gastroplastic surgery (body mass index (BMI): $43.6 \pm 1.3\,kg/m^2$; age: 41.6 ± 2.3 years). OAT was obtained at the level of the greater omentum and SAT from the subcutaneous abdominal fat. Microarray gene expression analysis and confirmation of gene expression by real-time PCR were carried out on mesothelial cells and cell fractions prepared from 5 OAT of the 11 patients (BMI: $42.3 \pm 2.4\,kg/m^2$). Adipose samples of the six other obese subjects (BMI: $44.9 \pm 2.9\,kg/m^2$) were used to perform immunohistology and gene expression analysis in the three cell fractions that is the mesothelial cell-enriched fractions (MEF), the AEF and the SVF. Real-time-PCR analysis performed on genes of the whole OAT or SAT was made on samples collected from all of the 11 obese subjects.

The three cell fractions, that is AEF, SVF and the mesothelial cell population, were prepared from fresh OAT digested for 20 min at 37°C with 0.1% collagenase A (Roche Biomedical, Basle, Switzerland) and then sieved on 250 μm filters to remove undigested pieces of tissue containing endothelial cells.[19] The filtrate was centrifuged 10 min at 1500 r.p.m. The upper layer containing floating adipocytes was collected and washed twice with 1% bovine serum albumin (BSA) (Sigma-Aldricht, Basle, Switzerland) to obtain the AEF, while the pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM)/F12 medium, supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich) and sieved on a 30-μm filter. The filtrate was centrifuged 5 min at 1000 r.p.m. and the pellet, representing the SVF, was frozen at −80°C. Clumps of mesothelial cells present on the 30-μm filter were detached by washing the filter with culture medium and further purified, as previously described.[16,20] Briefly, the medium was centrifuged and the pellet was resuspended in DMEM/F12 medium supplemented with 10% FCS. The cell suspension was gently layered onto 10 ml PBS (phosphate-buffered saline; Sigma-Aldricht) containing 1 mM calcium chloride and 5% BSA (Sigma-Aldricht). After 10 min at room temperature, 2 ml of the top layer was removed and the remaining 9 ml was centrifuged. The pellet was resuspended in 1 ml DMEM/F12 medium with 10% FCS and the cell suspension was layered onto a PBS/calcium chloride/BSA mix as mentioned above. At the end of the second decantation, 2 ml of the top layer was discarded, the remaining solution was centrifuged and the pellet, representing the MEF, was immediately frozen at −80°C. For the microarray study, mesothelial cells were seeded in a Petri dish containing a 10% FCS DMEM/F12 medium and frozen at confluency before RNA preparation.

Confluent cultures of MEF exhibited a homogeneous cobblestone monolayer morphology with no apparent contamination of other cell types (not shown). Previous studies using this method of purification showed that the MEF was depleted of endothelial cells as determined by specific immunostaining.[16,20] The quality of our MEF preparations was further checked by measuring the expression of P selectin, a specific endothelial cell marker,[21] using real-time PCR. The expression levels of this marker were very low (cycle threshold, $C_t > 30$) and similar to those measured in the SVF and AEF (AEF: 33.4 ± 0.91 C_t; SVF: 34.0 ± 1.54 C_t; MEF: 32.6 ± 0.29 C_t; $n = 5$), suggesting the absence of endothelial cell enrichment in the MEF.

In order to detect the potential presence of remaining mesothelial cells in the SVF, the expression of specific

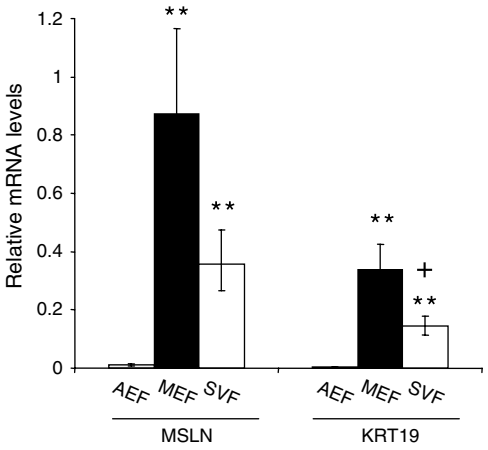

Figure 1 Relative mRNA expression of mesothelial cell markers in different cellular fractions. Keratin 19 (KRT 19) and mesothelin (MSLN) expression were measured by real-time PCR in the adipocyte- (AEF), the stromal vascular- (SVF) and the mesothelial (MEF)-enriched fractions prepared from OAT. Data are the mean ± s.e.m. of six biopsies collected from obese patients. Values were normalized with RPL-p0 and those statistically different to AEF or MEF are indicated by $^{**}P<0.01$ or $^{+}P<0.05$, respectively.

markers of this cell type[22,23] was measured by real-time PCR. Keratin 19 (KRT19) and mesothelin (MSLN) were found to be expressed in the SVF, but at much lower levels than in MEF (Figure 1) and they were not detected at all in AEF. These results indicate that SVF, used for the gene expression analysis, contained remaining mesothelial cells.

RNA preparation

About 0.3 g frozen adipose tissue was used to prepare total RNAs. After tissue homogenesation in Lysing Matrix tubes (Q-Biogen; Illkirch, France), samples were delipidated by adding 1 volume of chloroform and centrifuged for 5 min at 4000 r.p.m. at 20°C. RNA was extracted from the supernatant using the RNeasy mini kit (Qiagen; Basle, Switzerland) according to the procedure described by the manufacturer.

Total RNAs were prepared from the SVF and MEF using the Nucleospin RNAII kit (Macherey Nagel, Duren, Germany) according to the protocol of the manufacturer. In order to obtain enough good quality RNA to perform the microarray experiments, cells from the MEF were plated and RNA was prepared from confluent cells. A delipidation procedure was applied to the AEF before RNA extraction. Briefly, the cell homogenate was centrifuged for 5 min at 4000 r.p.m., the floating lipid phase was then discarded and 1 volume of chloroform was added to the aqueous phase in order to remove lipids from the homogenate. After centrifugation for 5 min at 4000 r.p.m., the aqueous phase was collected and incubated for 10 min at 37°C. RNAs were then extracted using the Nucleospin RNAII kit (Macherey Nagel).

RNAs prepared from the different fractions were monitored with the Agilent 2100 Bioanalyser (Agilent Biotechnologies, Boeblingen, Germany) and consistently demonstrated high-quality RNA (28S/18S ratio less than 3 and an RNA integrity number more than 8).

Microarray hybridization and data analysis

Microarray gene expression analysis was performed on cell fractions derived from each OAT, that is AEF, SVF and plated confluent mesothelial cells in order to obtain enough RNA of good quality. A total of 50 ng RNA from each of the different cell fractions was amplified using the Microarray Amplification Target Kit (Roche) before cRNA synthesis. cRNA synthesis, hybridization and scanning procedures were performed as previously described.[24] cRNAs were purified with the Microarray Purification Target Kit (Roche) and individually hybridized to the Affymetrix HG-U133 A GeneChip (Affymetrix UK, High Wycombe, UK), containing almost 45 000 probe sets which represents more than 39 000 transcripts.

Fluorescence values from the scans were analyzed with Affymetrix Gene Expression Analysis Software (MAS 5.0). The average difference intensity on each chip was normalized using natural logarithm transformation and quantile normalization. A mathematical method, termed the global error assessment model, was applied to the raw GeneChip data for the selection of differentially regulated genes.[24] Probe IDs with *P*-values below 10^{-6} were selected for function analysis. Annotation of the selected probe IDs and their clustering into biological functions were performed using the Panther database and gene classification (http://panther.appliedbiosystems.com). We focused our analysis on inflammation-related genes clustered in the Panther database on the 'immunity and defense' biological function.

Analysis of mRNA expression by real-time PCR

Reverse transcription (RT) was performed using 0.3 μg total RNA, prepared from the three cellular fractions (AEF, SVF and MEF or confluent mesothelial cells) or from OAT and SAT, using the first strand cDNA synthesis kit for real-time PCR (AMV; Roche Biomedical) with oligo $d(T)_{15}$ as primer. Real-time RT-polymerase chain reaction analyses were performed in a fluorescent temperature cycler (GeneAmp PCR 5700 Sequence Detection System; Applied Biosystems, Rotkreuz, Switzerland) as previously described.[25] Sequences of primers are listed in Table 1. Values were normalized to ribosomal large protein-P0 (RPL-p0) expression, which was previously reported to be one of the reference genes with the highest expression stability in human adipose tissue.[26] The effects of treatments on gene expression were evaluated by calculating the relative expression level as follows: $2^{\text{mean } C_t \text{ genes of interest} - \text{mean } C_t \text{ RPL-p0}}$ using the raw C_t values. The standard errors of the means (s.e.m.) were calculated using the following formula $2^{\text{SQRT(MS error} \times 2/n)}$, where n is the number of experiments and MS the residual mean square error calculated by the analysis of variance (ANOVA).

Immunohistochemistry

Omental and SAT samples collected from obese women ($n=5$) were immediately fixed in 10% paraformaldehyde, dehydrated, paraffin embedded and sectioned (5 μm sec-

tions). Sections were dewaxed before being stained with hematoxylin and eosin. Immunohistochemical detection of IL-18 (Santa Cruz Biotechnologies, Heidelberg, Germany; 10 μg/ml), CD68 (Dako Cytomation, Trappes, France; 1/400) and mast cell tryptase (MCT; Dako Cytomation; 1:1000) was performed using the avidin-biotin alkaline phosphatase method according to the manufacturer's instructions (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). Vector Red substrate (Vector) was used for visualization of IL-18 and MCT, and Vector Black substrate was used to visualize CD68. Tissues were previously stained with positive control antibodies such as CD31 (Nako, 1/1000) and vimentin (Nako, 1/500) to ensure that the tissue antigens were preserved and accessible for immunohistochemical analysis (not shown). Only tissues that were positive for CD31 and vimentin staining were used. The negative control consisted of performing the entire immunohistochemical procedure on adjacent sections in the absence of primary antibody. Slides were imaged with a DVC 1310C digital camera coupled to a Nikon microscope.

Statistical analysis

Significant differences of gene expression measured by real-time PCR were calculated using a two-factor ANOVA. An α-value of 0.05 was used as the threshold for significance. Significance ANOVA results were followed by *a posteriori* multiple comparisons to define the significance of the effects.

Statement of ethics

We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this study. The protocol of this study was approved by 'Ethical committee of the faculty of biology and medicine, Lausanne University'.

Table 1 Primer sequences used for gene expression analysis by real-time PCR

Gene	*Sequences (5′-3′)*	*Size (bp)*	*Accession no.*
IL-1β	Reverse: CATCAGCTTCAAAGAACAAGTCCATC Forward: CTGAGTCGCCAGTGAAATG	68	NM_000576
IL-18	Reverse: CTTCACAGAGATAGTTACAGCCATACCT Forward: GCACCCCGGACCATATTTATT	77	NM_001562
KRT-19	Reverse: CGATGTGCGAGCTGATAGTGA Forward: CCAGCCGCGACTTGATGT	70	NM_002276
MSLN	Reverse: GACTGAGCGCCTTCAAATCC Forward: GCCTTGCTTTCCAGAACATGA	95	NM_005823
SELP	Reverse: CTTATTAACATGTAGTTCTGAGCATTTCAC Forward: CTTCAGGACAATGGACAGCAGTA	70	NM_003005
RPL-P0	Reverse: TCGAACACCTGCTGGATGAC Forward: CCACGCTGCTGAACATGCT	66	NM_053275

Abbreviations: IL, interleukin; KRT-19, keratin-19; MSLN, mesothelin; RPL-P0, ribosomal phosphoprotein p0; SELP, P-selectin.

Results

Expression of inflammatory mediators in mesothelial cells

Microarray analysis indicated that 1897 and 2412 genes were differentially regulated in mesothelial cells when compared with the SVF and the AEF, respectively. Clustering analysis of these genes identified 227 and 216 as inflammation-related genes with a different expression level in mesothelial cells compared with SVF and AEF, respectively. Some of these genes were associated with biological processes linked to inflammation as shown in Figures 2a and b. In both comparisons, the 'chemokine- and cytokine-mediated signaling pathway' was one of the biological processes with the highest number of genes (Figures 2a and b). We focused our

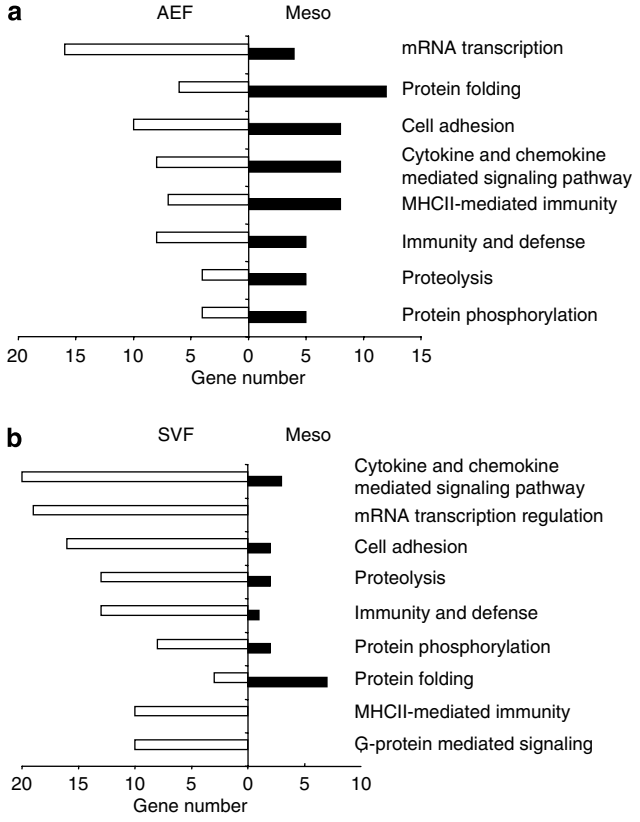

Figure 2 Distribution of inflammation-related genes differentially expressed in mesothelial cells. Genes differentially expressed in mesothelial cells compared with adipocyte- (AEF) or stromal vascular (SVF)-enriched fractions were determined by statistical analysis performed on individual fractions ($P<10^{-6}$), as described in the 'Methods' section. Genes belonging to the 'immunity and defense' biological function were selected and clustered using the Panther gene classification tool. The graph represents the number of genes differentially regulated in mesothelial cells compared with (**a**) AEF or (**b**) SVF in each biological process.

Table 2 Cytokine- and chemokine-related genes differentially regulated in mesothelial cells compared with the SVF

Gene name	*Gene symbol*	*Fold meso/SVF*
Interleukin 13 receptor, $\alpha 2$	IL13RA2	12.74
Interleukin 6 signal transducer	IL6ST	2.66
TNF receptor-associated factor 3	TRAF3	2.50
Interleukin 11 receptor, α	IL11RA	−2.86
Tumor necrosis factor (ligand) superfamily, member 14	TNFSF14	−3.75
Chemokine (C-X-C motif) ligand 1	CXCL1	−4.01
Tumor necrosis factor receptor superfamily, member 7	TNFRSF7	−4.34
Interleukin 16	IL16	−4.41
Interleukin 1 receptor antagonist	IL1RN	−7.83
Chemokine (C-C motif) ligand 2	CCL2	−7.97
Interleukin 6 receptor	IL6R	−10.03
Tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	−10.07
Interleukin 10 receptor, α	IL10RA	−11.26
Chemokine (C-C motif) ligand 18	CCL18	−16.47
Tumor necrosis factor receptor superfamily, member 1B	TNFRSF1B	−17.18
Triggering receptor expressed on myeloid cells 1	TREM1	−17.57
Chemokine (C-C motif) ligand 4	CCL4	−19.30
Colony stimulating factor 3 receptor	CSF3R	−21.11
Chemokine (C-X-C motif) ligand 2	CXCL2	−22.38
Interleukin 1, beta	IL1B	−24.22
Chemokine (C-X-C motif) ligand 3	CXCL3	−39.24
Chemokine (C-C motif) ligand 5	CCL5	−93.92
Interleukin 8	IL8	−111.15

Abbreviations: meso, mesothelial cells; TNF, tumor necrosis factor; SVF, stromal vascular fractions. Genes differentially expressed in mesothelial cells as compared with SVF were determined by statistical analysis performed on individual fractions ($P<10^{-6}$), as described in the 'Methods' section.

Table 3 Cytokine- and chemokine-related genes differentially regulated in mesothelial cells compared with the AEF

Gene name	*Gene symbol*	*Fold meso/AEF*
Interleukin 18	IL18	21.18
Interleukin 13 receptor, $\alpha 2$	IL13RA2	14.59
Tumor necrosis factor (ligand) superfamily, member 14	TNFSF14	12.01
Interleukin 4 receptor	IL4R	4.87
Tumor necrosis factor receptor superfamily, member 8	TNFRSF8	4.81
Interleukin 6 signal transducer	IL6ST	2.99
Chemokine (C-X-C motif) ligand 1	CXCL1	2.51
Interleukin 10 receptor, β	IL10RB	2.50
Interleukin 16	IL16	−2.68
Chemokine (C-C motif) ligand 4	CCL4	−3.53
Interleukin 1, beta	IL1B	−3.83
Chemokine (C-X-C motif) ligand 2	CXCL2	−4.85
Interleukin 8	IL8	−5.37
Chemokine (C-X-C motif) ligand 10	C7	−10.41
Chemokine (C-C motif) ligand 18	CCL18	−11.82
Tumor necrosis factor superfamily, member 10	TNFSF10	−24.46

Abbreviations: AEF, adipocyte-enriched fraction; meso, mesothelial cells. Genes differentially expressed in mesothelial cells as compared with AEF were determined by statistical analysis performed on individual fractions ($P<10^{-6}$), as described in the 'Methods' section.

analysis on genes belonging to this cluster and encoding for potential inflammatory mediators.

When compared with SVF genes, 23 cytokine and chemokine-related genes were differentially expressed in mesothelial cells (Table 2). Only three were upregulated in mesothelial cells, of which the IL-13 receptor and the IL-6 signal transducer were also found to be upregulated when compared with AEF (Table 3). Most of the genes with a higher expression in SVF, when compared with mesothelial cells, encoded for cytokine receptors or chemokines. IL-8 had the strongest expression in SVF when compared with mesothelial cells (Table 2).

Comparison of gene expression with the homogeneous population of adipocytes (AEF) shows that among the 16 genes differentially regulated, 8 were upregulated in mesothelial cells (Table 3). Genes with a higher expression in AEF encoded essentially for chemokines. The ILs-1β, -8 and -16 found to be upregulated in SVF (Table 2) also had a higher expression in AEF, compared with mesothelial cells (Table 3). Most of the genes with a higher expression in mesothelial cells, compared with AEF, encoded for cytokine receptors (Table 3). Interestingly, IL-18 that was the only interleukin upregulated in mesothelial cells was the most expressed gene from this cluster (+21.1-fold; Table 3). Since IL-18 was not identified as being differentially expressed in SVF, compared with mesothelial cells, it appears that this cytokine had similar expression levels in these two cell preparations.

This observation was confirmed by analysis of IL-18 expression level performed using real-time PCR. Indeed, IL-18 had a stronger expression level in mesothelial cells, compared with AEF (16.0-fold; Figure 3a), whereas its expression was similar when compared with SVF (1.1-fold). In contrast, IL-1β was expressed at a lower level in mesothelial cells when compared with either AEF (−17.9-fold) and SVF (−236.1-fold; Figure 3a), which was in agreement with the microarray data.

For reasons of RNA quality and quantity (see 'Methods' section) the transcriptomics study was performed on plated mesothelial cells, and we checked whether the MEF, derived directly from adipose tissue of six other obese subjects, also expressed IL-18. Figure 3b shows that MEF presented the highest IL-18 expression level when compared with either AEF or SVF. Although IL-18 expression was similar between SVF and plated mesothelial cells (Figure 3a), it tended to be higher in MEF compared with SVF (+1.9-fold; Figure 3b). The level of IL-1β in MEF was still lower compared with SVF, but it was significantly higher than that measured in AEF (+3.9-fold; Figure 3b).

Expression of IL-18 in OAT and SAT

The expression of IL-18 and IL-1β was measured by real-time PCR in OAT and SAT derived from 11 obese patients. IL-18

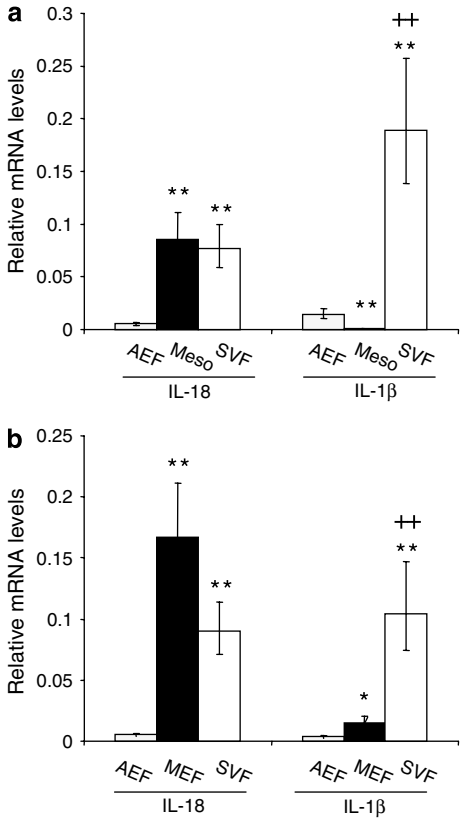

Figure 3 Relative mRNA expression of interleukins in different cellular fractions. Interleukin-18 (IL-18) and IL-1β expression were measured by real-time PCR in the adipocyte (AEF), the stromal vascular (SVF) and plated mesothelial cells (Meso; **a**) or the mesothelial (MEF; **b**)-enriched fractions prepared from OAT. Data are the mean$\pm$s.e.m. of five (**a**) or six (**b**) biopsies collected from obese patients. Values were normalized with RPL-p0 and those statistically different to AEF or MEF are indicated by *($P<0.05$), **($P<0.01$) or $^{++}$($P<0.01$), respectively.

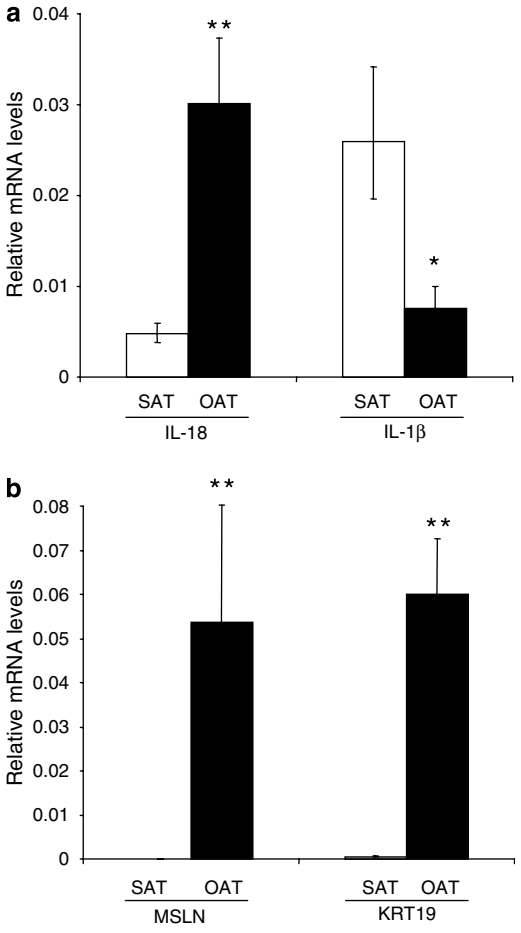

Figure 4 Expression of interleukins and mesothelial cell markers in omental (OAT)- and subcutaneous adipose tissue (SAT). The expressions of interleukin-18 (IL-18; **a**), IL-1β (**a**) and keratin-19 (KRT 19; **b**) and mesothelin (MSLN; **b**) were measured by real-time PCR in biopsies of OAT and SAT. Data are the mean$\pm$s.e.m. of 11 biopsies collected from obese patients. Values were normalized with RPL-p0 and those statistically different to SAT are indicated by *$P<0.05$ and **$P<0.01$.

was 6.3-fold more expressed in OAT than in SAT, whereas IL-1β was 3.4-fold less expressed (Figure 4a). As expected, the markers of mesothelial cells, that is MSLN and KRT19, were essentially expressed in OAT (Figure 4b).

The cellular location of IL-18 protein was determined by immunohistochemistry in OAT and SAT obtained from five obese subjects. A positive staining for IL-18, visualized either by light or fluorescence microscopy, was observed mainly in cells located between adipocytes in both fat depots (Figures 5a, b, e and f). The negative control, with only secondary antibodies, did not show any staining with light or fluorescence microscopy in either tissue (Figures 5c, d, g and h). In OAT, but not SAT, a bright staining pattern was observed on mesothelial cells surrounding the tissue (Figures 5a and b). At a higher magnitude ($\times 400$) these cells were easily identified as mesothelial cells (Figures 6a and b), as previously described in the literature.[14,27] Adipocytes were not stained by the IL-18 antibody in either depot, confirming the RT–PCR results. The identification of IL-18-positive cells, located between adipocytes and observed in both fat depots, was performed at higher magnification and visualized with light microscopy. Neutrophils, easily recognized by their multi nuclei, were shown to be positive for IL-18 protein in OAT and SAT (Figure 7b detection in OAT). In both fat depots, mast cells, identified with a specific marker, that is the mast cell tryptase antibody (Figure 7e), were also positive for IL-18 (Figure 7d in OAT, SAT not shown). However, macrophages, identified by CD68 labeling (Figure 7h), did not show any staining for IL-18 antibodies in either OAT or SAT (Figure 7g in OAT, SAT not shown).

Discussion

Omental and SAT are known to present differences in gene expression profiles, metabolic and biochemical properties.[28]

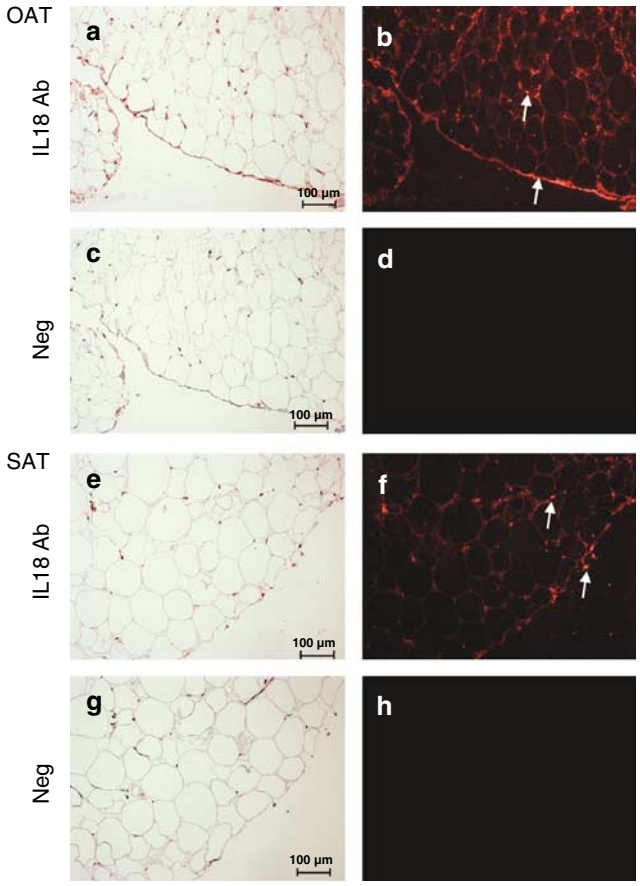

Figure 5 Immunostaining of interleukin-18 (IL-18) in omental adipose tissue (OAT) and subcutaneous adipose tissue (SAT). OAT and SAT were immunostained for IL-18 protein (IL-18 Ab; **a**, **b**, **e**, **f**) or with the secondary antibody alone (Neg; **c**, **d**, **g**, **h**), as a negative control. Staining was visualized by light microscopy (photographs in the right column) or a rhodamine excitation fluorescent filter at an identical intensity (photographs in the left column). Results are representative of OAT and SAT preparations collected from five different obese patients.

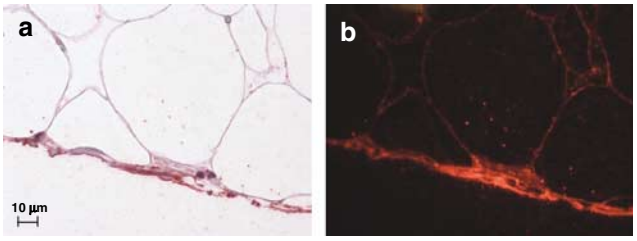

Figure 6 Immunostaining of IL-18 in mesothelial cells in OAT. OAT was immunostained for IL-18 protein (**a** and **b**). The negative control (secondary antibody alone) did not exhibit any staining (fluorescence or red staining by light microscopy; not shown). Results are representative of OAT preparations collected from five different obese patients.

Demonstration of the presence of mesothelial cells in OAT, but not in SAT,[14,15,16] also indicates a different cellular environment in these two depots which may contribute to the distinct characteristics of these tissues. To our knowledge, mesothelial cells of human OAT have not yet been characterized.

In the current study, we investigated the potential role of mesothelial cells to express inflammatory mediators in OAT. Analysis of the pattern of inflammation-related genes in mesothelial cells, compared with AEF and SVF, revealed that these cells expressed several cytokine receptors and related intracellular signaling molecules, as well as the proinflammatory cytokine IL-18, at higher levels. On the other hand, the level of some genes such as IL-1β and IL-8, that have previously been reported to be mainly produced by resident macrophages in OAT,[11] were either low, or absent, in mesothelial cells. In contrast, the IL-13 receptor alpha 2 subunit and the IL-6 signal transducer were strongly expressed in mesothelial cells when compared with SVF and AEF. The peritoneal mesothelial cells have been shown to secrete TNF-α, IL-6 and IL-10 after stimulation with lipopolysaccharide.[29]

Proinflammatory IL-18, which has recently been reported to be produced by adipose tissue[7–9] and found to be positively associated with visceral obesity and insulin resistance,[2,3,4] appears to be preferentially expressed in mesothelial cells. Although its expression in these cells was similar to that measured in SVF, containing immune cells and also remaining mesothelial cells, IL-18 mRNA levels were about 20-fold higher in these mesothelial cells than in the AEF, previously reported to express this cytokine.[7,8] These data are in agreement with a recent study, performed in 22 obese subjects, which reports a stronger IL-18 secretion in the adipose tissue matrix.[9]

Immunohistochemical analysis, performed on intact tissues, confirmed that IL-18 protein was present in the mesothelial cells surrounding OAT but not in SAT, which does not contain this cell type. However, IL-18 was not detected in adipocytes from any of the adipose depots, which is in contrast with previous studies that have detected IL-18 protein in adipocytes of human adipose tissues with ultrasensitive ELISA kits.[7,8] This discrepancy may be attributed to a lower sensitivity of the immunohistochemistry methods, necessary for localizing cytokine production in intact tissues, as compared with the ELISA assays.

In contrast with our data, showing the absence of IL-18 expression in resident macrophages of adipose tissue, macrophages obtained after differentiation of monocytes prepared from human blood were reported to produce this cytokine.[30] This discrepancy might be explained by the heterogeneous functions of macrophages that are influenced by their local environment, as previously demonstrated between testis and the peritoneum.[31] Neutrophils and mast cells were identified to be cellular sources of IL-18 in both adipose depots. The expression of IL-18 in these immune cells, and in contaminating mesothelial cells, may contribute to the relatively high expression of this cytokine measured in SVF.

We show that IL-18 mRNA was more expressed in OAT than in SAT. These data are in agreement with a recent study

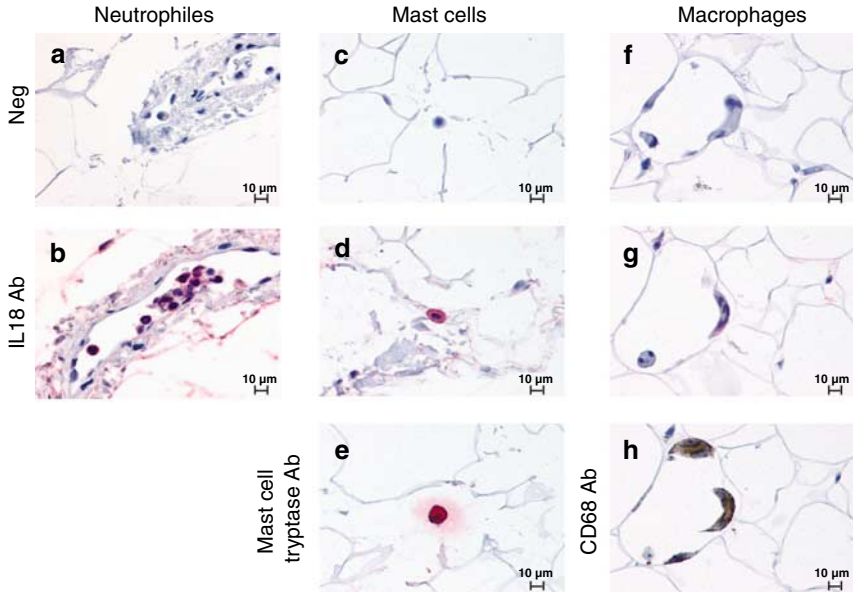

Figure 7 Immunolocation of interleukin-18 (IL-18) synthesis in immune cells by immunostaining. OAT, prepared as described in the 'Methods' section, was immunostained either for IL-18 (**b**, **d**, **g**), mast cell tryptase (**e**), CD68 (**h**) or with the secondary antibody alone (**a**, **c**, **f**), as a negative control. Results are representative of OAT preparations collected from five different obese patients.

showing that this cytokine was released more by OAT than by SAT sampled from obese subjects.[9] Although the relative contribution of mesothelial cells in the production of IL-18 in OAT remains to be determined, it is tempting to speculate that these cells are responsible for the higher IL-18 expression in OAT versus SAT.

IL-18 is recognized as a central regulator of the innate and acquired immune response.[32] This proinflammatory cytokine plays an early role in the inflammatory cascade by stimulating the production of TNF-α and, secondarily, IL-6.[32] This function of IL-18, may directly associate it with the development of insulin resistance through the inhibitory effects of TNF-α and IL-6 on the insulin action in adipocytes.[33,34] The precise function of IL-18 in human adipose tissue remains to be defined.

By demonstrating that mesothelial cells were able to express cytokines and their receptors in OAT, this study identified a potential new player in the regulation of the overall inflammatory process observed in obese individuals. Thus, this cell type may represent a cellular target for controlling the production of inflammatory mediators in this specific fat depot. Further studies are needed to better understand the overall function of the mesothelium in adipose tissue and its interactions with the different cell types present in OAT.

Acknowledgements

We are grateful to Dr DM Mutch (INSERM U755, Hôpital Hôtel Dieu, Paris, France), Dr K Acheson and F Raymond (Nestlé Research Center, Lausanne, Switzerland) for fruitful discussions and critically reading the manuscript, and C Verdumo (University Hospital CHUV, Lausanne, Switzerland) for expert technical assistance.

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