Altered fat differentiation and adipocytokine expression are inter-related and linked to morphological changes and insulin resistance in HIV-1-infected lipodystrophic patients

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Objective: To achieve a better understand of the pathophysiology of HIV-related lipoatrophy, we compared the mRNA expression of adipocytokines in fat samples from patients and healthy HIV-seronegative controls together with fat morphology and we studied the relationship between changes in fat morphology, adipocytokine expression, markers of adipose tissue differentiation and whole body insulin sensitivity.

Design: Cross-sectional analytical study.

Subjects and methods: The mRNA expression of adipocytokines and transcriptional factors in fat samples from 26 patients with peripheral lipoatrophy (all under antiretroviral therapy associating protease inhibitor and nucleoside-analogue reverse transcriptase inhibitors) and from 16 non-HIV-infected controls was measured by real time quantitative RT-PCR. Fat morphology was assessed histologically on a subgroup of 10 patients and six controls: collagen fibres by Sirius Red staining, apoptosis by the TUNEL technique, vessels by smooth muscle α-actin staining and macrophages by CD68 staining. Insulin resistance was assessed by using the homeostasis model assessment.

Results: The patients' fat showed higher values of apoptosis (P=0.005), fibrosis (P<0.05), vessel density (P=0.001) and macrophage infiltration (P<0.05) than the controls' fat, together with lower adiponectin and leptin mRNA levels and higher interleukin (IL)-6 and tumour necrosis factor (TNF)α mRNA levels. TNFα and IL-6 expression correlated positively with the level of apoptosis (P=0.05 and P<0.05, respectively) and negatively with CCAAT-enhancer binding protein (C/EBP)α (P<0.001 and P<0.05, respectively). Apoptosis correlated negatively with the expression level of sterol-regulatory-element-binding-protein-1c (SREBP1c) (P=0.01) and C/EBPα (P=0.01) whilst the vessel density correlated negatively with SREBP1c (P<0.005), C/EBPα (P=0.001) and β (P=0.001). Adiponectin and leptin expression correlated positively with each other, and also with adipogenic marker expression and overall insulin sensitivity. These relationships were also present when the patient group was studied separately. Finally, fat morphological abnormalities correlated positively with whole body insulin resistance.

Conclusions: Adipose tissue from patients with HIV-1-related lipoatrophy shows increased apoptosis, together with decreased adipocyte differentiation. Increased TNFα and IL-6 expression could be a major phenomenon linking these alterations. Decreased adiponectin and leptin expression, which may result from decreased adipocyte differentiation, could be involved in the observed whole body insulin resistance.

Introduction

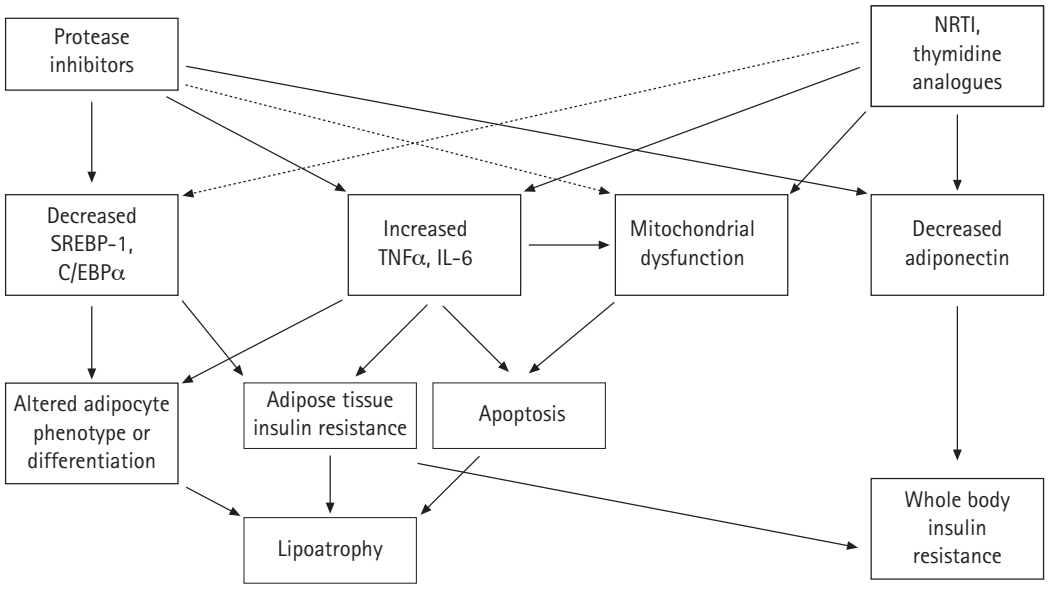
A high proportion of HIV-1-infected patients on antiretroviral therapy develop lipodystrophy, characterized by peripheral fat wasting, visceral fat redistribution and metabolic alterations with dyslipidemia and insulin resistance [1–4]. Although the lipodystrophy syndrome is multifactorial, protease inhibitors (PIs) and nucleoside reverse transcriptase inhibitors (NRTIs) have been clearly implicated in cohort studies and *in vitro* experiments. NRTIs, and especially thymidine analogues, have been implicated in peripheral lipoatrophy. Their combination with PIs results in an increased incidence and severity of this phenotype [5]. *In vitro*, PIs alter adipocyte differentiation and insulin sensitivity and induce apoptosis [6,7]. NRTIs reduce adipocyte lipid content and increase apoptosis [8] and, when combined with PIs, affect several adipocyte functions [8,9]. Therefore, the two classes of molecules have deleterious effects at the adipocyte level. They could act in synergy and play a role in clinical lipoatrophy.

An increasing number of studies report data on fat tissue from HIV-1-infected patients [10–17]. An altered fat morphology has been reported by two groups, with modified mitochondrial structure and numbers together with increased apoptosis [10,11,13]. We have previously reported that subcutaneous adipose tissue from patients with HIV-1-related lipoatrophy exhibits marked changes in adipogenesis, with decreased mRNA concentrations of the main adipogenic transcription factors [sterol-regulatory-element-binding-protein-1 (SREBP-1), peroxisome proliferators-activated receptor (PPAR)γ and CCAAT-enhancer binding protein (C/EBP)α], as well as lower adipocyte insulin sensitivity [12]. However, these alterations had not been linked to morphological changes in adipose tissue.

In addition to its metabolic functions, adipose tissue secretes a variety of adipocytokines. Leptin and adiponectin act through endocrine mechanisms in the liver and muscles, controlling insulin sensitivity [18,19]. Interleukin (IL)-6 and tumour necrosis factor (TNF)α probably act mainly at the local level. TNFα induces adipocyte dedifferentiation, insulin resistance and apoptosis, while IL-6 induces insulin resistance in adipocytes and reduces their differentiation *in vitro* [20–22]. Altered expression of these cytokines might thus be involved in some of the observed adipose tissue alterations in HIV-infected patients. We have previously observed lower leptin expression and higher TNFα expression in fat from HIV-infected patients with lipodystrophy than in healthy controls [12]. Very recently, the increased TNFα expression was confirmed in lipodystrophic compared with non-lipodystrophic HIV-infected patients [15,16] and an increased expression of IL-6 together with a decreased expression of adiponectin was reported [15,17].

Here we compared histological adipose tissue abnormalities and adipocytokine expression in HIV-1-infected subjects and studied the relationship between morphological alterations in adipose tissue, adipocytokine expression, adipocyte differentiation and whole body insulin sensitivity. Our findings allow us to propose this hypothetical scheme (Figure 1).

Figure 1. Pathogenesis of lipodystrophy



Materials and methods

Subjects

All the subjects studied had been previously presented [12]. Briefly, the HIV-1-infected group consisted of 26 patients treated with both PI and NRTI (80% with stavudine) who had developed peripheral lipoatrophy and who had undergone subcutaneous abdominal liposuction with fat re-injection into the cheeks, using Coleman's technique [23]. An additional abdominal fat sample was stored in liquid nitrogen or fixed in formol or Carson solution (Millonig's phosphate-buffered formalin). All the patients gave their written consent to participate in the study.

The patients were comprised of 21 men and five women, with a mean age of 45 years (range 36–62) and a mean body mass index (BMI) of 23.3 kg/m^2 (16.5–28.3). CD4+ cell counts were above 200/µl in all but three of the patients (145–165/µl). Nineteen patients had no detectable HIV-1 RNA at the time of the study and the remaining seven had viral load values between 730 and 135 000 copies/ml. The mean known duration of HIV infection was 11 years (2–19). Fifteen patients were on indinavir and nine were on nelfinavir; 21 were on stavudine and 19 on lamivudine. The mean duration of antiretroviral combination therapy was 37 months (15–48).

The control group consisted of 16 healthy HIV-1-seronegative non-diabetic and non-obese subjects (eight women and eight men). The mean age of the control subjects was 34 years (24–69) and their mean BMI was 24.1 kg/m^2 (19.0–31.3). Fat from control subjects was obtained during abdominal plastic surgery (liposuction) or from normal volunteers involved in a nutritional study with subcutaneous fat biopsies (needle fat aspiration) and immediately frozen.

Fat morphology studies involved a subgroup of 16 subjects (10 patients and six controls). The mean age and BMI of the HIV-1-infected patients was 43 years (37–53) and 24.0 kg/m^2 (21.3–26.7). All the patients were treated with stavudine plus either lamivudine (n=8), didanosine (n=1) or abacavir (n=1) and plus indinavir (n=7), nelfinavir (n=3) or ritonavir (n=1). The mean age and BMI of the controls was 49 years (28–69) and 24.8 kg/m^2 (19.8–30.0), with no statistical difference between the two groups.

Adipose tissue histology

Light microscopy was performed by a histopathologist on 10% zinc-formol-fixed paraffin-embedded 5-µm tissue sections stained with hemalum-phloxine and Sirius Red to detect collagen fibres. For immunocytochemical studies, we used an automated immunostainer, Optimax plus (BioGenex Laboratories, Inc., Calif., USA and an avidin–biotin immunoperoxidase method with 3-amino-9-ethylcarbazole chromogene (AEC) (Vector Laboratories, Inc., Calif., USA) or peroxidase-labelled secondary antibody followed by a peroxidase-labelled tertiary antibody before AEC revelation. A prestaining heat-based epitope-retrieval technique was used prior to staining for several antibodies, with sodium citrate pH 6.0 or water as buffer. Appropriate positive and negative control tissues were stained using the same methods. The following monoclonal and polyclonal antibodies were used: CD45 (1/10; Dako Corp, Calif., USA) a lymphocyte and neutrophil marker; CD68 (1/300; Dako) a macrophage marker; Ki67, which stains replicative nuclei; anti-smooth-muscle α actin (1/50; Dako), which labels vascular smooth muscle cells; and anti-mitochondria (1/50, BioGenex). Apoptosis was measured by the terminal deoxynucleotidyl transferase dUTP-digoxigenin nick-end labelling method (TUNEL) using the Apoptag kit (Oncor, Gaithersburg, Md., USA).

Each fat specimen was examined with a semi-automatic image analysis system (Mercator, La Rochelle, France). Firstly, the surface area of three regions chosen at random was measured. Then, in each region, adipocytes, apotag-positive nuclei and total nuclei (except vessels) were mapped and counted. The density of CD45-, CD68- and Ki67-positive cells and anti-smooth muscle actin-positive vessels was evaluated as the average of 10 fields (×40).

Fat samples for ultrastructural studies were fixed in Carson solution, rinsed in 0.1 M cacodylate buffer and then post-fixed in 1% osmium tetroxide for 1 h. After rinsing in cacodylate buffer, fragments were dehydrated in graded alcohol series and embedded in epoxy resin. Semi-fine sections (0.5 µm) were stained with toluidine blue. Ultrastructure sections (60 nm) were contrasted with uranyl acetate and lead citrate and examined on Geol 100, CX-2 electron microscope.

mRNA assays

Fat tissue RNA was extracted using the RNeasy total RNA kit (Qiagen, Courtaboeuf, France). The yield of total RNA was 2.1 µg (0.7–4.8) per 100 mg of tissue. The mRNA concentrations of PPARγ, C/EBPα, C/EBPβ, SREBP-1c, glucose transporter 4 (GLUT4), hormone-sensitive lipase (HSL), leptin, TNFα and β2-microglobulin were determined by reverse transcription followed by competitive polymerase chain reaction amplification (RT-cPCR); adiponectin and IL-6 mRNAs were quantified by real-time PCR. Real-time PCR was performed with a Light cycler® device (Roche-Boehringer, Meylan, France) using a SYBR Green® (Finnzymes, Espoo, Finland) detection signal. Quantification was obtained with reference to a plasmid dilution range that contained the human mRNA target sequence. Each quantification was

performed twice and each run was validated by the specificity of the PCR (Tm evaluation) and by the slope and error obtained for the different plasmid dilutions. The primer sequences were as follows: IL-6: forward primer 5′-AGCCCTGAGAAAGGAGACATGTAACA AG-3′ and backward primer 5′-TTCTGCAGGAACTG GATCAGGACTTT-3′; adiponectin: forward primer 5′-CAGAGATGGCACCCCTGGTG-3′ and backward primer 5′-TTCACCGATGTCTCCCTTAG-3′. The results were expressed as attomoles (amol/μg) of total RNA. β2-microglobulin was used as an internal standard for mRNA expression and all the mRNA results were expressed as amol/μg of total RNA and as ratios relative to β2-microglobulin mRNA expression.

Insulin resistance

Insulin resistance was assessed by calculating the homeostasis model assessment (HOMA) index defined as fasting plasma glucose (mmol/l) × fasting plasma insulin (μU/ml)/22.5 [24].

Statistical analysis

All results are presented as means ±SE or mean with range. Differences between groups were determined using the non-parametric Mann–Whitney U test. The significance of correlations was determined using the non-parametric Spearman's rank correlation test. The threshold of significance was set at P=0.05.

Results

Adipose tissue shows major morphological alterations

The population of small adipocytes is higher in patients than in controls [12]. In addition, we observed an increased number of apoptotic nuclei in fat from patients: 17.5% (4.3–30.2) versus 4.5% in controls [(0.7–7.2); P=0.005]. Blood vessels were more numerous in fat from patients [30 per field (5–47)] than from controls [three per field (1–4); P=0.001] (Figure 2A). The proportion of fibrosis was higher in fat from patients [10% (1–30)] than from controls [2% (0–8); P<0.05] (Figure 2B). Marked mitochondrial labelling was present in the cytoplasm of some adipocytes in the patients' fat (Figure 2C). Interestingly, these labelled adipocytes were small, with an increased cytoplasm often containing small fat droplets adjacent to the large unilocular fat vacuole or small residual fat vacuoles. These cells (Figure 2C; inset) were adipocytes and not lipid-laden macrophages since labelling by CD68 was negative (Figures 2D; inset). Control fat contained large adipocytes that showed no mitochondrial staining. The number of macrophages was higher in fat from patients [14.8 per field (1.6–25.0)] than from controls [3.2 per field (1.1–8.0), P<0.02]. Lipogranulomas were often seen, with macrophages surrounding a fat droplet lying free in connective tissue (Figure 2D). No difference was found between the two groups as regards the number of CD45 positive cells [10.5 per field (4.3–18.0) and 7.4 per field (2.0–16.1), P=0.23] in fat from patients and controls, respectively. Cell proliferation was evaluated by labelling replicative nuclei with Ki67 – few nuclei were labelled in the fat from both patients and controls (not shown), arguing against a process of active regeneration.

The proportion of apoptotic nuclei correlated positively with blood vessel density (r=0.63, P=0.01) and fibrosis (r=0.51, P=0.05). The proportion of small adipocytes (less than 70 μm) correlated strongly with blood vessel density (r=0.70, P<0.01). Overall, the altered fat morphology in the HIV-1-infected group was characterized by fields of small adipocytes with increased cytoplasm enriched in mitochondria and surrounded by increased stroma with fibrosis, blood vessel density and apoptosis.

Ultra-structural studies performed in 14 patients revealed small adipocytes in 13, and stromal fat droplets in all the patients. Small adipocytes were present in the fat of two out of six controls, while stromal lipid droplets were found in only one control.

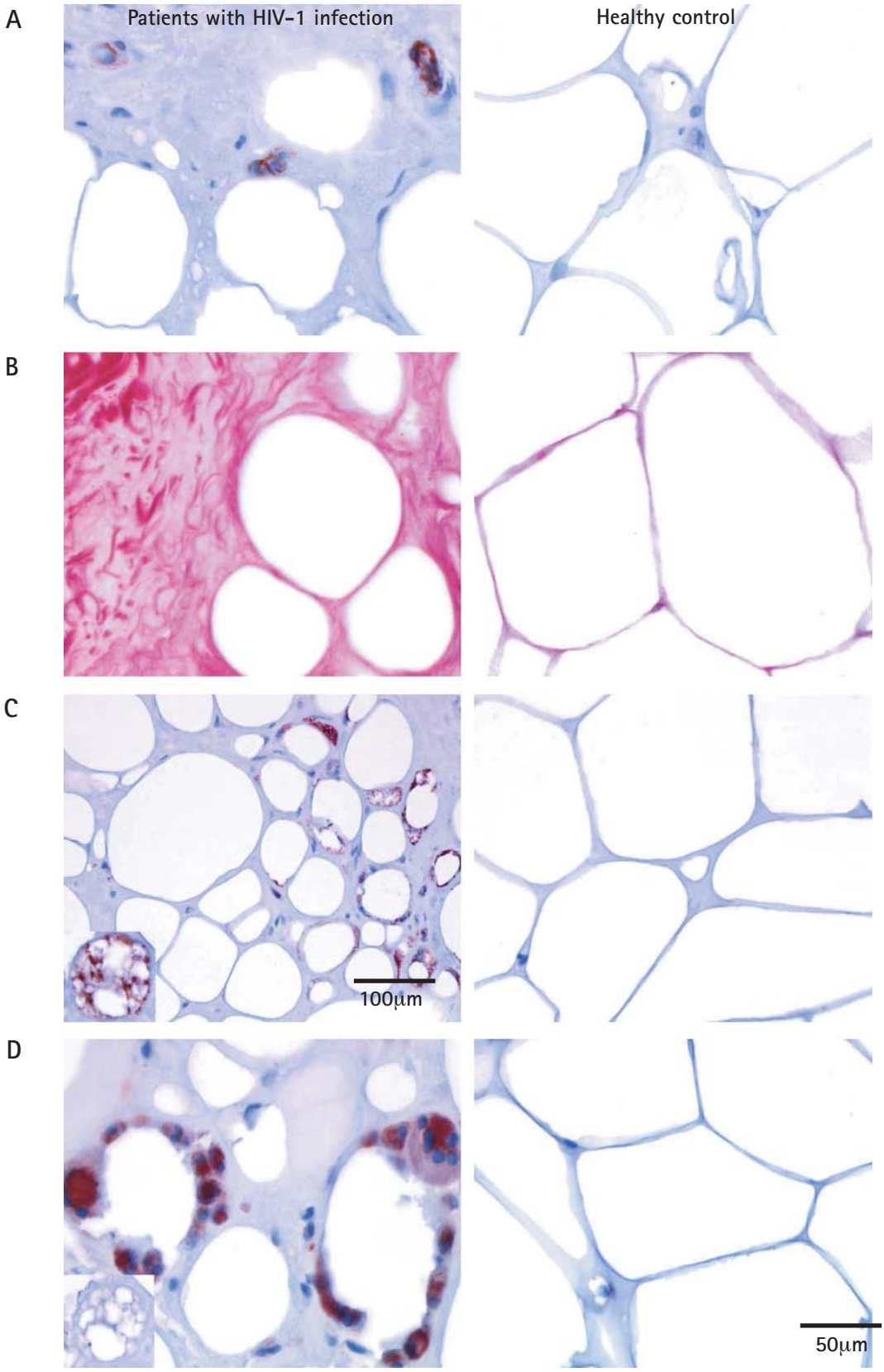
Adipocytokine expression

All the results are presented as absolute levels in the patient and control groups and as ratios relative to β2-microglobulin, which was used as an internal control (Table 1). These ratios were used for all the data presented below. The mRNA concentration of leptin was markedly lower in fat from patients than controls as previously reported [12]. We found here that the mRNA concentration of adiponectin was 2.1-fold lower in fat from patients than from controls (Table 1). The mRNA concentrations of leptin and adiponectin correlated positively with each other (Table 2). We found that the mRNA concentration of both adiponectin and leptin correlated positively with the expression of all the adipogenic transcription factors tested previously [12], the strongest correlation being with C/EBPα (Table 2).

The mRNA concentration of IL-6 was fourfold higher in fat from patients than in fat from controls (Table 1), as previously observed for TNFα [12]. The expression of TNFα correlated negatively with that of SREBP1c, PPARγ, C/EBPα and β while adiponectin and leptin were positively correlated with all these adipogenic transcriptional factors. Otherwise IL-6 was only inversely and significantly correlated with C/EBPα (Table 2).

When we studied the HIV-infected group separately, we found a significant positive correlation between adiponectin and C/EBPα (r=0.522, P<0.05), C/EBPβ

Figure 2. Typical histology of subcutaneous abdominal adipose tissue from an HIV-1-infected patient and a healthy control



(A) Blood vessels (anti-smooth-muscle α actin labelling); **(B)** fibrosis (Sirius Red staining); **(C)** mitochondria (anti-mitochondria labelling), inset: an adipocyte with multiple fat droplets; **(D)** macrophages with a lipogranuloma structure (CD68 labelling), inset: absence of labelling with CD68 of the adipocyte shown in (C) inset. The magnification was the same for all pictures including insets except for Figure 1C left which magnification was twofold lower.

Table 1. Comparison of mRNA gene expression from adipose tissue in controls (*n*=16) and HIV-infected patients (*n*=26) expressed as attomol/μg RNA or per β2-microglobulin mRNA gene expression

	Controls	HIV patients	Controls	HIV patients
	mRNA (amol/μg RNA)	mRNA (amol/μg RNA)	mRNA /β2 mRNA expression × 100	mRNA /β2 mRNA expression × 100
SREBP1c	26.0 ±3.3	2.8 ±0.7¶	18.6 ±2.4	2.0 ±0.6¶
C/EBPα	82.1 ±11.7	26.6 ±3.7¶	53.9 ±6.5	17.2 ±3.1¶
C/EBPβ	29.7 ±3.4	15.4 ±1.9∫	17.5 ±1.4	10.0 ±1.7ǂ
PPARγ	16.1 ±2.4	5.1 ±1.0∫	12.8 ±2.8	2.9 ±0.6¶
HSL	212.0 ±26.1	145.0 ±16.2*	149.8 ±19.2	87.9 ±10.6ǂ
LPL	72.2 ±11.2	45.7 ±5.6*	44.2 ±7.1	29.4 ±5.0*
GLUT4	33.7 ±6.2	6.6 ±0.9¶	26.7 ±6.1	4.0 ±0.6¶
Adiponectin	287.0 ±22.5	113.1 ±17.1†	206.3 ±20.5	96.1 ±14.0∫
Leptin	4.32 ±0.73	0.28 ±0.07¶	3.34 ±0.71	0.16 ±0.03¶
IL-6	0.64 ±0.18	2.36 ±0.55*	0.40 ±0.11	1.61 ±0.58*
TNFα	0.048 ±0.007	0.125 ±0.014§	0.033 ±0.004	0.070 ±0.008∫

The study was performed in 42 subjects except for IL-6, LPL and C/EBPβ (*n*=30–34).
*P<0.05; †P<0.01; ǂP<0.005; §P<0.001; ∫P<0.0005; ¶P<0.0001.

Table 2. Correlation between adipose tissue parameters and adipocytokine mRNA

	Adiponectin	Leptin	IL-6	TNFα
SREBP1c	r=0.576	r=0.647	r=–0.305	r=–0.527
	P<0.001	P<0.0001	P=0.10	P<0.001
C/EBPα	r=0.693	r=0.660	r=–0.396	r=–0.548
	P<0.0001	P<0.0001	P=0.05	P<0.001
C/EBPβ	r=0.583	r=0.344	r=–0.174	r=–0.354
	P<0.001	P=0.05	P=0.86	P<0.05
PPARγ	r=0.484	r=0.562	r=–0.379	r=–0.572
	P<0.005	P<0.001	P=0.09	P<0.001
HSL	r=0.521	r=0.478	r=–0.387	r=–0.234
	P<0.005	P<0.005	P=0.05	P=0.13
LPL	r=0.601	r=0.432	r=–0.219	r=–0.102
	P=0.0005	P=0.01	P=0.24	P=0.56
GLUT4	r=0.696	r=0.632	r=–0.234	r=–0.549
	P<0.0001	P<0.0001	P=0.20	P<0.001
Adiponectin	–	r=0.559	r=–0.036	r=–0.407
		P<0.001	P=0.84	P<0.05
Leptin	–	–	r=–0.326	r=–0.318
			P=0.08	P<0.05
IL-6	–	–	–	r=–0.257
				P=0.16

The study was performed in 42 subjects except for IL-6, LPL and C/EBPβ (*n*=30–34). All the gene mRNA expression was related to that of β2-microglobulin.

(r=0.511, P<0.05), GLUT4 (r=0.443, P<0.05) and LPL (r=0.490, P<0.05) mRNA expression while TNFα was inversely correlated with PPARγ and C/EBPα (r=–0.407, P<0.05 and r=–0.412, P<0.05, respectively).

There was no difference in adipose tissue gene expression between male and female subjects in both controls and patients groups for all the genes studied except for leptin. Indeed, female patients exhibited a higher mRNA expression than male patients (0.289 ±0.082 vs 0.126 ±0.034, P<0.05). However, leptin expression remained markedly decreased as compared with controls in both male (P<0.0001) and female groups (P=0.005).

When we compared male controls and patients or female controls and patients, the same significant variations as those observed in the whole group were found for SREBP1c, PPARγ, C/EBPα and β, HSL, GLUT4, adiponectin, leptin and TNFα.

Correlations between morphological alterations of adipose tissue and adipocytokine expression

We found numerous tight correlations between the diverse aspects of fat morphological changes (apoptosis, fibrosis and blood vessel density) and the different components of the underlying molecular events, namely adipocytokine expression and factors involved in fat differentiation.

The adipocytokines appeared to be involved in fat morphological changes, as the mRNA expression of IL-6 and TNFα correlated positively with apoptosis (Table 3). TNFα transcript levels also correlated with vessel density (Table 3). Conversely, leptin transcript expression correlated negatively with apoptosis, fibrosis and blood vessel density (Table 3), while adiponectin correlated only and negatively with blood vessel density.

Finally, a strong negative correlation was observed between the expression of several adipogenic transcription factors, including C/EBPα and SREBP1c, and both apoptosis and blood vessel density (Table 3).

Correlations between altered adipocytokine expression and insulin resistance

HIV-infected patients had a higher HOMA score than controls (4.9 ±0.7 vs 1.4 ±0.20, $P<0.0001$) confirming that HIV-infected patients presented a state of whole body insulin resistance. We have previously found a positive correlation between TNFα mRNA concentrations in fat samples and insulin resistance and a positive correlation between leptin mRNA expression and insulin sensitivity [12]. We observed that the rate of TNFα and leptin relative to β2-microglobulin were highly correlated with HOMA (r=0.531, $P<0.005$ and r=–0.670, $P<0.0001$ respectively) in accordance with previous results [12]. Accordingly, we found a positive correlation between IL-6 mRNA expression and both HOMA (r=0.529, $P<0.01$) and fasting plasma insulin (r=0.476, $P<0.05$). By contrast, we found a negative correlation between adiponectin mRNA concentrations and both HOMA (r=–0.535, $P<0.01$) and insulin levels (r=–0.507, $P=0.01$). This emphasizes the involvement of adipocytokines in insulin sensitivity in humans, and particularly in HIV-1-infected patients. Moreover, we found a significant correlation between HOMA and apoptosis (r=0.782, $P<0.005$), fibrosis (r=0.632, $P<0.05$) and the number of vessels (r=0.880, $P<0.001$).

Table 3. Correlation between adipose tissue alterations and adipocytokine and transcription factor gene expression in controls ($n=6$) and HIV-infected patients ($n=10$)

	Apoptosis	Fibrosis	Vessels
IL-6	r=0.647	r=0.197	r=0.482
	$P<0.05$	$P=0.46$	$P=0.07$
TNFα	r=0.517	r=0.126	r=0.515
	$P=0.05$	$P=0.48$	$P=0.05$
Adiponectin	r=–0.671	r=–0.688	r=–0.873
	$P<0.01$	$P<0.01$	$P<0.001$
Leptin	r=–0.649	r=–0.693	r=–0.771
	$P=0.01$	$P=0.01$	$P<0.005$
SREBP1c	r=–0.648	r=–0.371	r=–0.739
	$P=0.01$	$P=0.15$	$P<0.005$
PPARγ	r=–0.261	r=–0.068	r=–0.507
	$P=0.31$	$P=0.79$	$P=0.05$
C/EBPα	r=– 0.648	r=–0.769	r=–0.878
	$P=0.01$	$P<0.005$	$P=0.001$
C/EBPβ	r=–0.480	r=–0.629	r=–0.823
	$P=0.06$	$P<0.05$	$P=0.001$

All the gene mRNA expression was related to that of β2-microglobulin.

Discussion

This is the first comprehensive study of morphological and molecular alterations in subcutaneous adipose tissue from HIV-1-infected lipodystrophic patients treated with NRTI–PI combinations. However, an important limitation of its cross-sectional design together with the lack of a treated HIV-1-positive control group without lipodystrophy, is that it does not allow us to distinguish between potential effects of HIV or its treatment. In addition, no conclusions can be drawn concerning the role of specific antiretroviral drug classes. We observed striking morphological alterations in fat from patients as compared with healthy controls. We confirmed the increased proportion of small adipocytes [10–13,25], the higher rate of apoptosis [10] and the presence of stroma containing fibrosis and vessels [11,25]. Interestingly, these morphological alterations correlated with one another and also with abnormal adipocytokine expression, markers of altered adipocyte differentiation and insulin resistance.

We report here that the mRNA concentrations of C/EBPα and SREBP1c, two key transcription factors involved in adipocyte differentiation and insulin sensitivity, correlate strongly and negatively with apoptosis and increased blood vessel density. Altered cytokine expression could represent a possible link between differentiation and apoptosis. *In vitro* and animal studies have shown that TNFα, which mainly acts through autocrine/paracrine mechanisms, can promote adipocyte dedifferentiation, resistance to insulin and apoptosis [20]. While TNFα production by white adipose tissue is low in physiological conditions, it can be markedly increased in animal models of obesity [20]. A strong set of arguments suggests the involvement of TNFα in the adipose tissue changes associated with HIV-1-related lipodystrophy. We have previously observed higher mRNA concentrations of TNFα in fat from lipodystrophic patients [12]. This result has been

recently confirmed when HIV-infected lipodystrophic patients were compared with non-lipodystrophic HIV infected patients [15,16]. Likewise, higher circulating levels of TNFα and its soluble receptors have been reported by us and others in lipodystrophic patients as compared with controls [26–28]. An accumulation of circulating T cells primed for TNFα synthesis has been reported in patients with HIV-1-related lipodystrophy, and was linked to circulating lipid abnormalities [29].

Interestingly, we found that TNFα expression correlated negatively with the expression of adipogenic factors and positively with apoptosis. Together with other published data, this suggests that TNFα could be involved in adipocyte dedifferentiation and in apoptosis in fat of lipodystrophic patients.

We report an increased IL-6 expression in fat from lipodystrophic patients as recently reported by two groups [15,17]. In addition, we observed a negative correlation between IL-6 and C/EBPα expression and a strong positive correlation between IL-6 expression and apoptosis. This suggests that IL-6 could also play a major role at the local level, through paracrine/autocrine mechanisms, as previously reported in insulin-resistant obese non-diabetic and type 2 diabetic subjects [30]. *In vitro* studies suggest that this cytokine could alter adipocyte differentiation [21,22]. However, data are lacking to link increased IL-6 expression to adipocyte dedifferentiation or apoptosis.

Antiretroviral drugs could be responsible for the increased expression and secretion of these cytokines. Recent studies indicate that not only PIs but also thymidine analogues can induce the expression of TNFα and IL-6 by cultured 3T3F442A adipocytes ([31,32] and Lagathu *et al.*, personal results). It is therefore conceivable that PIs and NRTIs can increase the expression of TNFα and IL-6 in adipocytes, resulting in decreased differentiation and increased apoptosis.

We found that patients' adipose tissue contained an increased number of macrophages, which surrounded adipocytes in lipogranuloma-like structures. These macrophages are probably activated, expressing IL-6 and TNFα. Therefore, the increased IL-6 and TNFα expression observed in patients' fat could also derive partly from macrophages. As IL-6 and TNFα act through autocrine and paracrine mechanisms, these cytokines could be responsible for adipocyte dysfunction, whatever their cellular origin.

We observed an increased level of fibrosis and a markedly increased blood vessel density in patients' fat. This could reflect a process of tissue reparation after elimination of apoptotic adipocytes by macrophage phagocytosis. This tissue is enriched in blood vessels, suggesting that angiogenesis could also be involved in the remodelling process. We found that the mRNA concentration of leptin correlated strongly and negatively with blood vessel density. This is in line with a recent study showing that leptin can ablate adipose tissue by inducing a loss of adipose vasculature [33]. Likewise, leptin has been shown to induce the expression of the negative angiogenesis signal angiopoietin-2 [33]. Further studies are required to explain the increased angiogenesis in lipodystrophic patients' fat.

Altered mitochondrial biogenesis could play a role in increased apoptosis and could result from altered differentiation. The ability of NRTIs, and particularly thymidine analogues, to decrease mtDNA has been reported and linked to peripheral lipoatrophy [11,13]. Morphological studies have shown the presence of increased numbers of mitochondria with an altered ultrastructure in lipoatrophic adipose tissue [10,11,25]. We found that adipocytes with mitochondria-rich cytoplasm were small, and often contained multiple fat droplets or a very small fat vacuole, in accordance with previous studies [11,25]. As we found only scarce replicating nuclei in patients' fat, this aspect is probably due to toxicity rather than regeneration. A prominent role of NRTIs in this process was recently reported [11,13]. The responsibility for NRTIs and histological alterations in some patients is suggested by Nolan *et al.* who observed detectable improvement in tissue toxicity in one of three cases after switching d4T for zidovudine [13].

Metabolic alterations in lipodystrophic HAART-treated patients are frequently associated with insulin resistance, but the underlying mechanisms are unclear. However, in patients with HAART-related lipodystrophy, insulin resistance at the level of adipose tissue was revealed by the increased level of circulating free fatty acids [34,35], which have been shown to induce insulin resistance in human liver and muscle [36]. This could result from the effects of TNFα or IL-6 on adipocytes.

The role of leptin and adiponectin in insulin sensitivity has been reported several times. Serum adiponectin and leptin levels are decreased in human genetic syndromes with generalized lipoatrophy and insulin resistance: the metabolic disorders in these patients are markedly improved by leptin replacement therapy [37]. More recently, we and others showed a reduction in serum adiponectin and leptin levels in HIV-infected patients on HAART [26,27]. In addition, adiponectin and sTNF-R1 levels were related to metabolic alterations and insulin resistance, suggesting a role of these cytokines in insulin sensitivity.

A decreased mRNA concentration of adiponectin in fat from patients with HIV-related lipodystrophy was recently described, and was found to correlate with insulin resistance [14,15]. Accordingly, we found that adiponectin mRNA expression correlated negatively with insulin resistance and insulin levels in our

population. These data argue for a role of adipocytokines in insulin resistance in this setting. In addition, insulin resistance was related to morphological alterations suggesting a close relationship between adipose tissue dysfunction and whole body insulin resistance in lipodystrophic HIV patients. The decreased expression of adiponectin could result from drug toxicity: PIs and NRTIs were both found to decrease adiponectin mRNA expression in 3T3F442A adipocytes ([32] and Lagathu *et al*., personal results).

In conclusion, we show here that some morphological and molecular alterations in adipose tissue from lipodystrophic HIV-1-infected patients are interrelated. We had previously obtained evidence that antiretroviral treatment including PIs and NRTIs can inhibit adipocyte differentiation and induce insulin resistance, in part by decreasing SREBP-1 expression [12]. We recently found that, *in vitro*, some PIs alter lamin A/C maturation, resulting in abnormal nuclear lamina stability which, in turn, is probably responsible for altered SREBP-1 nuclear location and function [7,38]. The observed increase in TNFα and IL-6 expression could result from exposure to both PIs and NRTIs and lead to altered adipocyte differentiation and insulin sensitivity, as well as increased apoptosis, ultimately leading to lipoatrophy. Drug-induced mitochondrial dysfunction might also contribute to this phenotype. Finally, a drug-induced decrease in adiponectin secretion, together with an increase in free fatty acid release by insulin-resistant adipose tissue, could also be involved in whole body insulin resistance and metabolic disorders (Figure 1).

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