Original article

Mitochondrial RNA and DNA alterations in HIV lipoatrophy are linked to antiretroviral therapy and not to HIV infection

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Background: The aim of this study was to assess the effect of antiretroviral therapy (ART) versus HIV on mitochondria in fat.

Methods: Subcutaneous fat was collected from 45 HIV-infected patients on ART with lipoatrophy, 11 HIV-infected ART-naive patients and nine healthy controls. Three mitochondrial transcripts: NADH dehydrogenase subunit 1 (ND1), cytochrome B (CYTB) and NADH dehydrogenase subunit 6 (ND6) genes were quantitated using TaqMan® probes and normalized to nuclear-encoded ribosomal L13.

Results: ND1/L13 and CYTB/L13 were lower in HIV-positive patients on ART with lipoatrophy versus ART-naive patients (3.4 versus 7.2 [P=0.017] and 2.5 versus 4.6 [P=0.006], respectively). No difference was found between ART-naive patients and controls (P>0.70). ND6/L13 was similar between all groups. Dual-energy X-ray absorptiometry-measured limb fat and mitochondrial DNA in fat were also lower in HIV-positive patients on ART with lipoatrophy versus HIV-infected, ART-naive patients (4,382 versus 7,662 g [P=0.02] and 726 versus 1,372 copies/cell [P=0.03], respectively), but no difference was found between ART-naive and controls. In a multiple regression analysis, limb fat correlated with all three mitochondrial RNA, whereas mitochondrial DNA did not correlate with mitochondrial RNA or limb fat.

Conclusions: In contrast to ART-naive patients, HIV-positive patients on ART with lipoatrophy had significant depletion in mitochondrial DNA in fat and mitochondrial RNAs. This suggests that mitochondrial toxicity in lipoatrophy could be driven by ART and not by HIV itself. In addition, mitochondrial RNA abnormalities, and not mitochondrial DNA depletion, could be a key driving force behind lipoatrophy.

Introduction

Mitochondrial abnormalities have been described in HIV-infected patients with peripheral fat loss or lipoatrophy [1–4]. Thus far, the pathogenesis of mitochondrial dysfunction in HIV patients remains unclear and the degree to which antiretroviral therapy (ART), specifically nucleoside reverse transcriptase inhibitors (NRTIs), is responsible as opposed to HIV infection itself is not known. HIV infection could indeed contribute to mitochondrial dysfunction leading to lipoatrophy, as HIV induces apoptosis. It was shown that viral protein R can target mitochondria and induce apoptosis via a specific interaction with the permeability transition pore complex and the adenine nucleotide translocator [5,6]. Nie et al. [7] have demonstrated that HIV-1 protease specifically cleaves procaspase 8 to create a novel fragment termed casp8p41, which independently induces apoptosis. The novel fragment casp8p41 is specific to HIV-1 protease-induced death, but not to other caspase-8-dependent death stimuli [7]. Thus, antiretroviral agents might not explain all of the changes in body fat compartments and deleterious, or perhaps synergistic, effects induced by HIV and its accompanying inflammatory responses have been advocated as playing a role in the pathogenesis of HIV lipoatrophy [8,9]. Lower indices of subcutaneous fat in HIV-infected patients have been noted in both pre- and post-highly active ART in comparison with uninfected controls [10].
Although mitochondrial DNA (mtDNA) depletion has been frequently described in the fat of HIV-infected patients with lipoatrophy [1–4], limited data exist on mitochondrial RNA (mtRNA) and its relationship to changes in mtDNA and peripheral fat. Several factors including NRTI use, lower nadir CD4+ T-cell count, older age and higher body mass index have been shown in HIV-infected individuals to correlate with mitochondrial disease, whether in the form of hyperlactataemia or lipoatrophy [11,12]. To our knowledge, in the HIV-infected population no studies have concomitantly investigated mtRNA and mtDNA as well as objective quantitative assessments of lipoatrophy. Also, no such study has included both ART-naïve and uninfected groups as controls, and none investigated the relationship of mtRNA and mtDNA to HIV disease and treatment factors. Thus, in this study, we measured mtRNA and mtDNA levels in adipose tissue of an HIV-infected lipoatrophy group; an HIV-infected, ART-naïve group and HIV-uninfected group in order to explore the relationship of these mitochondrial indices to HIV and treatment factors.

Methods

Study design/population

This is a single-site, cross-sectional, controlled observational study. Patients were enrolled between May 2005 and April 2007. We studied a group of HIV-infected adults referred to the Case HIV Metabolic Center (Cleveland, OH, USA) for assessment of lipoatrophy. For the HIV-positive patients on ART with lipoatrophy, the inclusion criteria were HIV infection, age ≥18 years, receipt of stable ART containing a thymidine analogue NRTI for at least 6 consecutive months prior to study entry, HIV-1 RNA <50 copies/ml and clinical lipoatrophy at study entry. Clinical lipoatrophy was defined by self reports completed by the study patients on fat loss in at least two areas: face, arms, legs and buttocks, and confirmed by the investigator. We also included an HIV-positive, ART-naïve group that enrolled patients who had documented HIV infection and no prior ART. As controls, we enrolled an HIV-negative, healthy control group who were undergoing cosmetic surgical procedures and who were similar in age to the HIV-positive groups. Exclusion criteria included current opportunistic infections, renal or hepatic impairment, coagulation problems, abnormal prothrombin time and partial thromboplastin time tests or platelets <75×10^3/mm³, diagnosis of diabetes, active endocrine disorders and history of recent use of hormonal therapies. All patients were enrolled at the John T Carey Special Immunology Unit of Case Medical Center (Cleveland, OH, USA) and signed a written informed consent approved by the Institutional Review Board of University Hospitals Case Medical Center (Cleveland, OH, USA).

Study evaluations

Study evaluations in HIV-infected patients included clinical examination, blood sampling and a whole body dual energy X-ray absorptiometry (DEXA) scanning for body fat composition (Hologic 4500, Bedford, MA, USA). Blood was drawn in a fasting state (after at least 8 h fast) for lipid panel, insulin, glucose and lactate levels. Lactate levels were all drawn in a fasting state, without a tourniquet or fist clenching, and were immediately processed. The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated from fasting plasma glucose and insulin values [13]. An experienced surgeon performed excisional biopsies of subcutaneous fat from the lower abdomen of all study patients under local anaesthesia (using lidocaine without epinephrine). All biopsies were collected with the written informed consent of patients and with the approval of the Institutional Review Board of University Hospitals Case Medical Center.

Mitochondrial DNA measurements

Analysis of mtDNA was conducted by absolute quantitative real-time PCR, as previously described [3,14]. DNA was extracted from subcutaneous adipose tissue biopsies (stored at -70°C in RNAlater® (Sigma–Aldrich, St Louis, MO, USA) using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA). Total DNA was quantified in ng/µl using a UV-Vis ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the integrity of DNA was verified by agarose gel electrophoresis. Standardization of real-time PCR was performed using LightCycler FastStart DNA Master Plus SYBR Green I (Roche Applied Sciences, Indianapolis, IN, USA) with a LightCycler instrument (Roche Applied Sciences). A dilution series ranging from 10¹ to 10³ copies/cell of the control plasmid containing 90 base pairs mtDNA NADH dehydrogenase subunit II (mitochondrial) and 98 base pairs Fas ligand (genomic) gene was prepared for each standard [15]. The mitochondrial primers (Idaho Technologies BioChem, ID, USA), mtDIR (CAC AGA AGC TGC CAT CAA GTA) and mtREV (CCG GAG AGT ATA TTG TTG AAG AG) were specific for a region of the mitochondrial protein NADH dehydrogenase subunit II (mitochondrial) and mtDNA. The nuclear genomic primers (Idaho Technologies BioChem, GenDIR (GGC TCT GTG AGG GAT ATA AAG AAG AG) and GenREV (CAA ACC ACC CGA GCA ACT AAT CT) were specific for the nuclear region of the genome encoding for Fas ligand. Each sample and standard were run in duplicate (20 µl reaction volume) containing SYBR Green Master Plus Mix (FastStart Taq DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye, MgCl₂), 10 pM mitochondrial or genomic forward and reverse primers and approximately 10 ng DNA from sample. PCR
Mitochondrial RNA measurements

Total RNA was isolated from subcutaneous adipose tissue biopsies stored at -70°C in RNALater® (Sigma–Aldrich) using RNeasy Lipid Tissue Kit (Qiagen Inc.). RNA quality was checked and quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Purified RNA was reverse transcribed to cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Sciences) on a PCR thermal cycler instrument (Eppendorf, Westbury, NY, USA). Oxidative phosphorylation gene expression was then measured using real-time PCR on a LightCycler with a LightCycler TaqMan Master Kit (Roche Applied Sciences). Standard curves ranging from 10^5 to 10^7 copies were prepared from a single recombined plasmid containing the NADH dehydrogenase I (ND1), NADH dehydrogenase VI (ND6), cytochrome B (CYTB) and the nuclear housekeeping ribosomal L13 gene [16]. Each different transcript was quantified in a separate reaction to avoid non-specific amplifications and to ensure maximum reliability. Each reaction was conducted in duplicate (20 µl reaction volume) containing approximately 1 µl cDNA from sample, TaqMan Master Mix (FastStart Taq DNA polymerase, reaction buffer, dNTP mix, MgCl₂), 0.5 µM L13 or CYTB or ND1 or ND6 primer (Idaho Technologies BioChem), 0.2 µM corresponding (L13, CYTB, ND1 or ND6) probe (Sigma–Aldrich) and double distilled H₂O. Sequences of gene-specific primers and probes were customized according to Galluzzi et al. [17]. PCR cycling conditions were 95°C for 15 min followed by 40–45 cycles of 95°C for 10 s (denaturation), 57°C for 15 s (annealing) and 72°C for 15 s (extension). Following the final PCR cycle, a step of 37°C for 30 s allowed the instrument to cool to room temperature. Fluorescence of each transcript was detected at a wavelength of 530 nm. Quantification of ND1, ND6 and CYTB copies were calculated by the ratio of each mitochondrial transcript mean concentration to the mean L13 concentration for each sample.

Statistical methods

Demographics, clinical characteristics and mitochondrial parameters are described for each individual study group and HIV-related characteristics are described for HIV-infected patients only. Continuous measures are described by medians and ranges and nominal variables are described with percentages. Continuous measures were compared using Wilcoxon rank-sum tests and nominal variables using χ² analysis or Fisher’s exact test as appropriate.

The relationships between the outcome measures of mtRNAs and variables of interest were examined. Spearman’s rank correlation coefficients were calculated for each of the variables with each of the mtRNA measures. As the correlations with limb fat mirrored the correlations with limb fat adjusted to body mass index (limb fat/BMI), we elected to present only the correlations with the latter variable. Regression models were developed separately for each of the mtRNA measures and for mtDNA separately in a two-stage approach. The goal was to include disease, treatment and biological variables in the same model (correcting for age, BMI and limb fat), while being aware of the sample size limitations. In the first stage, four models were constructed: one including race and gender, one including disease-related variables, one including treatment-related variables and the last including laboratory values. Variables were chosen either on the basis of the bivariate results or because they were known to have clinical significance. Each model also included age at study entry, BMI and limb fat. Any variable from each of the first stage models that was significant at the P≤0.2 level or, lacking that, the most significant variable in the model, was included in the second stage analyses. All analyses were carried out using SAS Version 9/1 (The SAS Institute, Carey, NC, USA). The level of significance was set at 0.05.

Results

Study population

Between May 2005 and April 2007, 65 individuals enrolled in this study: 45 patients with established HIV lipoatrophy on ART; 11 HIV-infected, ART-naïve patients and nine uninfected controls. Table 1 summarizes the demographics and baseline characteristics of all study participants. There were 45 (69%) males enrolled and 54% of participants were Caucasian. Overall, the median age and BMI were 45 years and 26 kg/m² in the HIV-positive group versus 43 years and 40 kg/m² in the HIV-negative group (P=0.87 and P=0.002, respectively, for between-group differences in age and BMI). HIV-positive patients on ART with lipoatrophy were receiving ART containing at least one NRTI for a median (range) duration of 43 years and 40 kg/m² in the HIV-negative group (P=0.87 and P=0.002, respectively, for between-group differences in age and BMI). HIV-positive patients on ART with lipoatrophy were receiving ART containing at least one NRTI for a median (range) duration of 43 years and 40 kg/m² in the HIV-negative group (P=0.87 and P=0.002, respectively, for between-group differences in age and BMI). HIV-positive patients on ART with lipoatrophy were receiving ART containing at least one NRTI for a median (range) duration of 43 years and 40 kg/m² in the HIV-negative group (P=0.87 and P=0.002, respectively, for between-group differences in age and BMI).
and the nadir CD4+ T-cell count was lower in the HIV-positive with lipoatrophy on ART group compared with the ART-naive group (576 [67–1,427] cells/mm3 versus 194 [275–434] cells/mm3 [P < 0.001] and 199 [30–544] cells/mm3 versus 275 [194–325] cells/mm3 [P < 0.0001], respectively). All HIV-positive patients with lipoatrophy on ART had HIV-1 RNA < 50 copies/ml. By contrast, the ART-naive group had a median HIV-1 RNA of 31,850 (364–>100,000) copies/ml.

Between-group comparisons of mitochondrial and metabolic indices

Table 2 summarizes the metabolic and mitochondrial results in all study groups. The HIV-infected patients with lipoatrophy on ART group had higher fasting triglycerides, cholesterol and HOMA-IR when compared with the ART-naive group (576 [67–1,427] cells/mm3 versus 194 [275–434] cells/mm3 [P < 0.001] and 199 [30–544] cells/mm3 versus 275 [194–325] cells/mm3 [P < 0.0001], respectively). Other than leg fat, levels of mtDNA in fat were lower in the ART-naive group versus the lipoatrophy group (726 [194–3,091] copies/cell versus 1,372 [532–2,721] copies/cell; P = 0.03). No difference was found in mtDNA in fat between ART-naive and healthy controls (P = 0.79).

The expression of ND1, ND6 and CYTB were all different, although they strongly correlated among each other (for example, correlation between ND1 and ND6 r = 0.90; P < 0.0001; between ND1 and CYTB r = 0.96; P < 0.0001). In each of the groups, ND1 was always expressed at higher values than ND6 and CYTB, and the ratio of ND1/ND6 was preserved and similar in all groups (P > 0.54). However, ND1/CYTB was lower in the ART-naive group when compared with the lipoatrophy
Mitochondrial alterations in HIV lipoatrophy

Table 2. Mitochondrial and metabolic parameters in HIV-infected patients and uninfected controls

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<tr>
<td>Total cholesterol, mg/dl</td>
<td>182 [110–305]^†</td>
<td>150 [101–165]^†</td>
<td>–</td>
</tr>
<tr>
<td>High-density lipoprotein cholesterol, mg/dl</td>
<td>34 [14–68]</td>
<td>28 [19–57]</td>
<td>–</td>
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<tr>
<td>Triglycerides, mg/dl</td>
<td>168 [46–1,522]†</td>
<td>96 [49–420]^‡</td>
<td>–</td>
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<tr>
<td>HOMA-IR</td>
<td>2.3 [0.1–8.1]^n</td>
<td>0.8 [0.1–4.7]^‡</td>
<td>–</td>
</tr>
<tr>
<td>DEXA-limb fat, g</td>
<td>4,382 [2,158–12,734]^n</td>
<td>7,662 [2,268–18,313]^n</td>
<td>–</td>
</tr>
<tr>
<td>Total lean body mass, g</td>
<td>60,584 [24,035–81,067]^§</td>
<td>48,415 [30,087–71,474]^§</td>
<td>–</td>
</tr>
<tr>
<td>Fat mtDNA, copies/cell</td>
<td>726 [194–3,091]^§</td>
<td>1,372 [532–2,721]^§</td>
<td>1,344 [588–2,677]^§</td>
</tr>
<tr>
<td>ND1/L13</td>
<td>3.4 [0.01–16.6]^n</td>
<td>7.2 [3.0–15.4]^‡</td>
<td>9.1 [5.0–18]</td>
</tr>
<tr>
<td>ND6/L13</td>
<td>0.93 [0.01–8.9]</td>
<td>1.9 [0.4–13.3]</td>
<td>1.9 [0.4–18]</td>
</tr>
<tr>
<td>CYTB/L13</td>
<td>2.5 [0.03–17]^n</td>
<td>4.6 [1.9–22.3]^‡</td>
<td>7.3 [0.5–13]</td>
</tr>
</tbody>
</table>

Values are medians (range). *P<0.05 for comparison between HIV-positive antiretroviral therapy (ART)-naive patients and uninfected controls. †P<0.001 and ‡P<0.01 for comparison between HIV-positive patients with lipoatrophy and HIV-positive, ART-naive groups. BMI, body mass index; DEXA, dual energy X-ray absorptiometry; HOMA-IR, homeostasis model assessment of insulin resistance; mt, mitochondria.

Discussion

To our knowledge, this is the first study that assessed mtRNA expression along with mtDNA levels in the fat tissue of HIV-infected patients with lipoatrophy that included an ART-naive group and an HIV-negative control group. Few prior studies have assessed the effects of ART on mtRNAs [16–20] and only two [16,18] were performed on HIV-infected patients. In this report, we show that both mtRNA and mtDNA are significantly decreased in HIV-infected adults with lipoatrophy when compared with HIV-infected, ART-naive patients and uninfected control individuals. Interestingly, no differences were found between the HIV-positive, ART-naive group and HIV-negative controls, suggesting that these mitochondrial disturbances are the result of ART and not HIV infection itself. Earlier reports have suggested...
that HIV infection itself might cause mtDNA decline in ART-naïve patients, but this was shown mostly in peripheral blood mononuclear cells and not in adipose tissue [21,22]. In this study, the different groups were closely matched by age. This matching is crucial for the success of any such trial aimed at studying the effect of HIV and/or treatment on mitochondrial indices in HIV-infected adults, because increasing age could be associated with more mitochondrial abnormalities [23], which could significantly confound the study results in the absence of careful matching. Also, our HIV-negative group had significantly higher BMI, which makes our results even stronger as obesity has been linked to mitochondrial abnormalities [24]; therefore, our population could have attenuated the observed differences in mitochondrial abnormalities between the studied groups.

The depletion of mtDNA levels in ART-treated HIV patients with lipoatrophy has been consistently described [1–4,25]. However, mitochondrial dysfunction is not always associated with mtDNA depletion [26,27] and severe mtDNA depletion has been reported in asymptomatic individuals [28,29]. Thus, additional mechanisms for mitochondrial alterations in HIV beyond mtDNA depletion are likely, as recently suggested [26,30]. To our knowledge, two prior small studies assessed mtRNA levels in adipose tissue of HIV-positive patients with lipoatrophy and HIV-uninfected controls [16,18]. None of these studies included DEXA evaluations to measure limb fat and neither enrolled HIV-positive, ART-naïve patients. The mtRNA levels in these studies were also reduced in HIV-positive patients when compared with HIV-negative controls, but no HIV-positive group without lipoatrophy was included, making it impossible to differentiate between the effects of HIV infection versus those of ART.

The importance of mtRNA alterations, independently of mtDNA depletion, has been suggested by prior cell culture work. Galluzzi et al. [17] have previously shown that NRTIs can induce a significant decrease in mtRNA levels in cell lines even before any noticeable mtDNA depletion. Similarly, d’Amati et al. [19] have shown a significant disruption of mitochondrial cristae and alteration of mtRNA, but no change in mtDNA levels after 4 weeks of zidovudine treatment of mouse muscle cells. In our study, the lack of correlation between mtRNA alteration and mtDNA levels suggest that the alteration in transcription is not secondary to changes in mtDNA, but is rather a primary effect of therapy. Interestingly, only the mitochondrial heavy chain transcripts, ND1 and CYTB, were decreased in treated HIV-positive patients with lipoatrophy and not the light chain transcript ND6. This suggests that mitochondrial heavy strand transcription is affected, but not light chain transcription. In addition, the fact that limb fat/BMI correlated with mtRNA but not mtDNA also suggests that mtRNA alteration might indeed be the primary mitochondrial alteration that occasionally coexists with mtDNA depletion. The study reported by Mallon et al. [20] supports this hypothesis. Mallon et al. reported a significant decrease in mtRNA production after 2 weeks of NRTI in healthy HIV-uninfected adults [20]. This occurred before any changes in mtDNA levels of fat mass.

On regression analysis, only limb fat and limb fat/BMI correlated with all three mtRNA measurements whereas HIV disease factors, like CD4+ T-cell count and duration of HIV infection, did not. This again supports our observation that the mtRNA alterations in HIV-positive patients with lipoatrophy are linked to the use of ART. The duration of thymidine NRTIs correlated with fat mtDNA levels, although PI duration was also independently correlated. Limb fat/BMI did not correlate with mtDNA levels.

As expected, ART-treated HIV patients with lipoatrophy had higher fasting triglycerides, cholesterol and HOMA-IR when compared with ART-naïve individuals. Additionally, correlation analysis showed that HDL cholesterol correlated with all three mitochondrial transcripts. Mitochondrial function is sensitive to fat [31], insulin and glucose levels [32]. The mitochondria could become uncoupled due to the increased fat, and this corresponds with decreased transcription. Insulin resistance is associated with decreased ATP production. Thus, there might be multiple etiologies for the mitochondrial dysfunction observed in HIV-infected patients.

One limitation of our study is the lack of a control group of ART-treated HIV patients without lipoatrophy. We have recently learned that it is difficult to fully exclude lipoatrophy by clinical assessment alone in HIV patients. Indeed, studies have shown that despite the lack of clinical diagnosis of lipoatrophy, HIV-infected women and men have less peripheral fat when compared with gender- and age-matched, HIV-negative individuals [33,34], suggesting that clinical lipoatrophy might only be the extreme form of peripheral fat loss in HIV patients and that the subclinical form could be more common than previously thought. Thus, a group of HIV-treated patients without lipoatrophy is challenging to enrol, but would need to be explored in future studies. In addition, incorporating detailed mitochondrial indices in future ART-switch studies of patients with lipoatrophy would be helpful in dissecting the effect of ART in general from that of lipoatrophy.

In summary, a significant decrease in mtDNA levels and in mtRNAs were found in HIV-positive patients on ART with lipoatrophy, but not in ART-naïve individuals. This strongly supports that ART, and not HIV infection, plays a major role in the generation of mitochondrial disturbances in HIV lipoatrophy. The correlation found between limb fat/BMI and mtRNA and not
mtDNA support the fact that mtRNA alteration, and not mtDNA depletion, could be the primary mitochondrial disturbance in HIV lipodystrophy.

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Disclosure statement

GAM serves as a consultant and on the Speaker Bureau for GlaxoSmithKline, Bristol–Myers Squibb, Gilead and Abbott. MG serves as a consultant for Abbott and Oncolys Biopharma. The other authors declare no conflicts of interests.

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