0013-7227/07/$15.00/0
Printed in U.S.A.

Endocrinology 148(2):868–877

doi: 10.1210/en.2006-0687

Macrophage-Secreted Factors Impair Human Adipogenesis: Involvement of Proinflammatory State in Preadipocytes

Danièle Lacasa, Soraya Taleb, Mayoura Keophiphath, Alexandra Miranville, and Karine Clement

Institut National de la Santé et de la Recherche Médicale, Unité 755 Nutriomique (D.L., S.T., M.K., K.C.), 75004 Paris, France; University Pierre and Marie Curie-Paris 6 (D.L., S.T., M.K., K.C.), Faculty of Medicine Les Cordeliers, 75004 Paris, France; and AP-HP, Hôtel-Dieu Hospital (K.C.), Nutrition Department, 75004 Paris, France; and Cardiovascular Physiology Institute (A.M.), J.W. Goethe University, D-60385 Frankfurt/Main, Germany

Obesity is considered a chronic low-grade inflammatory state. The white adipose tissue produces a variety of inflammation-related proteins whose expression is increased in obese subjects. The nonadipose cell fraction, which includes infiltrated macrophages, is a determinant source of inflammation-related molecules within the adipose tissue. Our working hypothesis is that macrophage infiltration affects fat expansion through a paracrine action on adipocyte differentiation. Human primary preadipocytes were then differentiated in the presence of conditioned media obtained from macrophages differentiated from blood monocytes. Preadipocytes treated by macrophage-conditioned medium displayed marked reduction of adipogenesis as assessed by decreased cellular lipid accumulation and reduced gene expression of adipogenic and lipogenic markers. In addition to this effect, the activation of macrophages by lipopolysaccharides stimulated nuclear factor κB signaling, increased gene expression and release of proinflammatory cytokines and chemokines, and induced preadipocyte proliferation. This phenomenon was associated with increased cyclin D1 gene expression and maintenance of the fibronectin-rich matrix. Anti-TNFα neutralizing antibody inhibits the inflammatory state of preadipocytes positioning TNFα as an important mediator of inflammation in preadipocytes. Strikingly, conditioned media produced by macrophages isolated from human adipose tissue exerted comparable effects with activated macrophages, *i.e.* decreased adipogenesis and increased inflammatory state in the preadipocytes. These data show that macrophage-secreted factors inhibit the formation of mature adipocytes, suggesting possible role in limiting adipose tissue expansion in humans. (*Endocrinology* 148: 868–877, 2007)

OBESITY RESULTS FROM the expansion of white adipose tissue by adipocyte hypertrophy and formation of new adipocytes from precursor cells. Obesity is now considered a low-grade inflammatory disease, a feature shared with associated pathologies like type 2 diabetes and atherosclerosis (1, 2). Several experiments suggest that some molecules belonging to the broad panel of inflammatory factors (TNFα, IL-1β, IL-6, and so on) mostly originate from the nonadipose cell fraction, which includes infiltrated macrophages (3–5). The amount of infiltrated macrophages is closely related with adipose tissue mass (6–8). The inflammatory cells are both dispersed in the whole tissue or disposed in crown structures around some adipocytes (9). Weight loss leads to the improvement of the inflammatory profile (4) together with a significant reduction in the number of adipose tissue infiltrating macrophages in obese patients (9).

Bone marrow transplantation experiments in mice (6) suggest that the origin of adipose tissue macrophages is mainly from blood monocytes, which are in a proinflammatory state in obese subjects (10). Monocytes migrate to tissues where they differentiate into macrophages exhibiting tissue-specific functions (11). Once activated, macrophages produce a wide array of growth factors, cytokines, chemokines, and proteolytic enzymes (12). The role of macrophage infiltration and its derived products in adipose tissue biology and development in humans is mostly unknown.

Our working hypothesis is that macrophage infiltration affects fat expansion through a paracrine action on adipose differentiation. This process is characterized by extensive extracellular matrix remodeling with the disappearance of the fibronectin-rich matrix in the preadipocytes (13–15). During the differentiation program, the transcriptional factor CCAAT/enhancer-binding protein (C/EBP)β is transiently induced leading to activation of two master adipogenic transcription factors, peroxisome proliferator-activated receptor (PPAR)γ2 and C/EBPα. These factors positively regulate each other and then activate the transcription of genes involved in lipid metabolism. Regulation of adipocyte differentiation is exerted by various endocrine and autocrine factors (hormones, inflammatory cytokines, growth factors.), which mediate this process by acting on the synthesis and/or activity of adipogenic transcription factors (16, 17). In murine adipose cell lines, the proinflammatory cytokines IL-1β and TNFα strongly suppress adipogenesis (18, 19) through activation of the nuclear factor κB (NF-κB) pathway (20). Acti-

First Published Online November 2, 2006

Abbreviations: ATM, Adipose tissue macrophage; C/EBP, CCAAT/enhancer-binding protein; CM, conditioned media; FBS, fetal bovine serum; I-κB, inhibitor κB; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; PPAR, peroxisome proliferator-activated receptor; SVF, stroma vascular fraction.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

vation of this pathway leads to the release and nuclear translocation of NF-κB subunits resulting in an enhanced transcription of inflammatory markers (cytokines and chemokines) (21, 22).

It has been recently shown that secreted factors derived from the macrophage cell line increase the proinflammatory status of 3T3-L1 adipocytes and human preadipocytes (23–25). Because these effects could be due, at least in part, to the phenotype of transformed cells, we ought to develop an experimental cell model using primary human cells. The cells were cultured in presence of conditioned medium from human monocyte-derived macrophages, the most likely cell source for infiltrated macrophages in the adipose tissue. Because the phenotype of macrophages is subject to marked changes in response to inflammatory stimuli, monocyte-derived macrophages were stimulated with lipopolysaccharide (LPS) before harvesting the conditioned medium to test the effect of macrophagic activation. Finally, we were able to demonstrate that conditioned medium produced by macrophages isolated from human adipose tissue reproduced the effects of LPS activated monocyte-derived macrophages on preadipocyte differentiation.

Materials and Methods

Isolation of human preadipocytes

Subcutaneous adipose tissues biopsies were obtained from nonobese (body mass index < 30 kg/m^2) and young female patients undergoing elective surgery. None of the patients had diabetes or metabolic disorders and taking medications. This study was approved by the Ethical Committees of Hôtel Dieu (Paris, France). Human preadipocytes were isolated and cultured as described in (26, 27). Briefly, minced adipose tissue was digested by collagenase treatment. The digested material was filtered and centrifuged. The resulting pellet [stroma vascular fraction (SVF)] was resuspended in erythrocyte lysis buffer (154 mM NH_4Cl; 5.7 mM K_2HPO_4; and 0.1 mM EDTA, pH 7.0) at 250 g for 10 min. After washing in PBS, the SVF cells were suspended in DMEM-10% fetal bovine serum (FBS) and used for cell culture at passage 2 to eliminate nonpreadipocyte cell contamination as confirmed by negative staining for macrophage markers (Ham 56 and Mac-1).

Preparation of human blood monocyte-derived macrophages and conditioned medium: Blood from overweight (body mass index > 25 kg/m^2) female patients was immediately processed for plasma blood mononuclear cells isolation. Blood sample was layered on plasma blood mononuclear cell isolation medium (Amersham Biosciences, Little Chalfont, UK). Differentiation of monocytes to macrophages was conducted as previously described (28). Briefly, plasma blood mononuclear cells resuspended in 2 ml of RMPI medium containing 10% FBS were seeded at a density of 1×10^6 cells in six-well plates and allowed to differentiate for 7–8 d. Expression of specific macrophage markers (Ham 56/mac-1 staining and CD 68, CD 11b, CD 163 gene expression) was assessed to verify the degree of macrophagic differentiation (data not shown). Macrophage-conditioned media (CM) was prepared by incubating the monocyte-derived macrophages at 4×10^5 cells in 12-well plates in 1 ml of RMPI-10% FBS for 24 h. To test the effect of macrophage activation, the cells were incubated in the conditions described here for 24 h with 100 ng/ml LPS (from *Escherichia coli* 0127:B8; Sigma, St. Louis, MI) prior collecting the medium (Ac CM). The concentration of two inflammatory cytokines, TNFα and IL-6, were markedly increased in Ac CM *vs.* CM (TNFα: 52 ± 25 *vs.* 2465 ± 1069 pg/ml; IL-6: 62.7 ± 16.8 *vs.* 1301 ± 323 pg/ml, n = 3). Control medium was RPMI-10% FBS kept at 37 C for 24 h in the absence of macrophages. In some experiments, the 24-h culture RPMI medium obtained from human epithelial kidney cells (HEK 293) was tested. The conditioned media of the macrophages obtained from three to five individuals were pooled and stored at −80 C until used. Distinct pools were used for each culture experiment.

Preparation of adipose tissue macrophages and CM

Isolation of adipose tissue macrophage (ATM) from human adipose tissue stroma vascular fraction was performed as previously described (8). Isolated human SVF cells were obtained from sc adipose tissue biopsies as described previously. SVF cells were suspended in PBS/2% FBS/1 mmol/liter EDTA were incubated at room temperature for 15 min with CD34-positive selection cocktail followed by a 10-min incubation period with magnetic nanoparticles (Stemcell Technologies, Grenoble, France). The CD34-negative cell fraction was incubated with CD14-positive selection cocktail. The bead-coupled CD14+ cells were maintained for 24 h in 1 ml of ECBM (Promocell, Heidelberg, Germany) supplemented with 0.1% bovine serum albumin at a cell density of 4×10^5 cells in 12-well plates to obtain macrophage conditioned media (ATM CM), which was stored at −80 C before use.

Differentiation of human preadipocytes

Preadipocytes were cultured for 24 h in 1 ml of DMEM-10% FBS at a cell density of 10^5 cells per well in 12-well plates. The preadipocytes were then incubated with 0.25 ml of control RPMI, CM, or Ac CM (corresponding to 1×10^5 macrophages activated or not) and 0.75 ml DMEM/F12 induction medium (final concentration of 50 nM insulin, 100 nM dexamethasone, 0.25 mM inhibitor 1-methyl-3-isobutylxanthine, and 100 nM rosiglitazone) for 4 d (as described in Ref. 27). Next, this medium was replaced by 0.25 ml of control RPMI, CM, or Ac CM and 0.75 ml DMEM/F12 culture medium (final concentration of 50 nM insulin and 100 nM rosiglitazone). The medium was changed every 2 d until 10 to 12 d. For ATM CM, the experimental conditions were as described previously except that the proportion was 0.5 ml of ATM CM (corresponding to 2×10^5 AT macrophages) or control medium and 0.5 ml of culture medium. It should be noted that macrophage markers, Ham56 and Mac-1, staining tested negative in preadipocytes from the different experimental groups indicating the absence of remaining macrophages in cell culture.

Other cellular determinations

The cytotoxicity of macrophages conditioned media was assessed by measurement of lactate release in adipocytes culture medium (BioVision, Mountain View, CA). At the end of culture, the number of viable cells was measured using the MTS proliferation assay (Promega, Madison, WI) and by counting 4′-6 diamidino-2-phenyl indole-2HCl-stained nuclei. Differentiated preadipocytes were fixed with 4% paraformaldehyde for 10 min and Oil red O coloration was performed. After isopropanol extraction, quantification of lipids was performed by optical density measurements and normalized to cell protein levels as described in (29).

RNA preparation and real-time PCR

Differentiated preadipocytes were processed for RNA extraction using the RNeasy RNA Mini Kit (Qiagen, Courtaboeuf, France). Total RNAs (1 μg) were reversed transcribed using random hexamers and Supercript II reverse transcriptase (30). SYBR green primers for the tested genes are listed in Table 1. Real-time PCRs were conducted with 25 ng cDNA and both the sense and antisense oligonucleotides in a final volume of 20 μL using the SYBR green Taqman universal PCR mix (Applied Biosystems, Minneapolis, MN) monitored and assessed in a detection system instrument (Applied Biosystems) (30). All values were normalized according to 18S expression.

Western blot analysis

Cell protein extracts were prepared by cell lysing in RIPA buffer containing a cocktail of protease inhibitors (complete mini; Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (10 mM Naf and 1 mM sodium orthovanadate). Chemokine and adipokine contents were assessed in preadipocyte culture media. Protein concentrations were determined using a modified Lowry assay (Pierce, Rockford, IL). Proteins from cell lysates or culture media were resolved on sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were stained by Ponceau red to verify equal loading and transfer. Next, membranes were probed overnight at

TABLE 1. List of primer sequences used for real-time PCR

Gene	Forward	Reverse
h Adiponectin	TGTGATCTTGGCTCACTGTC	CAGCTACTTGGGAGGCTGA
h aP2	CCTTTAAAAATACTGAGATTTCCTTCA	GGACACCCCCATCTAAGGTT
h CD36	TGCCTATTCTTTGGCTTAATGAG	TTACTTGACTTCTGAACATGTTTGC
h C/EBPα	AGCAGGAGAAGGCCAAGG	CCCGGGTAGTCAAAGTCG
h C/EBPβ	CTGGAGACGCAGCACAAG	ACAGCTGCTCCACCTTCTTC
h CCND1	GAAGATCGTCGCACCTG	GACCTCCTCCTCGGACTTCT
h FAS	ACAGGGACAACCTGGAGTTCT	CTGTGGTCCCACTTGATGAGT
h IL6	GCCCAGCTATGAACTCCTTCT	GAAGGCAGCAGGCAACAC
h IL8	AGACAGCAGAGCACACAAGC	ATGGTTCCTTCCGGTGGT
h Leptin	AGAAAGTCCAGGATGACACC	GACTGCGTGTGTGAAATGTC
h MCP-1	TTCTGTGCCTGCTGCTCAT	GGGGCATTGATTGCATCT
h PPARγ	CAGGAAAGACAACAGACAAATCA	GGGGTGATGTGTTTGAACTTG
h SREBP-1c	GGAGGGGTAGGGCCAACGGCCT	CATGTCTTCGAAAGTGCAATCC

h, Human.

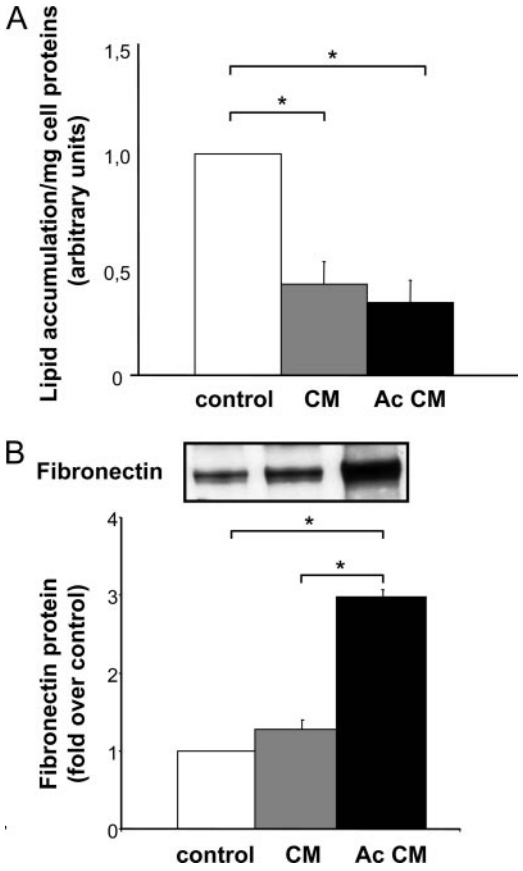

FIG. 1. Altered differentiation of human preadipocytes by macrophage CM. Preadipocytes were differentiated for 10–12 d with control, CM, or Ac CM. A, Oil red O quantification (mean ± SEM) in four independent cultures performed in quadruplicate are shown. B, Cell extracts were immunoblotted for fibronectin (*inset*). Graphs show quantification of the immunoblot signals. Mean ± SEM for three independent experiments. *, $P < 0.01$.

4 C with primary antibodies [monoclonal human fibronectin 610077; BD Transduction Laboratories, San Jose, CA; p65 NF-κB (9^3H1), phospho Ser 536 p65 NF-κB (7F1) and I-κBα; Cell Signaling Technology, Beverly, MA]. IL-6, IL-8, adiponectin, and monocyte chemotactic protein-1 antibodies were obtained from R&D Systems (Minneapolis, MN). Specific signals were detected with the ECL detection solution (Amersham Biosciences) and immediately exposed to x-ray films. Signals were quantified by densitometry.

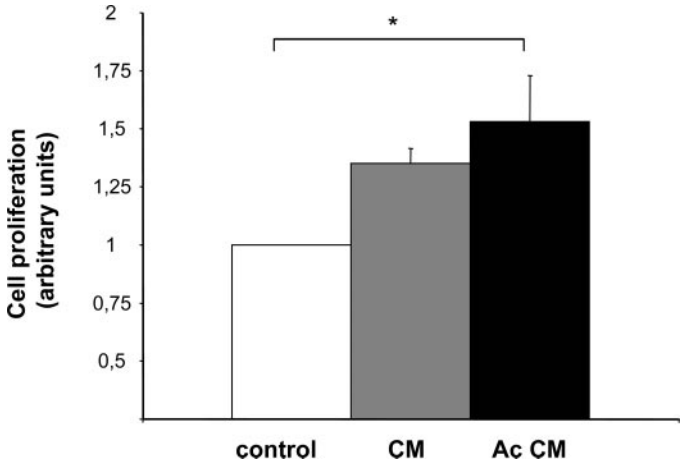

FIG. 2. Increased proliferation of human preadipocytes in presence of Ac CM. MTS proliferation assay was performed on preadipocytes differentiated for 10 d with control, CM, or Ac CM. Data are mean ± SEM of four separate experiments performed in quadruplicate. *, $P < 0.05$.

Immunofluorescence analysis

Preadipocytes were differentiated on glass coverslips in 24-well plates in the presence or not of CM or Ac CM. After 10 d, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. Coverslips were incubated with primary antibody for 1 h (monoclonal human fibronectin; BD Transduction Laboratories) and then with Cy2-conjugated antimouse IgG (Amersham Biosciences). Nuclei were stained with 4′-6 diamidino-2-phenyl indole-2HCl. Coverslips were examined with an Olympus BX 41 fluorescence microscope.

Measurements of cytokines in CM

Human IL-6 and TNFα immunoassays (R&D Systems) to measure cytokine concentrations in macrophage CM were performed according to the manufacturer's protocols.

Statistical analysis

Data are expressed as the mean ± SEM. The experiments were performed at least three times, each using preadipocytes from different human subjects and distinct macrophage CM. Statistical analysis was performed using a Student's *t* test. Comparisons between more than two groups were carried out using a one-way analysis of variance analysis in which $P < 0.05$ was considered statistically significant.

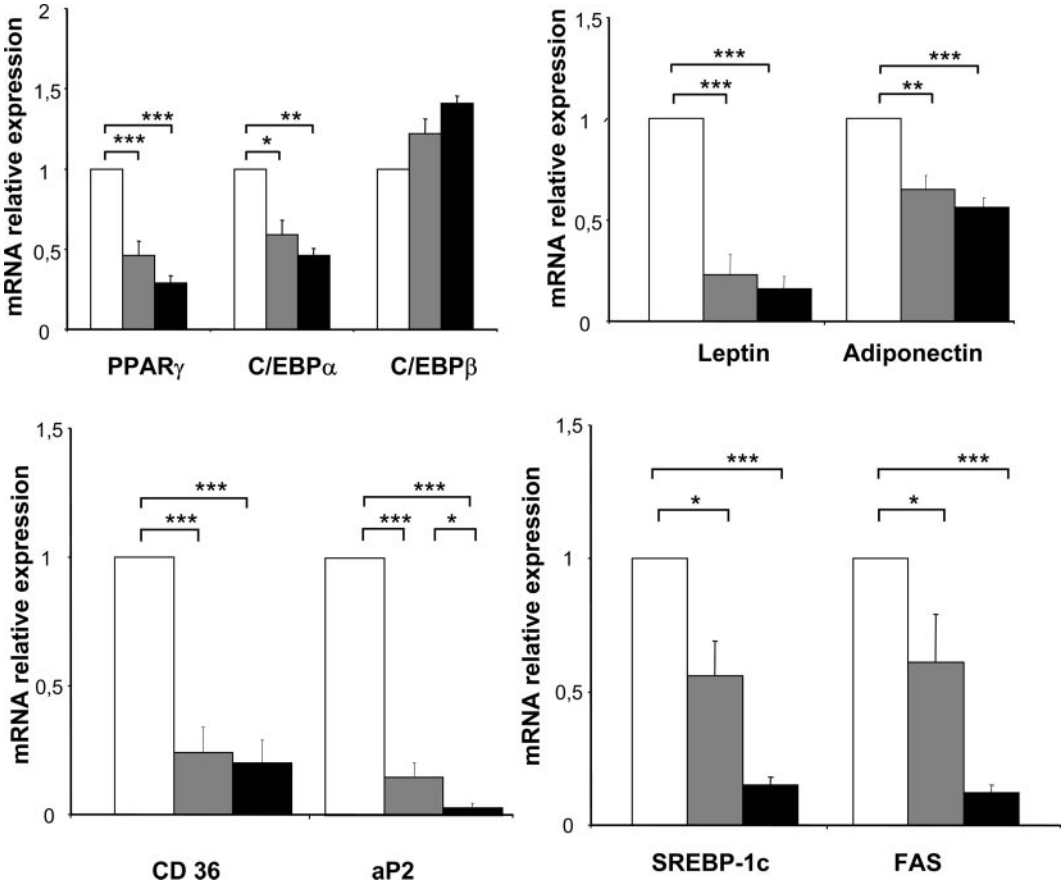

FIG. 3. Altered gene expression in human preadipocytes by CM or Ac CM. Preadipocytes were differentiated for 10 d in presence of control (*open bars*), CM (*gray bars*), or Ac CM (*black bars*). Adipose transcriptional factors, their target genes, adipokines, and lipogenic factors were quantified by real-time PCR. Data are mean ± SEM of six separate experiments in duplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

Human adipocyte differentiation is inhibited by macrophage-secreted factors

In a first series of experiments, human preadipocytes were cultured in the presence of control medium or conditioned medium from monocyte-derived macrophages without (CM) or after activation with LPS (Ac CM). The culture medium was supplemented with CM or Ac CM at d 1 and during the whole differentiation process. In control cultures, preadipocytes became spherical with lipid droplets in 75–80% of the cells at d 10–12. In contrast, preadipocytes cultured in the presence of CM or Ac CM showed a dramatic reduction of lipid droplets (−59% and −67%, respectively) (Fig. 1). These cells conserved a complete fibroblastic appearance. In addition, preadipocytes cultured in presence of Ac CM exhibited 3-fold more fibronectin-rich matrix than control or CM-treated cells as detected by immunofluorescence (data not shown) and Western blotting (Fig. 1).

The concentration of lactate measured in the culture medium at d 10, in three independent experiments, was similar in the absence or in presence of CM or Ac CM (data not shown), indicating minimal cytotoxicity of macrophage-conditioned media. The degree of adipose differentiation, as assessed by visual aspect of lipid droplet content, was unaffected by a conditioned media obtained from human epithelial kidney cells 293 cells (data not shown). This observation reinforces the specificity of the observed effect of macrophage CM on adipocyte differentiation.

The number of viable cells at the end of the differentiation process (10 d) was not significantly changed by the presence of CM. By contrast, 50% increased number of cells was counted in culture supplemented with Ac CM (Fig. 2). These data indicate that in the presence of CM or Ac CM, human preadipocytes failed to differentiate and continued to proliferate in presence of Ac CM.

Macrophage-secreted factors alter gene expression in human preadipocytes

To elucidate the molecular mechanisms involved in the impairment of adipogenesis induced by CM or Ac CM, we examined the gene expression levels of several specific adipose markers. As shown in Fig. 3, the gene expression of PPARγ2 and C/EBPα was diminished by 50% and 70%, respectively, in CM- and Ac CM-treated preadipocytes, whereas that of C/EBPβ remained unchanged. The expression of leptin and adiponectin was also reduced by 75% and 45%, respectively, with no further effect of Ac CM *vs.* CM. The inhibitory effect of CM or Ac CM on CD36 and aP2 gene expression was even more pronounced. The expression of SREBP-1c and FAS was decreased by 40% in CM-treated cells

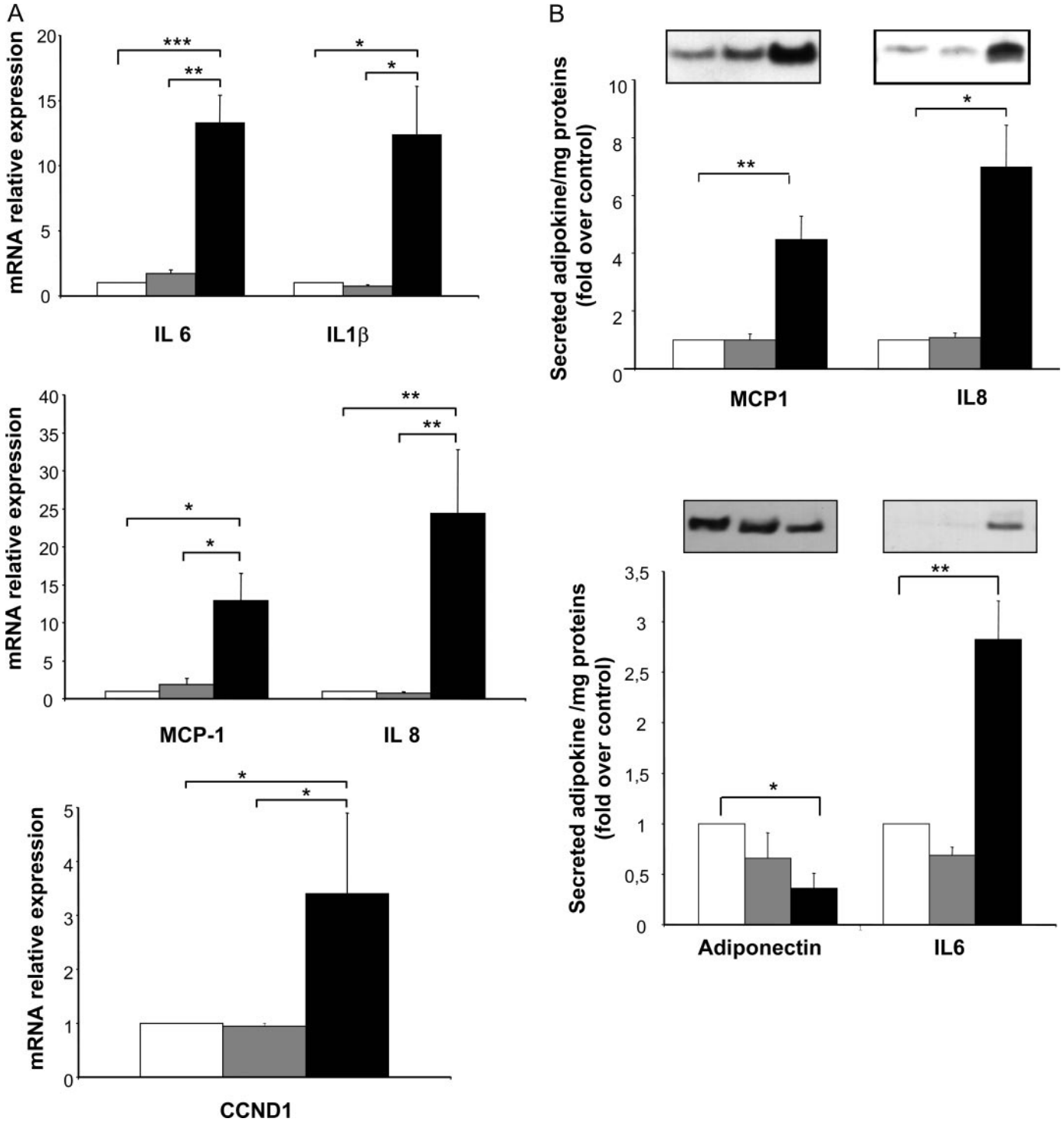

FIG. 4. Increased inflammatory markers in human preadipocytes in presence of Ac CM. Preadipocytes differentiated in the presence of control (*open bars*), CM (*gray bars*), or Ac CM (*black bars*) for 1 d and next were treated for 4 h additional by the corresponding media. A, Inflammatory markers were quantified by real-time PCR. B, The cells were placed in fresh DMEM/F12 medium with protease inhibitors. After 24 h, the media were immunoblotted for monocyte chemotactic protein-1, IL-6, IL-8, and adiponectin. Graphs show quantifications of the immunoblot signals or real-time PCR. Mean ± SEM from four independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and by 80% in Ac CM-treated preadipocytes. Of note, when the human preadipocytes were treated with CM or Ac CM exclusively during the first 4 d of culture, *i.e.* during the induction period, the expression of PPARγ, aP2, leptin, and adiponectin remains stable (data not shown) indicating a minimal effect on adipose differentiation in this experimental condition.

The effect of CM or Ac CM on the inflammatory state of the

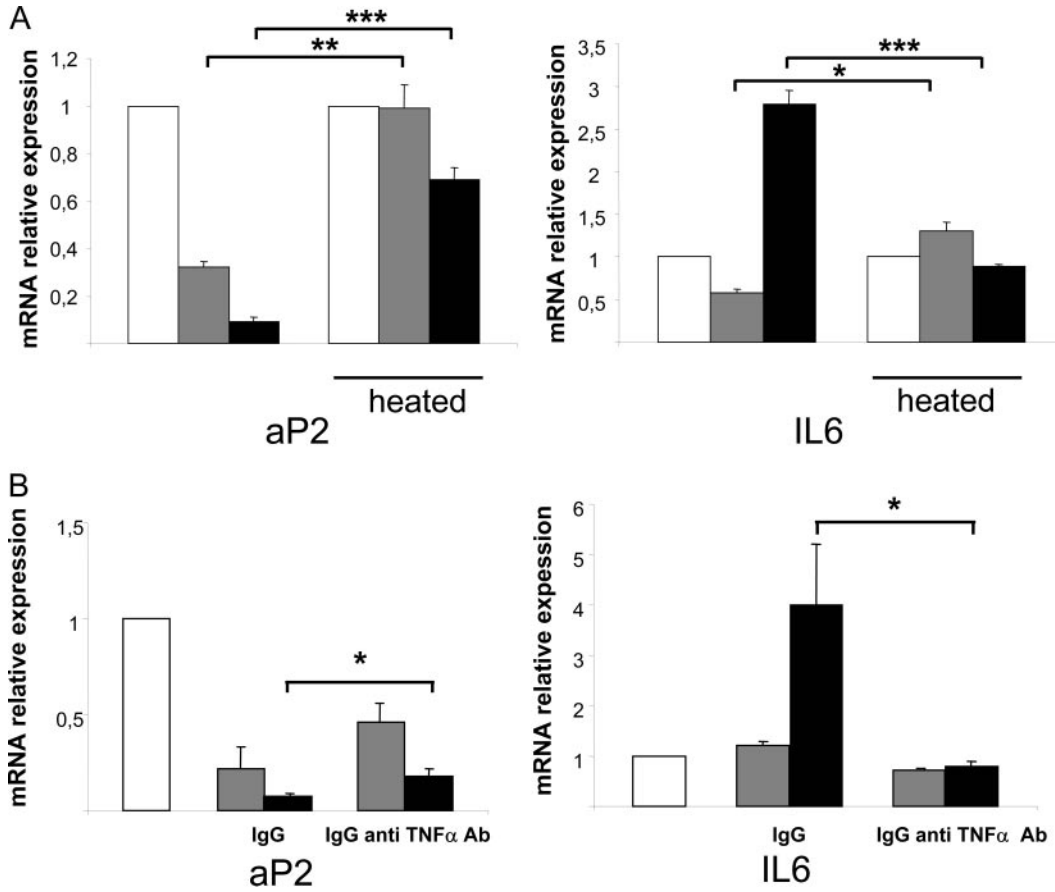

FIG. 5. Role of the macrophage-secreted TNFα on preadipocyte functions. A, Gene expression of human preadipocytes treated or not by native, heated macrophage CM. Preadipocytes were differentiated for 10 d in the presence of native or heated control (*open bars*), CM (*gray bars*), or Ac CM (*black bars*). AP2 and IL-6 mRNAs were quantified by real-time PCR. Data are mean ± SE from two separate experiments. B, Role of macrophage-secreted TNFα on preadipocyte phenotype. Preadipocytes were differentiated for 10 d with control (*open bars*), CM (*gray bars*), or Ac CM (*black bars*) in the absence of nonimmune IgG (IgG, nonimmune IgG, 2 μg/ml) or the presence of anti-TNFα antibody (IgG anti-TNFα Ab, 2 μg/ml). AP2 and IL-6 mRNAs were quantified by real-time PCR. Data are mean ± SEM from three separate experiments. *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$.

TABLE 2. Effects of increasing doses of CM (CM and Ac CM) on aP2 and IL-6 expressions by human preadipocytes

	aP2 (relative expression)	IL6 (relative expression)
Corresponding no. of macrophages (CM)		
0	1	1
0.5×10^5	0.75 ± 0.25	2.1 ± 1.1
1×10^5	0.41 ± 0.12	2.1 ± 0.9
2×10^5	0.42 ± 0.11	2.0 ± 0.2
Corresponding no. of activated macrophages (AcCM)		
0	1	1
0.5×10^5	0.80 ± 0.20	16.1 ± 4.8
1×10^5	0.33 ± 0.10	21.6 ± 3.7
2×10^5	0.18 ± 0.06	47.5 ± 15.8

Preadipocytes were differentiated for 10 d with an additional 4-h period in the presence of control or respective CM corresponding to various numbers of unactivated (CM) or activated macrophages (Ac CM). AP2 and IL6 mRNAs were quantified by real-time PCR. Data are the mean ± SEM from three to four experiments performed in duplicate.

preadipocytes was investigated by measuring the expression and release of selected proinflammatory markers, including the cytokines IL-1β, IL-6, and the chemokines monocyte chemotactic protein-1 and IL-8. Only Ac CM induced a very strong induction of these markers (15-fold for IL-1β, monocyte chemotactic protein-1 and IL-6 and 20-fold for IL-8) (Fig. 4A). In agreement with the gene expression findings, Ac CM- but not CM-treated preadipocytes exhibited markedly increased release of monocyte chemotactic protein-1 and IL-6 (5-fold) and of IL-8 (7-fold) (Fig. 4B). In contrast to the other markers, adiponectin decreased markedly in the culture medium in the presence of Ac CM.

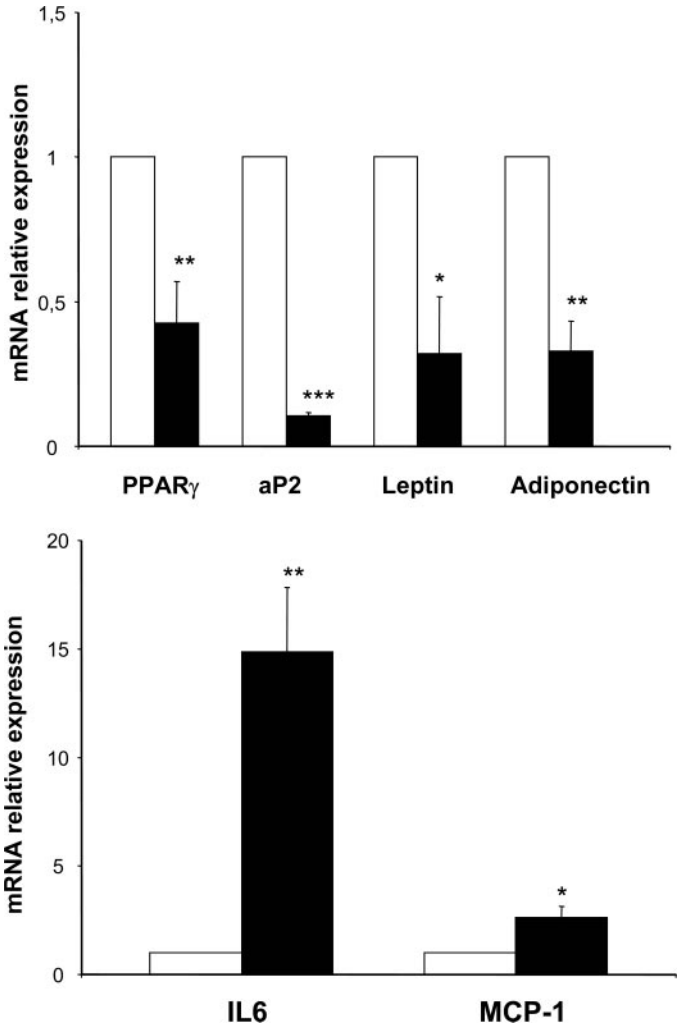

FIG. 6. Altered gene expression and increased inflammatory markers in presence of adipose tissue macrophage-secreted factors. Preadipocytes were differentiated in the presence of control (*open bars*) or ATM CM (*black bars*) for 10 d. Adipocyte (A) and inflammatory markers (B) were quantified by real-time PCR. Data are mean ± SEM of four separate cultures using two distinct ATM CM preparations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Nature of the macrophage-secreted factors involved in the alterations of preadipocyte functions

To determine the macrophage-secreted factors involved in the alteration of preadipocyte functions, CM was boiled to eliminate heat-sensitive factors. Heat denaturation markedly altered the inhibitory effects of CM or Ac CM on the gene expression of the adipose specific marker aP2. The IL-6 induction by boiled Ac CM was greatly reduced compared with native Ac CM (Fig. 5A). We next investigated the role of TNFα on preadipocyte functions by using anti-TNFα neutralizing antibody treatment. TNFα is mostly produced by macrophages and was recently shown to provoke inflammation changes in 3T3-L1 adipocytes (24). The inhibitory effect of CM or Ac CM on the aP2 gene expression was slightly corrected by blocking TNFα. In contrast, the strong induction of IL-6 by Ac CM was markedly inhibited by the anti-TNFα neutralizing antibody treatment suggesting an important role for TNFα in the preadipocyte inflammation (Fig. 5B). In addition, dose–response experiments using CM and Ac CM showed that Ac CM but not CM provoked a dose-dependent IL-6 induction. In contrast, aP2 gene expression was dose-dependently decreased by CM and Ac CM (Table 2).

Human adipose tissue macrophage-secreted factors decrease adipose marker gene expression

Next, we tested the effect of conditioned media of macrophages isolated from human adipose tissue (ATM CM). Because the effect of CM from monocyte-derived macrophages was observed with CM or Ac CM added at d 1 and during the whole period of differentiation, the same protocol was used to test the effect of ATM CM. The expression of adipocyte gene markers was clearly decreased in preadipocytes treated with ATM CM, including PPARγ2 (−65%), aP2 (−80%), leptin (−70%), and adiponectin (−65%) (Fig. 6). In addition, these preadipocytes also exhibited a strong inflammatory status evidenced by increased gene expression of IL-6 (16.5-fold) and monocyte chemotactic protein-1 (2.5-fold). These effects were strikingly similar to those elicited by Ac CM on the human preadipocytes (compare Fig. 6 with Figs. 3 and 4A).

NF-κB pathway is activated in preadipocytes in response to Ac CM

Because the NF-κB pathway is a key transcriptional regulator of inflammation, we hypothesized that CM and Ac CM activate this signaling system in the human preadipocytes. As shown in Fig. 7, the preadipocyte incubation with Ac CM, but not CM, rapidly increased by 2-fold the active form of NF-κB subunit, phospho NF-κB p65 (Ser 536). I-κBα protein, which sequesters the p50 and p65 NF-κB subunits in the cytosol, was decreased by 70% after Ac CM treatment. The protein expression of p50 NF-κB subunit precursor, p105, itself a NF-κB target (31), was increased by the chronic treatment of preadipocytes with Ac CM. These data indicate that Ac CM promotes NF-κB activation. The increased expression and release of IL-1β, IL-6, IL-8, and monocyte chemotactic protein-1 (Fig. 4), which are known NF-κB gene targets, supports the activation of this pathway in Ac CM-treated preadipocytes. In addition, the expression of cyclin D1, which is induced in response to NF-κB activation, was increased 2.5-fold in Ac CM-treated preadipocytes (Fig. 4A).

Discussion

By using an experimental approach in which human preadipocytes were differentiated in the presence of conditioned media from monocyte-derived or adipose tissue macrophages, we demonstrated that products secreted from macrophages deeply decrease the differentiation capacity of sc preadipocytes. The defective adipogenesis potential of human preadipocytes was clearly demonstrated by a significant decrease in the expression of two key genes of adipocyte differentiation (32, 33), PPARγ2 and C/EBPα, when preadipocytes are exposed to macrophage-derived factors all along the culture period. Molecules produced by macrophages rep-

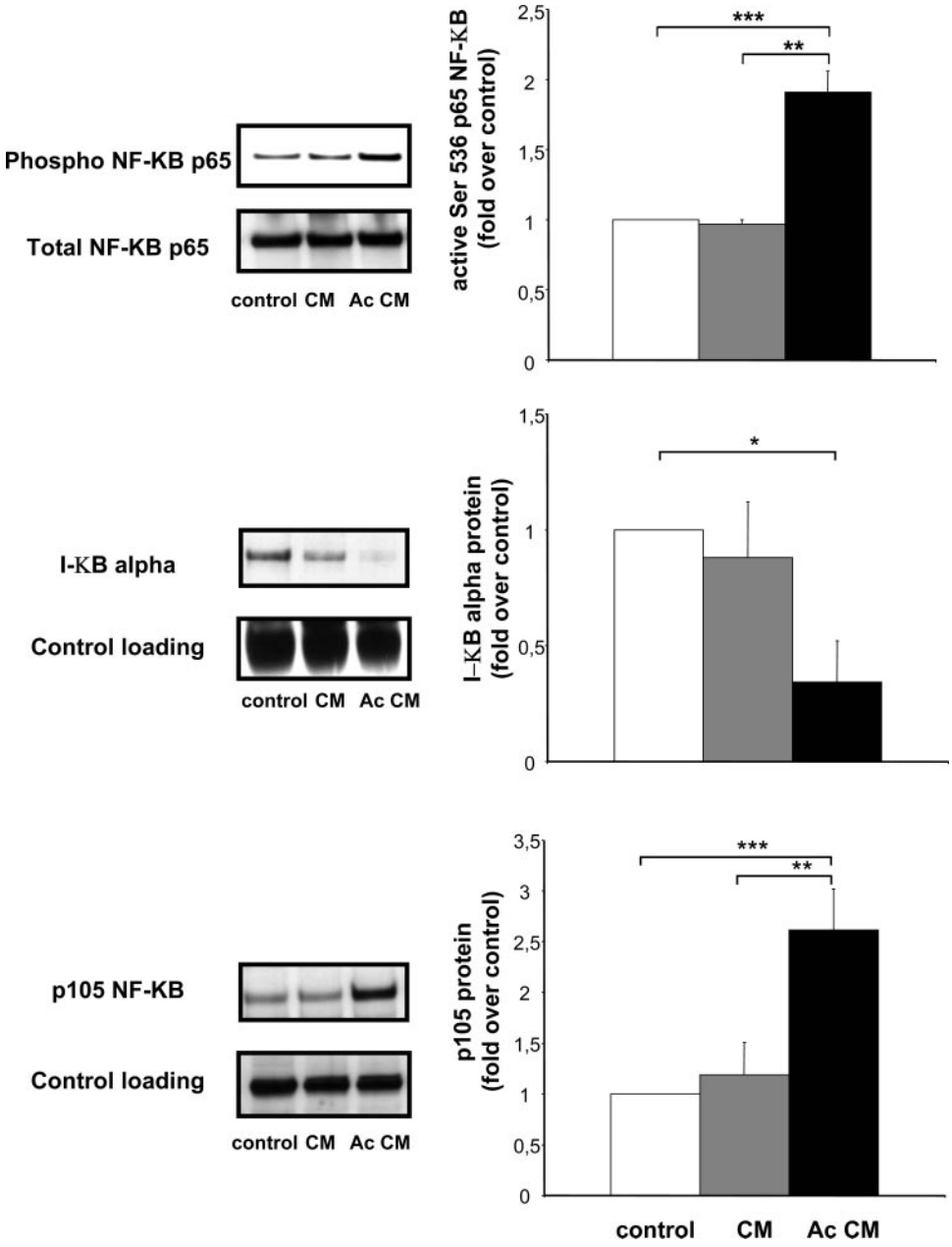

FIG. 7. NF-κB pathway activation of human preadipocytes in the presence of CM or Ac CM. Preadipocytes differentiated for 10–12 d with control, CM, or Ac CM were incubated for 15 min with control, CM, or Ac CM, respectively. Cell extracts were immunoblotted for phospho p65 NF-κB (ser 536), I-κBα, and p105 NF-κB. Control loading was total NF-κB or nonspecific bands as indicated. Graphs show quantification of the immunoblots. Mean ± SEM from four independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

resent a physiological cocktail of biomolecules probably acting synergistically on PPARγ2 and C/EBPα, both transcription factors known to function in a positive regulatory loop to activate adipogenesis (34). By contrast, C/EBPβ expression was unaffected, suggesting that this transcription factor is not critical for the effect of macrophage-derived factors on PPARγ2 and C/EBPα gene expression at this stage of human adipose differentiation.

Until now, the role of inflammation molecules on adipogenesis was addressed mostly on adipocyte rodent cell lines by exploring the individual effect of a limited number of cytokines. IL-1β and TNFα have been described as strong suppressors of adipogenesis (18–20) and these cytokines exert their inhibitory effects through suppression of PPARγ expression and activity via NF-κB activation (20, 35). However, macrophages produce a huge variety of biomolecules such as growth factors (transforming growth factor β, vascular endothelial growth factor), cytokines (TNFα, IL-1β, IL-6), proteolytic enzymes, and metabolites (reactive oxygen species, nitric oxide, and prostaglandins) (12) that needs to be precisely characterized in macrophages infiltrating human adipose tissue. The production of these inflammatory molecules is exacerbated in activated macrophages. It is plausible that a spectrum of biomolecules acting synergisti-

cally is involved in the inhibition of adipogenesis through various pathways. Here, we observed that Ac CM, but not CM, induced NF-κB activation in preadipocytes as well as the modulation of several target genes. This could be related to the high levels of TNFα and IL-6 produced by LPS-activated macrophages; also, other factors cannot be excluded. The preadipocyte inflammation induced by Ac CM could then self-perpetuate through increased secretion of inflammatory factors. Blocking TNFα totally inhibited the IL-6 mRNA induction by Ac CM. Because TNFα is mostly secreted by macrophages, we suggest that this cytokine is a major mediator of preadipocyte inflammation. However, it cannot be excluded that other macrophage-secreted factors may also be induced during the proinflammatory state of preadipocytes. In contrast, blocking TNFα did not totally reverse the defective adipogenesis provoked by CM and Ac CM indicating mostly a TNFα-independent process. However, the preadipocyte inflammation could aggravate the altered differentiation process because the inhibitory effects were more pronounced with Ac CM than for CM at least for some adipose-specific markers (aP2, SREBP1-c and FAS; Fig. 3).

During adipose differentiation of murine cell lines, extensive extracellular matrix remodeling takes place characterized by decreased expression of fibronectin (13–15). Moreover, culture of human preadipocytes on fibronectin matrix markedly inhibits adipogenesis (36). Activated macrophage secreted cytokines might participate to increase fibronectin. Such a role has been clearly demonstrated for TNFα in 3T3-L1 cells (19). In addition, fibronectin appears to promote cell proliferation through the induction of cyclin D1 (37), a cell-cycle entry protein, which is a target gene of NF-κB (38). Therefore, potential links involving activated NF-κB pathway, cyclin D1 and fibronectin, could be established between the inflammatory state and the increased proliferation of Ac CM-treated preadipocytes. Due to a scarcity of material, not all these parameters were assessed in preadipocytes cultured in presence ATM CM. Nevertheless, the marked increase in IL-6 and MCP-1 gene expression strongly suggests that adipose tissue macrophage secreted factors generate NF-κB activation-mediated inflammatory state in these cells alike. Of note, such molecular links are unlikely to operate in CM-treated preadipocytes, which display reduced adipogenesis, but no alteration in NF-κB activation, fibronectin levels, and cyclin D1 gene expression. Further experiments examining gene expression and canonical pathways are needed to provide information on the specific molecular mechanisms involved in this condition.

The significance of the proinflammatory state of preadipocytes and their defective engagement toward adipogenesis need to be understood, particularly in obesity, which is characterized by macrophage infiltration. In particular, signals favoring macrophage infiltration in adipose tissue remains poorly understood. Monocyte chemotactic protein-1 and IL-8, which play crucial role for the recruitment of immune cells in inflammatory zones, could be candidates. The role of monocyte chemotactic protein-1 was evidenced by recent data showing that CCR2 (monocyte chemotactic protein-1 receptor) deficiency reduced macrophage content of adipose tissue in obese mice (39). The monocyte chemotactic protein-1 and IL-8 releases by human adipose tissue, mainly attributed to the stroma vascular cells, are increased during obesity (40, 41). Our data clearly showed increased expression of IL-8 and/or monocyte chemotactic protein-1 by Ac CM- and ATM CM-treated preadipocytes. These chemokines, and probably others, produced by the inflammatory preadipocytes could participate with other stroma vascular cells to macrophage recruitment and maintenance in adipose tissue.

In summary, our data suggest that in adipose tissue, and particularly in the obesity context, the factors secreted by infiltrated macrophages inhibit the formation of new adipocytes and facilitate their proliferation. This phenomenon could represent an adaptive response to limit fat expansion in humans. However, it remains to be demonstrated whether the proliferative effect of activated macrophages on preadipocytes may represent a major determinant of resistance to weight loss or could lead to weight regain in obese subjects.

Acknowledgments

We thank Martine Pinçon-Raymond [Institut National de la Santé et de la Recherche Médicale (INSERM) Unité 505] for helpful discussions concerning ECM studies, Anne Bouloumié (INSERM Avenir) for providing ATM CM, Mièle Guerre-Millo and David Mutch for critical reading of the manuscript, and Dr. Franck Viguié (Cytogenetic Department, Hôtel Dieu) for providing immunofluorescence microscopy facilities. We thank Dr. Pierre Niccolo and Dr. Philippe Sellam (Surgery Department) for adipose tissue availability. We also acknowledge patients and physicians of the Nutrition department of Hôtel Dieu (Paris, France) for patients' recruitment.

Received May 23, 2006. Accepted October 25, 2006.

Address all correspondence and requests for reprints to: Danièle Lacasa, Institut National de la Santé et de la Recherche Médicale, Unité 755 EA 3502, Service de Nutrition Hôtel Dieu, 1 place du parvis Notre Dame, 75004 Paris, France. E-mail: daniele.lacasa@ea3502.org.

This work was supported by the French National Agency of Research (ANR, RIOMA No. ANR-05-PCOD-030-02) and grants from Alfediam/Antadir.

Author Disclosure Summary: All of the authors have nothing to disclose.

References

1. **Cottam DR, Mattar SG, Barinas-Mitchell E, Eid G, Kuller L, Kelley DE, Schauer PR** 2004 The chronic inflammatory hypothesis for the morbidity associated with morbid obesity: implications and effects of weight loss. Obes Surg 14:589–600
2. **Trayhurn P, Wood IS** 2004 Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br J Nutr 92:347–355
3. **Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW** 2004 Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology 145:2273–2282
4. **Clement K, Viguerie N, Poitou C, Carette C, Pelloux V, Curat CA, Sicard A, Rome S, Benis A, Zucker JD, Vidal H, Laville M, Barsh GS, Basdevant A, Stich V, Cancello R, Langin D** 2004 Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. FASEB J 18:1657–1669
5. **Curat CA, Wegner V, Sengenes C, Miranville A, Tonus C, Busse R, Bouloumie A** 2006 Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin. Diabetologia 49:744–747
6. **Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante Jr AW** 2003 Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 112:1796–1808
7. **Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H** 2003 Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 112:1821–1830
8. **Curat CA, Miranville A, Sengenes C, Diehl M, Tonus C, Busse R, Bouloumie**

A 2004 From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. Diabetes 53:1285–1292
9. **Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, Coupaye M, Pelloux V, Hugol D, Bouillot JL, Bouloumie A, Barbatelli G, Cinti S, Svensson PA, Barsh GS, Zucker JD, Basdevant A, Langin D, Clement K** 2005 Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. Diabetes 54:2277–2286
10. **Ghanim H, Aljada A, Hofmeyer D, Syed T, Mohanty P, Dandona P** 2004 Circulating mononuclear cells in the obese are in a proinflammatory state. Circulation 110:1564–1571
11. **Gordon S** 2003 Alternative activation of macrophages. Nat Rev Immunol 3:23–35
12. **Hume DA, Ross IL, Himes SR, Sasmono RT, Wells CA, Ravasi T** 2002 The mononuclear phagocyte system revisited. J Leukoc Biol 72:621–627
13. **Spiegelman BM, Ginty CA** 1983 Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes. Cell 35:657–666
14. **Rodriguez Fernandez JL, Ben-Ze'ev A** 1989 Regulation of fibronectin, integrin and cytoskeleton expression in differentiating adipocytes: inhibition by extracellular matrix and polylysine. Differentiation 42:65–74
15. **Antras J, Hilliou F, Redziniak G, Pairault J** 1989 Decreased biosynthesis of actin and cellular fibronectin during adipose conversion of 3T3-F442A cells. Reorganization of the cytoarchitecture and extracellular matrix fibronectin. Biol Cell 66:247–254
16. **Gregoire FM, Smas CM, Sul HS** 1998 Understanding adipocyte differentiation. Physiol Rev 78:783–809
17. **MacDougald OA, Mandrup S** 2002 Adipogenesis: forces that tip the scales. Trends Endocrinol Metab 13:5–11
18. **Petruschke T, Hauner H** 1993 Tumor necrosis factor-α prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. J Clin Endocrinol Metab 76:742–747
19. **Ruan H, Hacohen N, Golub TR, Van Parijs L, Lodish HF** 2002 Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-κB activation by TNF-α is obligatory. Diabetes 51:1319–1336
20. **Suzawa M, Takada I, Yanagisawa J, Ohtake F, Ogawa S, Yamauchi T, Kadowaki T, Takeuchi Y, Shibuya H, Gotoh Y, Matsumoto K, Kato S** 2003 Cytokines suppress adipogenesis and PPAR-γ function through the TAK1/TAB1/NIK cascade. Nat Cell Biol 5:224–230
21. **Dixit V, Mak TW** 2002 NF-κB signaling. Many roads lead to Madrid. Cell 111:615–619
22. **Chen LF, Greene WC** 2004 Shaping the nuclear action of NF-κB. Nat Rev Mol Cell Biol 5:392–401
23. **Permana PA, Menge C, Reaven PD** 2006 Macrophage-secreted factors induce adipocyte inflammation and insulin resistance. Biochem Biophys Res Commun 341:507–514
24. **Suganami T, Nishida J, Ogawa Y** 2005 A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor α. Arterioscler Thromb Vasc Biol 25:2062–2068
25. **Constant VA, Gagnon A, Landry A, Sorisky A** 2006 Macrophage-conditioned medium inhibits the differentiation of 3T3-L1 and human abdominal preadipocytes. Diabetologia 49:1402–1411
26. **Deslex S, Negrel R, Vannier C, Etienne J, Ailhaud G** 1987 Differentiation of human adipocyte precursors in a chemically defined serum-free medium. Int J Obes 11:19–27
27. **Hauner H, Entenmann G, Wabitsch M, Gaillard D, Ailhaud G, Negrel R, Pfeiffer EF** 1989 Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. J Clin Invest 84:1663–1670
28. **Patel L, Buckels AC, Kinghorn IJ, Murdock PR, Holbrook JD, Plumpton C, Macphee CH, Smith SA** 2003 Resistin is expressed in human macrophages and directly regulated by PPARγ activators. Biochem Biophys Res Commun 300:472–476
29. **Ramirez-Zacarias JL, Castro-Munozledo F, Kuri-Harcuch W** 1992 Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. Histochemistry 97:493–497
30. **Viguerie N, Clement K, Barbe P, Courtine M, Benis A, Larrouy D, Hanczar B, Pelloux V, Poitou C, Khalfallah Y, Barsh GS, Thalamas C, Zucker JD, Langin D** 2004 *In vivo* epinephrine-mediated regulation of gene expression in human skeletal muscle. J Clin Endocrinol Metab 89:2000–2014
31. **Ten RM, Paya CV, Israel N, Le Bail O, Mattei MG, Virelizier JL, Kourilsky P, Israel A** 1992 The characterization of the promoter of the gene encoding the p50 subunit of NF-κB indicates that it participates in its own regulation. EMBO J 11:195–203
32. **Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, Milstone DS, Spiegelman BM, Mortensen RM** 1999 PPARγ is required for the differentiation of adipose tissue in vivo and in vitro. Mol Cell 4:611–617
33. **Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, Gonzalez FJ, Spiegelman BM** 2002 C/EBPα induces adipogenesis through PPARgamma: a unified pathway. Genes Dev 16:22–26
34. **Brun RP, Spiegelman BM** 1997 PPARγ and the molecular control of adipogenesis. J Endocrinol 155:217–218
35. **Xing H, Northrop JP, Grove JR, Kilpatrick KE, Su JL, Ringold GM** 1997 TNFα-mediated inhibition and reversal of adipocyte differentiation is accompanied by suppressed expression of PPARγ without effects on Pref-1 expression. Endocrinology 138:2776–2783
36. **O'Connor KC, Song H, Rosenzweig N, Jansen DA** 2003 Extracellular matrix substrata alter adipocyte yield and lipogenesis in primary cultures of stromal-vascular cells from human adipose. Biotechnol Lett 25:1967–1972
37. **Danen EH, Yamada KM** 2001 Fibronectin, integrins, and growth control. J Cell Physiol 189:1–13
38. **Fu M, Wang C, Li Z, Sakamaki T, Pestell RG** 2004 Minireview: cyclin D1: normal and abnormal functions. Endocrinology 145:5439–5447
39. **Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, Ferrante Jr AW** 2006 CCR2 modulates inflammatory and metabolic effects of high-fat feeding. J Clin Invest 116:115–124
40. **Bruun JM, Lihn AS, Madan AK, Pedersen SB, Schiott KM, Fain JN, Richelsen B** 2004 Higher production of IL-8 in visceral vs. subcutaneous adipose tissue. Implication of nonadipose cells in adipose tissue. Am J Physiol Endocrinol Metab 286:E8–E13
41. **Bruun JM, Lihn AS, Pedersen SB, Richelsen B** 2005 Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT. J Clin Endocrinol Metab 90:2282–2289

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**