Human Adipose Tissue Macrophages: M1 and M2 Cell Surface Markers in Subcutaneous and Omental Depots and after Weight Loss

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Context: Macrophages accumulate in adipose tissue and possibly participate in metabolic complications in obesity. Macrophage number varies with adipose tissue site and weight loss, but whether this is accompanied by phenotypic changes is unknown.

Objective: The objective of the study was to characterize the activation state of adipose tissue macrophages in human obesity.

Design/Setting: We performed a single-center prospective study.

Participants/Interventions: Paired biopsies of sc and omental adipose tissue were obtained during gastric surgery in 16 premenopausal obese women (aged 41.1 $\pm$ 8.6 yr; body mass index 43.8 $\pm$ 3.4 kg/m^2). Subcutaneous adipose tissue biopsies were obtained 3 months later in obese subjects and in 10 nonobese women (aged 43.3 $\pm$ 3.5 yr; body mass index 22.5 $\pm$ 0.75 kg/m^2). The number of macrophages stained with CD40, CD206, and CD163 surface markers was determined by immunochemistry.

Main Outcomes: The number of CD40$^+$ macrophages significantly increased with obesity and in omental *vs.* sc adipose tissue in obese women. No significant changes in CD163$^+$ and CD206$^+$ macrophage counts was found with obesity and fat pad anatomical location. Three months after gastric surgery, the ratio of CD40$^+$ to CD206$^+$ macrophages was 2-fold lower than before surgery in the sc adipose tissue of obese subjects ($P < 0.001$) due to a concomitant decrease of CD40$^+$ and increase of CD206$^+$ macrophages counts.

Conclusion: We suggest that the activation state of adipose tissue macrophages is weighted toward M1 over M2 status in obese subjects and switch to a less proinflammatory profile 3 months after gastric bypass. **(*J Clin Endocrinol Metab* 94: 4619–4623, 2009)**

Acknowledged to be a low-grade inflammatory state, obesity favors the development of metabolic, cardiovascular, and liver diseases. Macrophages, which accumulate in obese adipose tissue in proportion to body fat mass, might contribute to obesity-linked inflammatory and metabolic complications (1–4). Macrophages accu-

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Abbreviation: QUICKI, Quantitative insulin sensitivity check index.

mulation varies with adipose tissue anatomical site, and increased macrophages infiltration in omental adipose tissue associates with liver fibroinflammation (5). Modification of energy balance and adipose tissue mass may not only influence the number of accumulating macrophages (4) but also favor changes in macrophage activation state. Tissue macrophages can exist in different activation states: either proinflammatory classically activated by interferon-γ or lipopolysaccharide, known as M1, or antiinflammatory alternatively activated by IL-13 or IL-4, known as M2. Depending on microenvironmental stimuli, M1- and M2-activated macrophages fulfill different functions through the production of pro- or antiinflammatory factors (6). We reported that weight loss increases the expression of antiinflammatory M2 macrophages genes (IL-10 and IL-1-Ra) in human adipose tissue, suggesting a phenotypic switch toward an antiinflammatory profile (4, 7). Recently sc adipose tissue macrophages were shown to express a mix of pro- and antiinflammatory factors in two distinct populations of normal to overweight women (8, 9).

To further characterize the phenotype of adipose tissue macrophages in human obesity, we used immunohistochemistry to quantify cells expressing selected M1 and M2 surface markers in normal-weight and massively obese subjects. Change in the number of M1 or M2 marker-labeled macrophages with adipose tissue site and in response to weight loss was assessed in obese subjects.

TABLE 1. Anthropometric and clinical measures

		Obese	
	Nonobese	Before surgery	3 months after surgery
n (gender)	10 (female)	16 (female)	16 (female)
Age (yr)	43.3 ± 3.5	41.1 ± 8.6	
Weight (kg)	58 ± 1.7[a]	124 ± 11	105.1 ± 10.4[b]
BMI (kg/m^2)	22.5 ± 0.75[a]	43.8 ± 3.4	37.1 ± 3.6[b]
Fat mass (%)[c]		45.5 ± 3.4	43.8 ± 3.6[b]
Fasting glucose (mM)		5.04 ± 0.56	5.02 ± 0.53
Fasting insulin (μU/ml)		13.1 ± 9.7	8.6 ± 4.5[b]
QUICKI		0.34	0.36[b]
Triglycerides (mmol/liter)		1.30 ± 0.86	1.03 ± 0.38
HDL cholesterol (mmol/liter)		1.39 ± 0.4	1.43 ± 0.41
Leptin (ng/ml)		60.7 ± 18.5	35.4 ± 15[b]

Data are means ± SEM. Differences between groups were tested by unpaired (lean *vs.* obese) or paired (before *vs.* after surgery) Student's *t* test. QUICKI = 1/[log fasting insulin (microunits per milliliter) + log fasting glycemia (milligrams per deciliter)]. BMI, Body mass index; HDL, high-density lipoprotien.

[a] $P < 0.05$ *vs.* obese; [b] $P < 0.05$ *vs.* before surgery; [c] fat mass was assessed by biphotonic absorptiometry (dual-energy x-ray absorptiometry).

Subjects and Methods

Human subjects

We enrolled 16 morbidly obese Caucasian premenopausal women eligible for laparoscopic Roux-en-Y bypass surgery included in a larger population previously described in detail elsewhere (10). We selected women without dyslipidemia, type 2 diabetes, or inflammatory or infectious diseases. They were weight stable for 3 months preceding surgery. Five women were on antihypertensive drugs before surgery and two of them had their treatment discontinued after weight loss. Ten lean women were recruited as a control group. Anthropometric and clinical measures are shown in Table 1.

Adipose tissue biopsies

In the obese group, biopsies of sc and omental adipose tissue were obtained during surgery. Three months later, a sc adipose tissue biopsy was collected in the same subjects by incision of the skin under local anesthesia (11). In the lean group, sc adipose tissue was sampled during programmed surgery for plastic surgery of abdominal wall, hernia or hysterectomy. The clinical investigation was approved by the Ethics Committees of Pitié-Salpêtrière Hospital. All subjects gave their written informed consent.

Immunomorphological analysis of adipose tissue

The biopsies were processed as described previously (4, 5). We used antibodies targeted to M1 and M2 macrophages subtypes, respectively, CD40 (R&D Systems, Minneapolis, MN) and CD163 (AbDSEROTEC, Düsseldorf, Germany) or CD206 (R&D Systems). HAM56 antibody (DakoCytomation, Trappes, France) was used for macrophage labeling. CD3 (Neomarker Microm, Francheville, France) and CD19 (AbDSEROTEC) antibodies were used for quantification of T lymphocytes and B lymphocytes, respectively. Immunohistochemical detection was performed with the avidin-biotin-peroxidase method and slides were counterstained with Mayer's hematoxylin. Labeled cells were identified by careful visual examination of the slides with increasing grades of magnification. The numbers of adipocytes and macrophages were counted in ten randomly chosen areas at ×40 magnification by two independent researchers. The number of macrophages was normalized to 100 adipocytes (5).

Statistical analysis

Data are shown as mean ± SEM. Differences between groups were determined by paired Student's *t* test (before *vs.* after surgery) or unpaired Student's *t* test with Welch's correction (obese *vs.* lean subjects). Differences were considered significant when $P < 0.05$. Statistical analysis was performed with JMP statistics software (SAS Institute, version 3.2.6, Cary, NC).

Results

Immunohistochemistry was used to quantify $CD40^+$, $CD163^+$, and $CD206^+$ cells on adipose tissue slides. Both CD40 and CD206 antibodies label cells dispersed in the parenchyma and arranged in crown-like structures (Fig. 1, A and B, *upper panels*). These cells were identified as mac-

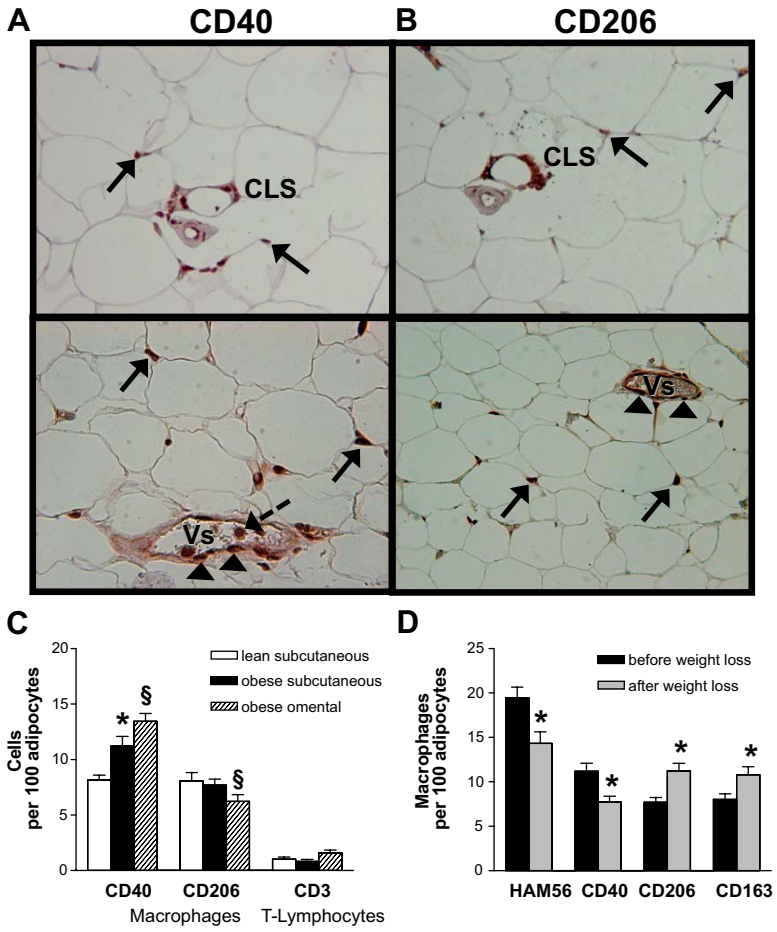

FIG. 1. Detection and quantification of CD40, CD206, CD163, and HAM56 positive cells in human adipose tissue: effect of obesity and weight loss. Immunopositivity for CD40 (A) and CD206 (B) in sc adipose tissue of obese subjects. *Black arrow*, Macrophage dispersed in parenchyma; *arrowhead*, endothelial cell; *hatched arrow*, intravascular cell. Magnification, ×40. CLS, Crown-like structure; *Vs*, vessel. C, Quantification of $CD40^+$ and $CD206^+$ macrophages and $CD3^+$ T lymphocytes in sc adipose tissue of lean (*white bar*) and obese subjects (*black bar*) and obese omental (*hatched bar*) adipose tissue. Data are means ± SEM. *, $P < 0.05$ *vs.* lean sc adipose tissue; §, $P < 0.05$ *vs.* omental adipose tissue. D, Quantification of HAM56, CD40, CD206, and CD163 positive macrophages in sc adipose tissue of obese subjects before (*black bar*) and 3 months after gastric bypass surgery (*gray bar*). Data are means ± SEM. *, $P < 0.05$ *vs.* before surgery.

rophages, based on HAM56 staining on a subset of serial slides (data not shown). The antibodies also label endothelial and intravascular cells (Fig. 1, A and B, *lower panels*) that were visually identified and not counted. CD40 is known to be expressed on $CD19^+$ B lymphocytes and $CD3^+$ T lymphocytes that are not easily distinguished from macrophages. Because we found virtually no $CD19^+$ cells (data not shown) and few amounts of $CD3^+$ cells (Fig. 1C) on the slides used for macrophage quantification, we estimated that $CD40^+$ macrophage miscounting due to this cross-reactivity was minimal.

The number of $CD40^+$ macrophages was significantly higher in obese than lean sc adipose tissue (Fig. 1C). In contrast, the numbers of both $CD206^+$ (Fig. 1C) and $CD163^+$ macrophages (nonobese: 7.2 ± 0.27; obese: 8.0 ± 0.62, $P = 0.229$) were not significantly changed by obesity. $CD206^+$ macrophage count was chosen to estimate the M1/M2 balance by the ratio $CD40^+$ to $CD206^+$ macrophage number. In sc adipose tissue, this ratio was 1.5-fold higher in obese (1.52 ± 0.17) than nonobese women (1.10 ± 0.11, $P = 0.047$).

The number of $CD40^+$ macrophages was higher in omental than sc adipose tissue of the same subject (Fig. 1C). An opposite but less marked effect was observed for $CD206^+$ macrophages. The CD40 to CD206 ratio measured in omental adipose tissue (2.40 ± 0.27) was 1.6-fold higher than in sc fat ($P = 0.002$).

To assess the effect of weight loss, obese subjects were investigated 3 months after gastric surgery. As expected, weight loss amounted 15% of initial body weight and was accompanied by significant improvement of insulin sensitivity illustrated by increased quantitative insulin sensitivity check index (QUICKI) value (Table 1). In sc adipose tissue, the number of $HAM56^+$ and $CD40^+$ cells decreased, whereas the number of $CD206^+$ and $CD163^+$ increased 3 months after gastric bypass (Fig. 1D). A 2-fold reduction of the CD40 to CD206 ratio (0.75 ± 0.082, $P = 0.002$) was observed compared with the presurgery value.

Discussion

To our knowledge, our study is the first to show that obesity, anatomical location, and weight loss induce significant changes in the number of adipose tissue macrophages expressing M1 or M2 surface markers. Our data suggest that the M1/M2 balance, estimated by the ratio of CD40 to CD206 macrophage number, is increased in obesity and omental *vs.* sc adipose tissue and reduced by weight loss.

These findings rely on the use of cell surface markers appropriate to detect M1 and M2 macrophages. Whereas it is admitted that CD163 and CD206 staining labels M2 macrophages, finding M1 immunohistochemistry marker is more challenging. Here we chose CD40, a macrophage surface receptor involved in proinflammatory pathways (12), whose cell surface expression is increased in blood monocyte-derived macrophages induced by interferon-γ (8). Additionally, immunofluorescence experiments revealed increased CD40 expression on *in vitro* differentiated human macrophages treated with lipopolysaccharide for 24 h, compared with IL-4-treated cells (data not

shown). Both CD40 and CD206 antibodies label several cell types on adipose tissue slides. This lack of cell selectivity was counterbalanced by a careful inspection of the slides, allowing the visual discrimination of the positive cells and identification of macrophages on location and cell shape. Although the CD40 antigen is also present on T lymphocytes (13) and B lymphocytes (14), these cells were found in very low abundance in human adipose tissue compared with macrophages and therefore interfere marginally with $CD40^+$ macrophages counting.

Our data not only confirm the presence of M2 stained cells in the adipose tissue of lean subjects as reported (8, 9) but also reveal the presence of M1 macrophages. Currently we cannot discriminate whether adipose tissue macrophages express concomitantly M1 and M2 surface markers or whether distinct macrophages subsets are present in the adipose tissue of lean individuals. Nevertheless, this observation suggests a physiological role for both pro- and antiinflammatory macrophages in healthy adipose tissue.

Previous studies using flow cytometry found a positive relationship between the proportion of $CD206^+$ cells in the stroma vascular fraction of adipose tissue and body mass index (8, 9). Here direct quantification of $CD206^+$ and $CD163^+$ macrophages on adipose tissue slides shows no significant difference between normal weight and obese women. These discrepant data might be accounted for by the distinct populations studied but also by the different methods used for adipose tissue processing and quantification of labeled cells. Notably, immunohistochemistry is a unique opportunity to discriminate visually the labeled cells in distinct areas and histological structures of adipose tissue.

When compared with sc adipose tissue, omental adipose tissue, recognized as the at risk depot for metabolic and cardiovascular diseases, showed increased $CD40^+$ and reduced $CD206^+$ macrophages. This M1-oriented phenotype in omental adipose tissue is in line with the idea that omental macrophages release proinflammatory mediators, like IL-6, into the portal vein (15), potentially linking omental inflammation to liver diseases (5). The mechanisms by which macrophages preferentially accumulate in the omental depot and acquire a proinflammatory phenotype in obesity remain to be deciphered. It is also unknown in humans whether proinflammatory macrophage accumulation in the adipose tissue contributes to or is the result of increased body fat mass.

Three months after gastric bypass, the amount of M2 macrophages increased, whereas the amount of M1 macrophages decreased in sc adipose tissue of obese subjects. Whether this feature is related to changes in adipose tissue biology or to altered energy balance remains to be investigated. From the present immunohistochemistry study, we cannot determine whether the M1/M2 switch induced by gastric surgery is related to change in individual cell polarization or due to the recruitment of M2 polarized cells from the circulation. In a mirror situation, studies in mice favor the hypothesis that high-fat diet-induced switch from M2 to M1 activation state of adipose tissue macrophages relies more on the recruitment of new macrophages from the circulation than on the conversion of resident macrophages (16, 17).

Gastric bypass-induced weight loss improves individuals' metabolic and inflammatory profile. Whereas M1 proinflammatory macrophage-derived factors induce insulin resistance and inflammation in preadipocytes and/or adipocytes (18, 19), the role of M2-oriented macrophages remains to be established. Genetically modified mice, with impaired M2 macrophage activation, are prone to diet-induced obesity and insulin resistance (20). Thus, it is tempting to speculate that modification of the M1/M2 balance toward a less proinflammatory state after weight loss contributes to the improvement of the metabolic condition. In our population limited in number, we were unable to detect any significant association between change in clinical and biological parameters and change in M1 or M2 macrophage counts or their ratio in the adipose tissue. Prospectively following up the macrophages phenotypic profile at longer term, notably during weight maintenance, will be of interest to evaluate whether the M2-oriented phenotype maintains over time. Although not demonstrated, it is plausible that the phenotypic switch in favor of an antiinflammatory microenvironment improves adipose cell biology and, in turn, contributes to the improvement of subjects' metabolic profile after gastric surgery.

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