Impaired Preadipocyte Differentiation in Human Abdominal Obesity

Role of Wnt, Tumor Necrosis Factor-α, and Inflammation

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OBJECTIVE—We examined preadipocyte differentiation in obese and nonobese individuals and the effect of cytokines and wingless-type MMTV (mouse mammary tumor virus) integration site family, member 3A (Wnt3a) protein on preadipocyte differentiation and phenotype.

RESEARCH DESIGN AND METHODS—Abdominal subcutaneous adipose tissue biopsies were obtained from a total of 51 donors with varying BMI. After isolation of the adipose and stromalvascular cells, inflammatory cells (CD14- and CD45-positive cells) were removed by immune magnetic separation. CD133-positive cells, containing early progenitor cells, were also isolated and quantified. The CD14- and CD45-negative preadipocytes were cultured with tumor necrosis factor (TNF)-α, interleukin (IL)-6, resistin, or Wnt3a with or without a differentiation cocktail.

RESULTS—The number of preadipocytes able to differentiate to adipose cells was negatively correlated with both BMI and adipocyte cell size of the donors, whereas the number of CD133-positive cells was positively correlated with BMI, suggesting an impaired differentiation of preadipocytes in obesity. Cultured preadipocytes, like freshly isolated mature adipocytes, from obese individuals had an increased expression of mitogen-activated protein 4 kinase 4 (MAP4K4), which is known to inhibit peroxisome proliferator–activated receptor-γ induction. TNF-α, but not IL-6 or resistin, increased Wnt10b, completely inhibited the normal differentiation of the preadipocytes, and instead induced a proinflammatory and macrophage-like phenotype of the cells.

CONCLUSIONS—The apparent number of preadipocytes in the abdominal subcutaneous tissue that can undergo differentiation is reduced in obesity with enlarged fat cells, possibly because of increased MAP4K4 levels. TNF-α promoted a macrophage-like phenotype of the preadipocytes, including several macrophage markers. These results document the plasticity of human preadipocytes and the inverse relationship between lipid storage and proinflammatory capacity. ***Diabetes*** **58:1550–1557, 2009**

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In adult humans, the increased adipose tissue mass in obesity mainly leads to an expansion of the size of the prevailing adipose cells (1,2). However, there is also a continuous turnover of the adipose cells, and thus recruitment of new adipocytes (2–4).

The development of insulin resistance and its complications are predominantly seen in conjunction with abdominal adipose tissue distribution and associated cell enlargement (hypertrophic or abdominal/upper-body obesity) rather than peripheral obesity, which is usually associated with a recruitment of new preadipocytes and thus small adipose cells (hyperplastic or peripheral/lower-body obesity) (5–7).

An important consequence of adipose cell enlargement is the development of local inflammation in the adipose tissue with infiltration of monocytes/macrophages (8,9). The reason for this is currently unclear but may be attributable to adipose cell death, where macrophages can be seen as scavengers of remaining debris and lipids (10).

In addition, adipose cell enlargement leads to increased secretion of cytokines and chemokines, which in turn attract monocytes/macrophages into the tissue (11–14). Both mechanisms may well be operative, and, in addition, local activation of the macrophages in the adipose tissue plays a key role (15), but the tissue factors involved are currently unknown.

Obesity-associated inflammation leads to highly dysregulated adipose tissue with an altered pattern of secreted adipokines and increased lipolysis (rev. in 14,16). Secretion of cytokines like tumor necrosis factor (TNF)-α in the adipose tissue impairs the differentiation of preadipocytes, reduces adiponectin secretion, and promotes a proinflammatory state, which in turn further promotes the local secretion of cytokines and chemokines (17,18). In addition, both interleukin (IL)-6 and TNF-α induce insulin resistance in the adipose cells at the level of insulin signaling and action because of reduced expression of insulin receptor substrate-1 and GLUT4 (17,19).

Obesity, both in animal models and in humans, is associated with an increase in different markers of inflammatory cells, such as CD68, macrophage inflammatory protein (MIP)-1α, EMR (epidermal growth factor–like module containing mucin-like hormone receptor), and ADAM-8 (a disintegrin and metalloproteinase domain-8) in the adipose tissue (rev. in 14,18,20). In fact, using such markers, Weisberg et al. (8) reported that up to 50% of the cells in the adipose tissue were positive for CD68 and thus could be classified as macrophages. They also reported that the CD68-positive cells were the major producers of the different cytokines studied (8).

Although there is no doubt that macrophages are present in the adipose tissue in obesity, it is highly unlikely that they could account for up to 50% of the cells. This raises the question of whether other cells present in the adipose tissue can also become positive for macrophage markers and under what conditions. However, several studies with human preadipocytes cultured in vitro have been unable to find these cells positive for CD68 or other macrophage markers (21). Nevertheless, both gene arrays and the report that 3T3-L1 cells injected into the peritoneal cavity of mice assumed a macrophage-like phenotype (22,23) support the close link between preadipocytes and macrophages.

Here, we asked whether human undifferentiated preadipocytes cultured in vitro could assume a macrophage-like phenotype if they were activated by proinflammatory molecules (TNF, IL-6, or resistin). During the course of the experiments, we also we found that the number of preadipocytes in subcutaneous abdominal adipose tissue that differentiated to adipose cells was negatively correlated with BMI as well as mean adipose cell size of the donors. A potential mechanism for this may be increased mitogen-activated protein 4 kinase 4 (MAP4K4) expression in preadipocytes from obese individuals because MAP4K4 inhibits peroxisome proliferator–activated receptor (PPAR)-γ activation as well as adipogenesis (24,25).

RESEARCH DESIGN AND METHODS

Abdominal subcutaneous adipose tissue was obtained from 51 different subjects by needle biopsy ($n = 45$) or bariatric surgery ($n = 6$). This procedure was approved by the ethical committee of the University of Gothenburg. Subjects were between 28 and 69 years of age and had a BMI (mean ± SE) of 28.8 ± 2.2 kg/m^2 (range 19.3–54.8).

Preadipocyte culture. Adipose tissue was digested with collagenase as previously reported and cell size measured (19). The medium under the isolated adipocytes was collected and centrifuged for 10 min at 200*g*. The cell pellet was washed twice and the erythrocytes were lysed with 155 mmol/l NH_4Cl for 5 min before seeding the cells in a 55-cm^2 petri dish. After 3 days, when the cells had started to proliferate, the progenitor or inflammatory cells were isolated with magnetic immune separation. The remaining cells were then cultured at 37°C with Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (1:1) with 10% fetal bovine serum (FBS), 2 mmol/l glutamine, 100 units/ml penicillin, and 100 μg/ml streptavidin. After 2 weeks cells were trypsinized and any remaining inflammatory cells removed by magnetic immune separation of CD14- and CD45-positive cells (Miltenyi Biotech, Bergisch Gladbach, Germany). The remaining preadipocyte fraction was seeded (10,000 cells/cm^2) and cultured in six-well plates (Nunc, Roskilde, Denmark). Cells were left untreated or grown in the presence of 5 ng/ml TNF-α, 20 ng/ml IL-6, 50 ng/ml resistin, or 10 ng/ml lipopolysaccharide for 10 days. In some experiments, 300 μmol/l oleic acid was added to the medium for 48 h after 10 days of culture.

Differentiation of the preadipocytes was induced with a differentiation cocktail consisting of 850 nmol/l insulin, 10 μmol/l dexamethasone, 0.5 mmol/l IBMX (isobutylmethylxanthine), 10 μmol/l pioglitazone, 33 μmol/l biotin, and 17 μmol/l pathenonate in DMEM/F12 supplemented with 3% FBS (vol/vol), 2 mmol/l glutamine, and antibiotics. After 3 days, the medium was changed to a medium containing only insulin, dexamethasone and pioglitazone in DMEM/F12, 10% FBS with glutamine, and antibiotics. (Human preadipocytes require the continuous presence of a thiazolidinedione to remain differentiated.) The cells were then left to differentiate for another 18 days with medium changed every other day. In some experiments 5 ng/ml TNF-α, 20 ng/ml IL-6, 50 ng/ml wingless-type MMTV (mouse mammary tumor virus) integration site family, member 3A (Wnt3a), or 50 ng/ml resistin was added to the differentiation medium.

Oil red O staining. To determine lipid accumulation, cells cultured in six-well plates were fixed with 10% formalin for 20 min and then stained with oil red O for 60 min. Optical density for oil red O was determined by washing the stained cells carefully, and, after removal of all water, 1 ml isopropanol was added to each well. The plates were left for 10 min and optical density measured at 510 nm. The absorbance of cells that had undergone differentiation was then related to that of undifferentiated cells (= 1).

Isolation of CD133-positive cells. After trypsin treatment, the total number of cells were counted in a hemocytometer and then labeled and isolated with magnetic beads against CD133. The CD133-enriched cell fraction was counted and the number of CD133-positive cells calculated.

Real-time PCR. RNA was isolated with RNeasy (Qiagen, Hilden, Germany), and 10 ng total RNA was used for cDNA synthesis using a high-capacity cDNA reverse-transcription kit (Applied Biosystems, Foster City, CA). An ABI Prism 7900 sequence detection system (Applied Biosystems) was used to analyze gene expression. On-demand primers and probes (Applied Biosystems) or custom-designed reagents were used. The sequences are available on request. For real-time PCR, 10 ng cDNA, 100 nmol/l probes, and 200 nmol/l of both reverse as well as forward primers were used and the procedure was performed as recommended by the manufacturer. Relative quantification of mRNA levels was plotted as the fold change compared with the basal state, using 18S rRNA as endogenous control (Applied Biosystems).

Enzyme-linked immunosorbent assay. Secreted cytokines and chemokines (IL-1β, IL-6, IL-8, and monocyte chemoattractant protein [MCP]-1) were determined in the cell culture medium by enzyme-linked immunosorbent assay (ELISA) techniques according to the manufacturer's instructions (Biosource, Carlsbad, CA). Secretion of granulocyte-macrophage–colony-stimulating factor (GM-CSF), serum amyloid A (SAA), and intercellular adhesion molecule (ICAM) was measured by multiplex electrochemiluminescence using Meso Scale Discovery technology (Gaithersburg, MD), and levels of TNF-α were measured by high-sensitivity human TNF-α Quantikine ELISA (R&D Systems, Minneapolis, MN).

Acetylated LDL uptake. Cells were washed with PBS and incubated with medium containing 10 μg/ml Dil-Ac-LDL (Invitrogen, Carlsbad, CA) for 4 h at 37°C. After incubation, cells were washed with PBS and fixed with 3% formalin for 10 min. Fluorescence was visualized using standard rhodamine excitation/emission filters. Human primary macrophages were used as positive controls.

Statistical analyses. All statistical analyses were performed with SPSS version 11 programs. Wilcoxon's nonparametric test for paired observations was used to evaluate differences between the different conditions. $P < 0.05$ was considered statistically significant.

RESULTS

Impaired differentiation of abdominal subcutaneous preadipocytes from obese subjects. We plated the remaining stromal-vascular cells after the CD14- and CD45-positive inflammatory cells had been removed by magnetic immune separation. After addition of the differentiation cocktail, the CD14- and CD45-negative cells accumulated lipids, and the expression of adiponectin and GLUT4 was increased (Fig. 1*A*), showing that these cells indeed were preadipocytes.

The number of preadipocytes that differentiated varied markedly between the patients and ranged from 20 to 90% (Fig. 1*B*). Interestingly, there was a highly significant negative correlation between BMI of the donor and number of preadipocytes differentiating to adipocytes, as reflected by oil red O accumulation (Fig. 1*C*). Although the results shown in Fig. 1*C* also included five type 2 diabetic individuals, the results were virtually identical when these were excluded from the analyses ($r = 0.594$, $P < 0.01$, $n = 20$, vs. $r = 0.549$, $P < 0.01$, $n = 15$). Negative correlation was also found between preadipocyte differentiation and average adipocyte size of the donors (Fig. 1*D*), which in turn is a marker of insulin sensitivity (5–8).

The negative correlation between BMI, cell size, and number of preadipocytes able to differentiate can either be attributable to a reduced number of preadipocytes in the abdominal adipose tissue in obesity and/or to an impaired ability of these cells to differentiate. One reason could be fewer progenitor cells, including mesenchymal stem cells, which undergo commitment to preadipocytes in vivo. To address this, we isolated CD133-positive cells because we (unpublished data) and others (26,27) have shown that this population contains pluripotent cells that can be differentiated to adipose, bone, and muscle cells.

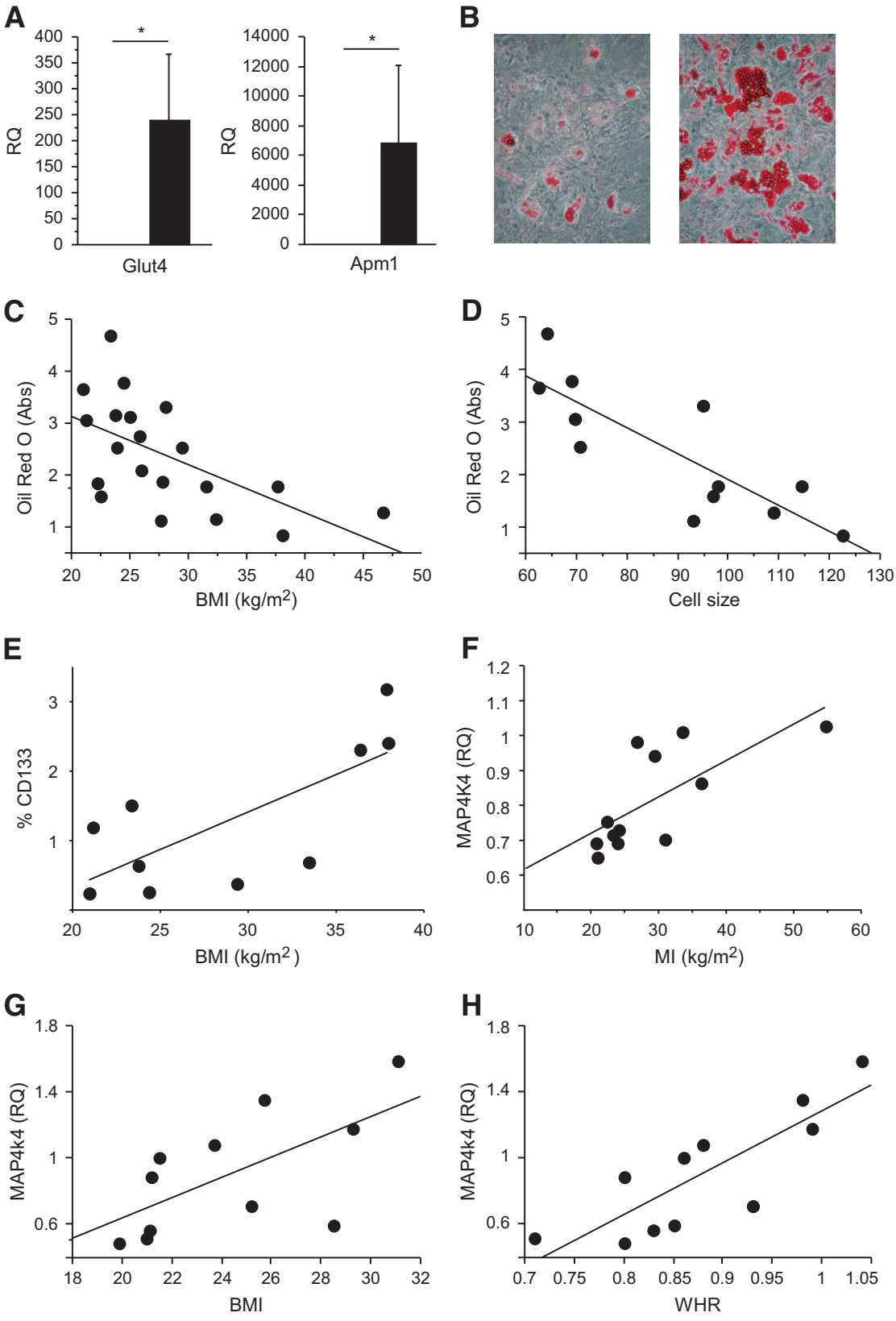

FIG. 1. Impaired differentiation of human preadipocytes from obese donors. *A*: mRNA expression of GLUT4 and adiponectin (Apm1) before (□ = 1) and after (■) adipocyte differentiation. Data are the means ± SE from six different subjects. *$P < 0.05$. *B*: Oil red O staining (absorbance) of primary human preadipocytes differentiated for 21 days from two different donors with varying percentage of differentiation. *C*: BMI of the preadipocyte donor is significantly and inversely correlated to the degree of preadipocyte differentiation ($R^2 = 0.59$, $P = 0.006$). *D*: Correlation between preadipocyte differentiation and mean adipocyte size of the donors ($R^2 = 0.69$, $P < 0.001$). *E*: The relative number of CD133-positive cells in the stromal vascular fraction is positively correlated with BMI of the donor ($R^2 = 0.51$, $P < 0.01$). *F*: mRNA levels of MAP4K4 in cultured preadipocytes in relation to BMI of the donors ($R^2 = 0.42$, $P < 0.05$). *G* and *H*: Correlation between MAP4K4 mRNA levels in isolated mature adipose cells and BMI ($R^2 = 0.41$, $P < 0.04$) (*G*) or waist-to-hip ratio (WHR) (*H*) of the donors ($R^2 = 0.67$, $P = 0.002$). Abs, absorbance; RQ, relative quantification.

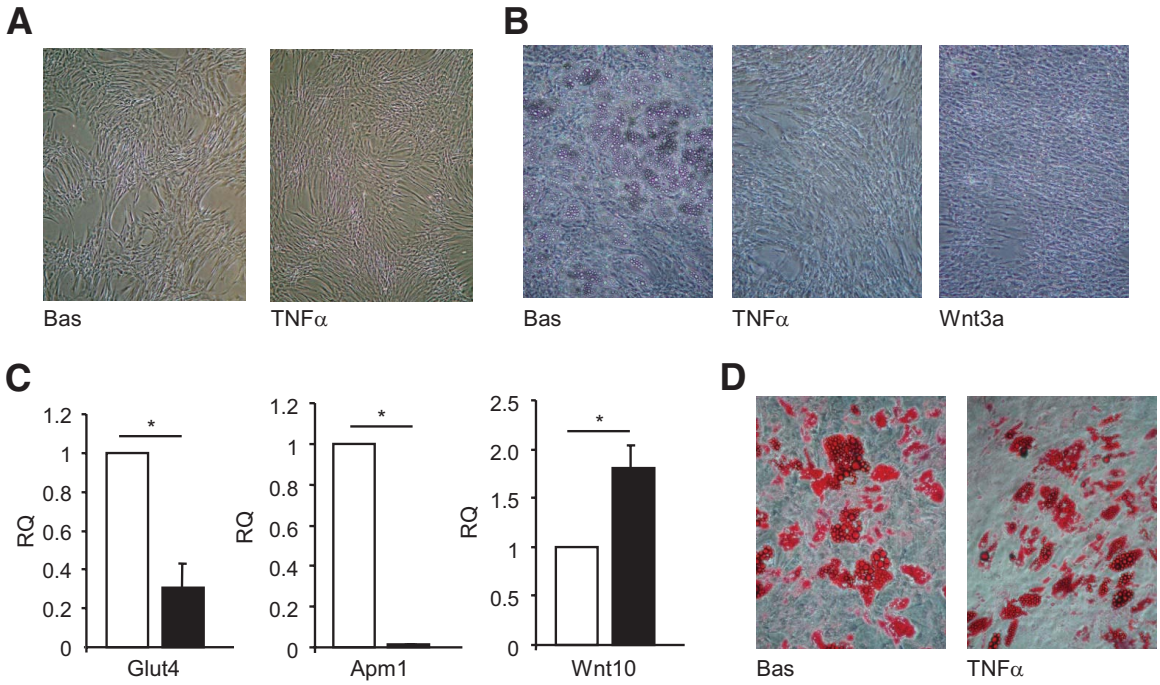

FIG. 2. TNF-α and Wnt3a inhibit the differentiation of human preadipocytes. *A*: **The presence of TNF-α induces proliferation of primary human preadipocytes.** *B*: **The presence of either TNF-α (5 ng/ml) or Wnt3a (~50 ng/ml) completely prevented normal adipocyte differentiation, as shown by oil red O staining.** *C*: **mRNA expression of GLUT4, adiponectin (Apm1), and Wnt10 after adipocyte differentiation for 21 days in the absence (□) or presence of TNF-α (5 ng/ml) (■). Data are the means ± SE from 11 different subjects. *$P < 0.05$.** *D*: **Oil red O staining of human preadipocytes differentiated for 21 days with or without preincubation with TNF-α (5 ng/ml) for 10 days. Bas, basal; RQ, relative quantification.**

Interestingly, the relative number of CD133-positive cells in the stromal vascular fraction was positively correlated with BMI ($r = 0.7141$, $P = <0.01$, $n = 10$) (Fig. 1*E*). This positive correlation seemed to plateau at higher BMI because we also studied an individual with BMI 66 kg/m^2 in whom 1.8% of the cells were CD133 positive. Thus, it appears that the pool of early adipocyte precursor cells is not reduced but, rather, that commitment is impaired and/or committed preadipocytes from obese individuals have an impaired ability to differentiate to the adipocyte phenotype.

We examined the expression of several different genes in the undifferentiated preadipocytes from obese versus nonobese individuals and found that expression of MAP4K4 in the preadipocytes was positively correlated with BMI ($r = 0.647$, $P < 0.05$) (Fig. 1*F*). This kinase has previously been shown to impair adipogenesis, to be induced by TNF-α, and to inhibit PPAR-γ expression (24,25). To examine whether this increase was induced by the culture procedure, we examined MAP4K4 mRNA levels in freshly isolated mature adipose cells. MAP4K4 was also increased in these cells and the expression correlated with both BMI (Fig. 1*G*) and the waist-to-hip ratio of the donor (Fig. 1*H*). Waist-to-hip ratio is a well-established marker of the metabolic complications of obesity, including insulin resistance (5–8). Thus, increased MAP4K4 seems to be an intrinsic property of the (pre)adipocytes in obesity characterized by enlarged fat cells.

TNF and Wnt3a, but not resistin or IL-6, inhibit human preadipocyte differentiation. When TNF-α was added, the CD14- and CD45-negative stromal vascular cells became more densely packed, suggesting a proliferating and/or antiapoptotic effect of TNF-α (Fig. 2*A*). However, no cell underwent normal differentiation with lipid accumulation (Fig. 2*B*), and this was also supported by the lack of induction of mRNA of the differentiation markers adiponectin and GLUT4 (Fig. 2*C*). Similar to TNF-α, addition of the Wnt ligand Wnt3a completely prevented the differentiation of these cells (Fig. 2*B*).

Addition of TNF-α to undifferentiated 3T3-L1 cells has been shown to activate the Wnt pathway and Wnt10b expression (18,28). We also found in human preadipocytes that Wnt10b was increased in the presence of TNF-α (Fig. 2*C*), whereas Wnt3a was not expressed (data not shown). In contrast to TNF-α, addition of either IL-6 or resistin did not prevent normal differentiation of the cells or increase gene expression of Wnt10b (data not shown). Thus, of the tested cytokines, TNF-α alone completely prevented the normal differentiation of human preadipocytes, activated Wnt10b expression, and, probably as a consequence, also seemed to enhance the proliferation of these cells.

The inhibitory effect of TNF-α is reversible. We then asked whether the inhibitory effect of TNF-α was transient or whether an associated activation of the Wnt pathway could contribute to a long-term inability of the preadipocytes to become adipose cells and thus explain this negative correlation with BMI. TNF-α was removed from the incubation medium after 10 days and differentiation induced with the normal cocktail. However, the effect of TNF-α seems to be rapidly reversible because the cells accumulated lipids to a similar degree as the control cells, which had not been exposed to TNF-α (Fig. 2*D*). Furthermore, they started to express differentiation markers as rapidly as the control cells, albeit at a lower level (data not shown).

Human preadipocytes assume a macrophage-like phenotype in the presence of TNF-α or lipopolysaccharide. We next examined the gene expression of undifferentiated preadipocytes that had been exposed or not exposed to TNF-α, IL-6, or resistin for 10 days. In the

TABLE 1
Effect of TNF-α, IL-6, and resistin on mRNA levels of macrophage-related genes in preadipocytes

		TNF-α		IL-6		Resistin	
	Basal	Mean	*P* value	Mean	*P* value	Mean	*P* value
GM-CSF	1.0 ± 0.0	50 ± 29	0.004	1.0 ± 0.1	NS	1.1 ± 0.1	NS
IL-1β	1.0 ± 0.0	305 ± 148	0.003	1.7 ± 0.4	NS	1.3 ± 0.2	NS
MIP-1α	1.0 ± 0.0	804 ± 432	0.003	0.9 ± 0.2	NS	1.1 ± 0.2	NS
TNF-α	1.0 ± 0.0	3.3 ± 0.4	0.003	1.1 ± 0.2	NS	1.5 ± 0.5	NS
CD68	1.0 ± 0.0	3.4 ± 0.9	0.03	1.0 ± 0.1	NS	1.1 ± 0.2	NS
MMP3	1.0 ± 0.0	59 ± 39	0.003	2.1 ± 0.8	NS	1.0 ± 0.1	NS

Data are means ± SE where basal is set to 1. Human preadipocytes in the stromal vascular fraction were cultured for 10 days with or without 5 ng/ml TNF-α, 20 ng/ml IL-6, or 50 ng/ml human resistin. $n = 11$.

presence of TNF-α, the cells assumed a marked proinflammatory phenotype associated with increased expression of cytokines and chemokines (Tables 1 and 2). Most importantly, genes that were not expressed or only expressed at very low levels in the basal state became activated by TNF-α. These included GM-CSF, IL-1β, MIP-1α, TNF-α itself, CD68, and matrix metalloprotease 3 (MMP3) (Table 1). Significant upregulation was also seen of proinflammatory molecules already expressed in the preadipocytes, like IL-6, IL-8, MCP-1, SAA, ICAM, MMP7, and toll-like receptor (TLR)-2, but not TLR4 (Table 2). Again, neither IL-6 nor resistin induced any significant changes in these genes (Tables 1 and 2).

We also measured the secretion of several cytokines and chemokines and found increases in secretion that paralleled the gene expression pattern (Fig. 3). Again, IL-1β, which was not secreted, and GM-CSF, which was secreted at very low levels in the nonstimulated cells, were clearly induced by TNF-α (Fig. 3). TNF-α also increased the secretion of IL-6, IL-8, MCP-1, SAA, and ICAM. However, we found no consistent secretion of TNF-α itself, as measured in the medium of cells where TNF-α had been removed from the medium after 10 days (data not shown), indicating a lack of activation of TACE (TNF-α converting enzyme). Thus, these partially transdifferentiated preadipocytes still lack endogenous TNF-α secretion.

We also tested whether lipopolysaccharide can alter the phenotype of these cells and found, in general, similar changes as with TNF-α. There were no additive effects of combining lipopolysaccharide and TNF-α, suggesting that they influenced the same pathways (data not shown).

We then examined whether the cells also became phagocytotic or expressed the scavenger receptor. However, we were unable to see the induction of a phagocytotic potential (data not shown). Although acetylated LDL uptake was slightly increased by the presence of TNF-α, only a few cells (<5%) were positive for LDL uptake. Thus, a complete transdifferentiation of the cells into macrophages, including phagocytotic potential, secretion of TNF-α, and expression of the scavenger receptor, was not seen.

Effect of oleic acid. Because TNF-α did not alter TLR4 expression, we asked whether the addition of fatty acids to the medium—a normal component in vivo—would further contribute to the transdifferentiation of the cells. Addition of 300 μmol/l oleic acid to the medium for 48 h increased lipid droplet formation in the cells but did not further alter the pattern of transdifferentiation seen in the presence of TNF-α alone (data not shown).

DISCUSSION

Many studies have shown that the secretion of IL-6, MCP-1, and other cytokines/chemokines by human adipose tissue mainly emanate from the stromal vascular cells (29,30). The finding that macrophages infiltrate the adipose tissue in obesity has led to the conclusion that these cells are the main source of cytokine/chemokine secretion (8,9), although recent studies have shown that mature adipose cells also secrete IL-6 and MCP-1 (11,29). The role of the inflammatory cells has been supported by reports that different markers of macrophages, such as CD68, are highly prevalent (up to 50% of the cells in the adipose tissue expressing this marker) and positively related to the size of the adipose cells (8).

CD68 and MIP-1α have been used in many studies to indicate the number/presence of macrophages in the adipose tissue, and a negative correlation between CD68 mRNA expression and insulin sensitivity in vivo has been found in humans (31). Thus, it appears that CD68 expression and other markers of macrophages in the adipose

TABLE 2
Effect of TNF-α, IL-6, and resistin on gene expression in undifferentiated human preadipocytes

		TNF-α		IL-6		Resistin	
	Basal	Mean	*P*	Mean	*P*	Mean	*P*
IL-6	1.0 ± 0.0	4.3 ± 0.7	0.003	1.0 ± 0.1	NS	1.2 ± 0.1	NS
IL-8	1.0 ± 0.0	777 ± 344	0.003	1.6 ± 0.2	NS	8.5 ± 7.9	NS
MCP-1	1.0 ± 0.0	6.2 ± 2.1	0.016	1.1 ± 0.2	NS	1.2 ± 0.2	NS
SAA1	1.0 ± 0.0	13.1 ± 6.1	(0.09)	1.1 ± 0.2	NS	1.1 ± 0.1	NS
SAA2	1.0 ± 0.0	7.7 ± 3.5	(0.09)	1.2 ± 0.3	NS	1.1 ± 0.1	NS
ICAM	1.0 ± 0.0	113 ± 107	0.003	1.3 ± 0.1	NS	1.4 ± 0.3	NS
MMP7	1.0 ± 0.0	3.9 ± 0.9	0.02	0.9 ± 0.2	NS	2.4 ± 0.8	NS
TLR2	1.0 ± 0.0	7.1 ± 0.7	0.003	0.9 ± 0.1	NS	1.5 ± 0.3	NS
TLR4	1.0 ± 0.0	2.5 ± 1.0	NS	1.0 ± 0.1	NS	1.2 ± 0.2	NS

Data are means ± SE where basal is set to 1. The same experimental conditions were used as in Table 1. $n = 11$.

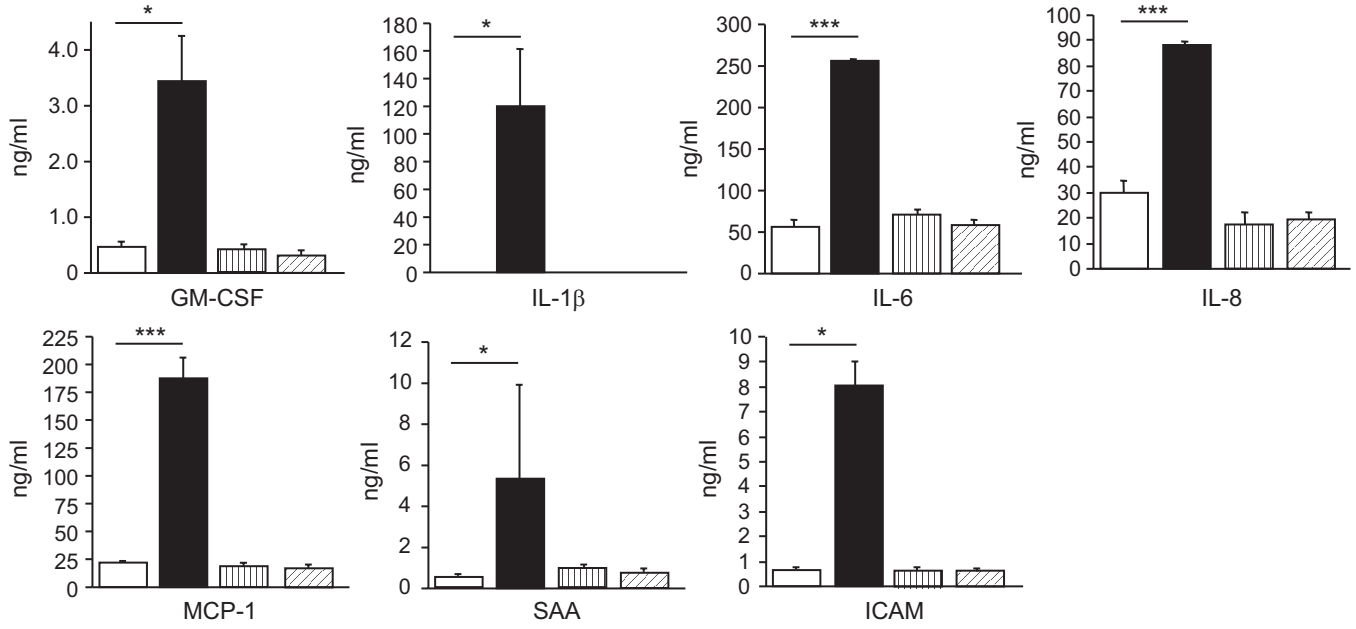

FIG. 3. Human preadipocytes assume a macrophage-like phenotype in the presence of TNF-α. Levels of secreted inflammatory molecules by human preadipocytes untreated (□) or treated with TNF-α (5 ng/ml) (■), IL-6 (20 ng/ml) (▥), or human resistin (50 ng/ml) (▨) for 10 days. Data are the means ± SE from six different subjects. *$P < 0.05$; ***$P < 0.001$.

tissue also reflect the associated clinical phenotype of the individuals. However, the current study clearly shows that CD68, MIP-1α, or other commonly used markers cannot be considered dependable indicators of the number of macrophages, but rather that they reflect the degree of inflammation in the tissue. In this context, it should be added that it is highly unlikely that any macrophages were present in the cultures as a confounding factor. First, we initially removed the CD14- and CD45-positive cells through magnetic immune separation of the cells, and, second, it is highly unlikely that any macrophages would have survived the culture procedures in this medium extending, in total, 3–4 weeks.

We examined the ability of human preadipocytes, obtained from the abdominal subcutaneous tissue of different individuals, to differentiate to adipose cells and the role of TNF-α, IL-6, and resistin on differentiation and phenotypic development. The salient findings were that *1*) there was a very large interindividual difference in the number of cells capable of differentiating into adipocytes, and the differentiation capability was negatively correlated with BMI as well as the average adipocyte size of the donors and, thus, insulin sensitivity; *2*) MAP4K4 expression in adipocytes and cultured preadipocytes was positively correlated with BMI; *3*) TNF-α, but not IL-6 or resistin, completely prevented the normal differentiation of the preadipocytes into adipose cells; and *4*) TNF-α promoted a partial transdifferentiation of the preadipocytes to assume a macrophage-like phenotype. This effect, at least under the short-term conditions used, was reversible and dependent on the continuous presence of TNF-α because the cells were not induced to secrete TNF-α themselves. Lipopolysaccharide also prevented the normal differentiation, as also previously reported (21), but lipopolysaccharide does not contribute above and beyond the effect of TNF-α. This latter point was also supported by our findings that activation of TLR4 with oleic acid (32) did not further enhance the TNF-α–induced partial transdifferentiation.

Our finding of a reduced number of preadipocytes in abdominal subcutaneous adipose tissue in obesity is in agreement with recent work by Permana et al. (33) and Tchoukalova et al. (34). These latter authors (34) also identified and quantified preadipocytes in the stromal-vascular cells through the positive expression of aP2, or FABP4 (fatty acid binding protein 4), but negative expression of CD68. Using these markers in freshly isolated cells, it was reported in a small study ($n = 3$) that obese individuals may have up to a 50% reduction in the apparent number of preadipocytes in the abdominal subcutaneous adipose tissue, but this was not seen in a peripheral (lateral thigh) depot (34). However, a problem with the interpretation of these data are the exclusion of CD68-positive cells, which would also exclude potential preadipocytes, which are inhibited by the proinflammatory environment. Thus, it is still an open question whether the number of preadipocytes is reduced in abdominal subcutaneous adipose tissue in obesity or whether the negative correlation with BMI and adipocyte size that we found mainly reflects an inability of the preadipocytes to undergo normal differentiation when the adipose cells expand in obesity. It should be emphasized that we cultured the preadipocytes in the presence of thiazolidinediones to fully promote adipogenesis. Thus, the differences in ability to differentiate cannot be related to lack of endogenous ligand formation.

In contrast to the reduced apparent number of preadipocytes, we found that the pool of potential precursor cells, identified through magnetic immune separation with the CD133 marker (26,27), was positively correlated to BMI. This suggests that there is a specific problem for preadipocytes in abdominal subcutaneous adipose tissue to differentiate into normal adipocytes and/or for the commitment of progenitor cells to preadipocytes. This is a very intriguing finding that could explain why there is differential lipid partitioning and also why abdominal obesity is closely linked to insulin resistance and the accumulation of ectopic fat in the liver and expanded fat

depots in the epicardial and visceral depots (rev. in 35). Our results also suggest that this inability is not attributable to the inhibitory effect of TNF-α per se on differentiation because we found that this effect is rapidly reversible and dependent on the continuous presence of TNF-α secreted by the macrophages. However, it may well be that chronically elevated TNF-α levels (or other unknown molecules) in the adipose tissue in obesity can, for instance, via MAP4K4 and/or Wnt signal activation, lead to a complete transdifferentiation or dedifferentiation of the preadipocytes to another phenotype, including inflammatory cells. Interestingly, we found that cultured preadipocytes from obese individuals had higher expression of MAP4K4, a kinase induced by TNF-α that inhibits the induction of PPAR-γ as well as adipogenesis (24,25). It is indeed intriguing that preadipocytes from obese individuals maintain elevated MAP4K4 expression, despite the long culture period. Interestingly, MAP4K4 expression in freshly isolated adipose cells was also positively related to BMI as well as the waist-to-hip ratio—a well-known marker of the metabolic abnormalities associated with obesity (5–7). Thus, this seems to be an intrinsic effect in adipose tissue in obesity and involves both preadipocytes and mature adipose cells.

The enlarged adipose cells in obesity become dysregulated (14,16) with reduced expression of many PPAR-γ–regulated genes, including GLUT4 and adiponectin. Instead, inflammatory genes are increased. This dysregulated state may be a consequence of reduced PPAR-γ activation, including the transrepression of proinflammatory genes. We do not currently understand the molecular mechanisms for this apparent partial loss of PPAR-γ activation, but ongoing studies where MAP4K4 is knocked down in human preadipocytes may shed new light on these intriguing findings.

An additional possibility that needs to be considered is that there is a slow, continuous turnover of adipose cells (~10% per year) (12) and that this depletes the number of available preadipocytes in the adipose tissue in obesity. However, this would seem to be particularly important for obesity characterized by hyperplastic adipose tissue, and this is usually not associated with abdominal adipose cell enlargement, insulin resistance, and inflammation.

We have previously described in 3T3-L1 preadipocytes that TNF-α completely prevents the normal differentiation and that it is associated with an activation of Wnt 10b and the Wnt signaling cascade (18,36). Similar to the current study, we did not find any effect of resistin or MCP-1 on Wnt signaling in 3T3-L1 cells (36), and thus this seems to be specific for TNF-α. A direct activation of the Tcf/Lef (T-cell–specific transcription factor/lymphoid-enhancer binding factor) nuclear receptor by TNF-α has also recently been reported (28). Thus, TNF-α maintains preadipocytes in an undifferentiated state while, concomitantly, it is able to drive the cells toward a macrophage-like phenotype.

Intriguingly, we found that TNF-α induced the expression and secretion of molecules that were not expressed or secreted by the preadipocytes, such as GM-CSF and IL-1β. In addition, the cells assumed a marked proinflammatory phenotype with the induction of both markers (SAA and ICAM) and effectors (IL-6, MCP-1, and IL-8) of inflammation. Overall, the cells became highly macrophage-like, with the exception of phagocytotic capacity, expression of the scavenger receptor, or secretion of TNF-α itself. Thus, the partially transformed and proinflammatory preadipocytes would seem to be an important source for maintaining, and enhancing, inflammation both in the adipose tissue in obesity as well as systemically because many of the secreted molecules are released to the bloodstream (SAA, ICAM, IL-6, IL-8, and MCP-1). Furthermore, the undifferentiated preadipocytes/macrophage-like cells started to induce and secrete GM-CSF. Thus, infiltrating macrophages can both drive inflammation in the adipose tissue/preadipocytes and also induce a milieu whereby bound monocytes will undergo differentiation to macrophages in the tissue.

To evaluate the potential quantitative importance of the undifferentiated human preadipocytes for MCP-1 secretion by the adipose tissue, we compared our results with those reported in the literature. MCP-1 secretion by adipose tissue from obese individuals was estimated by Dahlman et al. (11) to 23 ng/g tissue · h or 23 ng/10^6 cells · h, assuming an average cell size of ~0.8 μg/cell. In the current study, the secretion of MCP-1 by preadipocytes cultured in the presence of TNF-α was ~200 ng/2.5 × 10^5 cells · 48 h or ~17 ng/10^6 cells · h. Thus, activated preadipocytes may indeed be the major source of MCP-1 secretion by adipose tissue. Again, using the results reported by Dahlman et al. (11), it is clear that mature adipose cells only account for 2–5% of the MCP-1 produced by adipose tissue. Published data (29,30) for IL-6 and -8 secretion by adipose tissue also lead to the conclusion that undifferentiated preadipocytes can be the major source of secretion of these cytokines.

In conclusion, these findings underscore the importance of partially transdifferentiated preadipocytes as generators of inflammation in obesity. These effects appear to be specific to TNF-α; at least they are not shared by IL-6, MCP-1, or resistin. Furthermore, obesity with subcutaneous abdominal adipose cell enlargement is characterized by a reduced number of preadipocytes that can undergo differentiation. Understanding the mechanisms for this and the cross-talk between TNF-α and Wnt signaling is a priority because it may open up new possibilities to treat obesity with regards to inflammation, preadipocyte recruitment, and insulin sensitivity.

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