Mitochondrial and apoptotic in vitro modelling of differential HIV-1 progression and antiretroviral toxicity

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Objectives: Ex vivo analysis of mitochondrial function may reveal HIV progression and the impact of ART. We propose a mitochondrial and apoptotic in vitro model using Jurkat T cells incubated with plasma. The objectives of this study were to evaluate mitochondrial and apoptotic lesions in this model in relation to HIV progression, and to assess the effect of >1 year of standard non-thymidine-containing therapy.

Methods: This was a cross-sectional comparison among three age- and gender-matched groups (n = 19 × 3): healthy non-HIV-infected participants, HIV-infected long-term non-progressors (LTNPs) and standard antiretroviral-naive chronically infected patients [standard progressors (Sp1)], longitudinally evaluated before (Sp1) and after (Sp2) >1 year of efavirenz + tenofovir + emtricitabine therapy. We analysed mitochondrial DNA content by RT–PCR, mitochondrial function by spectrophotometry, mitochondrial protein synthesis by western blot analysis, mitochondrial dynamics by western blot analysis (MFN2), apoptotic transition pore formation by western blot analysis (VDAC-1) and mitochondrial membrane potential and annexin V/propidium iodide fluorescence by flow cytometry.

Results: There was a decreasing non-significant trend towards lower mitochondrial parameters for HIV-infected values with respect to uninfected control reference values. HIV progression (LTNP versus Sp1) was associated with decreased mitochondrial genetic, functional and translational parameters, which partially recovered after treatment intervention (Sp2). Mitochondrial fusion showed a trend to decrease non-significantly in Sp patients compared with LTNP patients, especially after therapy. All apoptotic parameters showed a trend to increase in Sp1 with respect to LTNP, followed by recovery in Sp2.

Conclusions: We proposed an in vitro model for mitochondrial and apoptotic assessment to test the effects of HIV infection and its therapy, resembling in vivo conditions. This model could be useful for clinical research purposes.

Keywords: in vitro model, HIV progressors, mitochondrial function, apoptosis

Introduction

Under no treatment, most HIV-infected patients show a progressive decrease in CD4+ T cell counts over time that is inversely related to plasma HIV RNA. ART suppresses HIV replication and prevents CD4+ T cell loss. A small subset of HIV-infected patients (1%–5%), named long-term non-progressors (LTNPs), spontaneously maintain undetectable HIV RNA in plasma and their CD4 cell counts may be preserved over time without antiretroviral (ARV) intervention.1 HIV may cause mitochondrial damage and promote apoptosis. Different degrees of evidence have been found in in vitro, cell and
animal models but also in the ex vivo analysis of different tissues of infected patients, including PBMCs.\textsuperscript{2,3} Given that (i) T lymphocytes are responsible for the immune defence capacity of the organism and that these cells constitute the main target cells for viral replication, consequently acting as both rescuers and prey for HIV infection, and (ii) mitochondria are the main organelles responsible for enhancing or triggering cell apoptosis, the hypothesis that mitochondrial status within T lymphocytes may determine differential HIV progression seems plausible. Some studies demonstrate that mitochondrial disturbances induced by HIV may lead to the death of T cells and promote faster progression of HIV infection. One of these studies demonstrated that the mitochondrial and apoptotic lesions in PBMCs of LTNPs were reduced with respect to naive standard progressor (Sp) subjects,\textsuperscript{9} demonstrating variability of both phenomena depending on the course of infection: LTNPs presented intermediate mitochondrial and apoptotic alterations with respect to uninfected healthy volunteers (lacking any lesion) and naive Sp subjects (presenting the highest level of mitochondrial and apoptotic alterations).\textsuperscript{6,7}

Several later studies indicated that mitochondria are important agents of differential progression of HIV infection. For instance, while some studies have failed to demonstrate that genetic variants in nuclear-encoded mitochondrial genes influence AIDS progression,\textsuperscript{5} others have reported that certain mitochondrial DNA (mtDNA) haplogroups (specifically J and USA) influence AIDS progression\textsuperscript{9} and specific disorders related to HIV infection.\textsuperscript{10}

ART may at least partially restore mitochondrial function by controlling HIV replication. However, some ARV agents may have mitochondrial toxicity. Although the capacity of the formerly used thymidine NRTIs to cause mitochondrial lesions is well established\textsuperscript{11–13} and has been postulated to be responsible for several adverse effects,\textsuperscript{14,15} it remains unknown whether therapies that do not include non-thymidine-NRTIs are safer. Even though the evolution of less-toxic drugs is the principal aim of the scientific, clinical, and pharmaceutical communities and current ARVs seem to be safer for the patient in most recent schedules, further information regarding the details of the mitochondrial toxicity associated with such combination treatments is needed. It is still unknown whether the current therapy combinations, such as ARV regimens including the two NRTIs tenofovir and emtricitabine and the NNRTI efavirenz have been associated with mitochondrial toxicity and secondary effects of medication, since controversial results have also been observed regarding the NNRTI efavirenz, which has been associated with cell apoptosis,\textsuperscript{16–18} although to a lesser extent than NRTI-derived agents.

Currently, there is no gold standard test to assess mitochondrial function in HIV-infected patients. Mitochondrial DNA (mtDNA) has been widely used, but the method requires cells and provides limited information on mitochondrial function.

We propose an in vitro model of mitochondrial analysis using Jurkat T cells incubated with plasma. Plasma samples can be easily obtained and preserved until assessment. We evaluated the results obtained in groups of different subjects according to whether they were HIV-infected, and, if HIV-infected, according to whether HIV replication was controlled either spontaneously or by a standard ARV regimen.

The present study aimed to: (i) establish an in vitro culture model for study based on the capacity of the plasma of different types of progressor patients to induce mitochondrial and apoptotic lesions; (ii) investigate the HIV-derived molecular mechanisms of mitochondrial and apoptotic lesions in the model in relation to the differential progression of HIV infection (LTNP versus Sp); and (iii) determine the mitochondrial and apoptotic effects of current therapeutic schedules (tenofovir + emtricitabine + efavirenz), which are presumably less toxic than the thymidine NRTI-containing ones in this model.

**Methods**

**Design**

This was a multicentric, controlled, cross-sectional and longitudinal study.

**Patients**

The present work consisted of: (i) a cross-sectional study including plasma samples from non-HIV-infected controls (n = 19), LTNP patients (n = 19) and standard ARV-naive progressors (Sp1 group); and (ii) a longitudinal study including plasma samples of the same Sp1 individuals (n = 19) after >12 months of ART, consisting of two NRTIs (tenofovir + emtricitabine) and one NNRTI (efavirenz) (Sp2 group).

All patients signed an informed consent form previously approved by the ethics committee of each participating centre. Sample recruitment was also approved at each centre and was performed with the collaboration of the infectious diseases departaments of several centres: Hospital General de Granollers (Granollers, Spain), Hospital Universitari Joan XXIII (Tarragona, Spain), Hospital de la Santa Creu i Sant Pau (Barcelona, Spain) and Hospital Clinic of Barcelona (Barcelona, Spain).

**Inclusion criteria**

A control group, matched for age and gender with the study groups, of healthy non-infected volunteers was included to obtain normal reference values within the general population.

- (i) LTNP group: patients presenting asymptomatic HIV infection, lacking opportunistic infections, not undergoing ARV therapy and with long-term non-progression of the disease were considered.\textsuperscript{16} Specifically, subjects with >15 years of infection, stable CD4+ T cell counts persistently >500 cells/mm\(^3\) and plasma viral load <5000 copies/mL, in the absence of ARV.
- (ii) Sp1 group: HIV-infected patients with standard progression before initiation of ARV (naive) and presenting with CD4+ T cell counts <350 cells/mm\(^3\) and viral load up to 35 000 copies/mL.
- (iii) Sp2 group: Sp1 subjects after receiving >12 months of tenofovir + emtricitabine + efavirenz ARV.

Exclusion criteria were a personal or family history of mitochondrial or neuromuscular disease or contact with drugs having known or potential toxicity for mitochondria (e.g. aminoglycosides, linezolid or antipsychotics).

**Clinical data**

Clinical and epidemiological variables of the patients and controls are shown in Table 1.

**Model for study**

After an overnight fast, 20 mL of whole blood from all patients and controls was collected in EDTA tubes and the plasma was obtained by 15 min of centrifugation at 1500 g.
Jurkat cells (of an immortalized T lymphocyte cell line from a patient with acute lymphocytic leukaemia) were cultured, grown to 250 000–500 000 cells/mL and incubated for two complete viral replication cycles (16 h), at identical passage numbers, in complete medium [RPMI + 1% (v/v) penicillin/streptomycin], containing 10% (v/v) plasma from the patients and controls. The experimental procedures always included a sample from each study group to be analysed in parallel. Immediately after this incubation, an aliquot of each sample was analysed by flow cytometry, while the remaining material was cryopreserved at −80 °C until molecular and biochemical analysis.

**Experimental assays**

**Protein quantification**

The BCA assay was used to calculate the total protein cell content (Pierce BCA Protein Assay Kit #23225; Thermo Scientific). Mitochondrial functional parameters were expressed in relation to the total amount of cell protein.

**mtDNA levels**

To quantify mtDNA content, total DNA was isolated by the phenol–chloroform method and RT–PCR was performed (Applied Biosystems) as reported elsewhere.19

**Mitochondrial function**

Spectrophotometry was performed to assess complex IV (CIV) and cytochrome c oxidase (COX) enzyme activity of the mitochondrial respiratory chain (MRC) and mitochondrial content by measuring citrate synthase (CS) enzyme activity.20–22 MRC enzyme activities were expressed as relative units, normalized for mitochondrial content, estimated by CS activity23 (COX/CS).

**Mitochondrial protein synthesis**

Western blot analysis was performed to quantify the mitochondrial-encoded subunit II of COX (COXII) and nuclear-encoded subunit IV of COX (COXIV) of the MRC by using 20 μg of total cell protein with 7%/13% SDS–PAGE and immunodetection with the specific corresponding antibodies.24

**Mitochondrial dynamics**

Western blot analysis was performed to quantify mitofusin-2 (MFN2) relative to β-actin by using 20 μg of total cell protein with 7%/13% SDS–PAGE and immunodetection using the mouse monoclonal anti-MFN2 antibody ab56889 (Abcam® UK) diluted 1/2000 in 1% milk.

**Apoptotic rate**

Quantification of early and late apoptotic rates was performed by using three different measures. First, voltage-dependent anion channel-1 (VDAC-1) protein content relative to β-actin content was assessed by western blot analysis, as previously described.24 Second, flow cytometry was used to assess mitochondrial membrane potential (MMP) using the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetravethyl benzimidazolo carbocyanine iodide (JC1), by quantifying cells with depolarized mitochondria as a percentage of the total number of cells.25 Finally, cells double-stained for annexin V and propidium iodide were quantified by flow cytometric analysis as a percentage of the total number of cells, as reported elsewhere.26

**Statistical analysis**

Statistical analysis was performed using the Mann–Whitney test for non-parametric variables and statistical significance was set at P<0.05.

**Results**

**Clinical data and participant characteristics**

We performed 76 in vitro assays to test 19 plasma samples from each study group (healthy controls, LTNP, Sp1 and Sp2) with identical Jurkat T cells.

Men predominated in the Sp1 and Sp2 groups, while in the LTNP and control groups the gender distribution tended to be balanced. As expected, CD4 counts and viral load in HIV-infected patients were different according to case definition (LTNP, Sp1 and Sp2).

### Table 1. Clinical and epidemiological data for patients and controls providing plasma

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>LTNP</th>
<th>Sp1</th>
<th>Sp2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size (n)</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Age, years (mean ± SEM)</td>
<td>41.06 ± 3.98</td>
<td>44.89 ± 3.06</td>
<td>37.58 ± 2.53</td>
<td>38.58 ± 2.53</td>
</tr>
<tr>
<td>Male (%)</td>
<td>46.15</td>
<td>47.36</td>
<td>78.94</td>
<td>78.94</td>
</tr>
<tr>
<td>ARV schedule</td>
<td>—</td>
<td>—</td>
<td>tenofovir + emtricitabine + efavirenz</td>
<td></td>
</tr>
<tr>
<td>Log_{10} viral RNA, copies/mL (mean ± SEM)</td>
<td>—</td>
<td>2.83 ± 0.25</td>
<td>4.44 ± 0.23</td>
<td>1.62 ± 0.017</td>
</tr>
<tr>
<td>CD4+ T cell count, cells/mm³ (mean ± SEM)</td>
<td>—</td>
<td>732.47 ± 55.94</td>
<td>478.00 ± 75.82</td>
<td>628.78 ± 35.22</td>
</tr>
</tbody>
</table>

aSignificantly higher in Sp1 than in LTNP and Sp2.
bSignificantly higher in LTNP and Sp2 than in Sp1.
Mitochondrial parameters

Most of the parameters tended to be consistently lower in HIV-infected patients than the reference values derived from the non-infected control group, although this was not statistically significant.

The mtDNA content was 30% lower in the Sp1 group than in the LTNP group and was 2% higher in the Sp2 group than in the Sp1 group (Figure 1).

Regarding mitochondrial function, the relative value of COX or CIV enzyme activities normalized per mitochondrion (CIV/CS ratio) in the Sp1 group was 58% of the value in the LTNP group and was 146% higher in the Sp2 group than in the LTNP group (Figure 2a).

Mitochondrial protein synthesis, expressed as the COXII/COXIV ratio, was 14% lower in Sp1 than in LTNP and was maintained in Sp2 with respect to the Sp1 level (Figure 2b).

Intergenomic coordination between mitochondrial and nuclear genomes, measured as the correlation between the levels of expression of the mitochondrial-encoded COXII and the nuclear-encoded COXIV, was positive, which was statistically significant in all groups (Figure 3).

Mitochondrial dynamics, measured as MFN2/β-actin expression, gradually decreased, being 24% lower in Sp1 and 44% lower in Sp2 than in LTNP (Figure 4).

Apoptotic parameters

The apoptotic parameter VDAC-1/β-actin content, an indicator of transition pore formation and apoptosis initiation, was 22% higher in Sp1 and 50% lower in Sp2 with respect to the LTNP group. LTNP and Sp2 values were within control reference values (Figure 5). Accordingly, the percentage of cells with depolarized mitochondria with respect to total number of cells, a marker of MMP loss and early apoptosis, was 10% higher in Sp1 and 14% lower in Sp2 with respect to LTNP. Sp2 values were within control reference values (Figure 6).

Finally, the percentage of cells with double staining for annexin V and propidium iodide with respect to the total number of cells, an indicator of advanced apoptotic status, was 8% higher in Sp1 and 1% lower in Sp2 with respect to the LTNP group. Values for all groups were within the control reference values (Figure 7).

In summary, there were no statistically significant differences in most of the mitochondrial and apoptotic parameters depending on HIV progression (LTNP versus Sp1) or treatment intervention (Sp1 versus Sp2). However, there was a trend towards differential levels of mitochondrial and apoptotic lesions, with an increase in the Sp1 group with respect to the LTNP group, and a partial recovery in the Sp2 group. This pattern was consistent for all parameters.
models to determine the underlying mechanisms of HIV progression, as well as to test the adverse effects of NRTI and toxic mechanisms. Distinct experimental models have been developed, most of them based on animal experimentation. Consequently, determining the pathophysiological effects of ARV on animals has become important in understanding the risks associated with current therapeutic approaches, in which mitochondrial toxicity is important. However, animal models present serious disadvantages, such as high costs, extensive animal facilities and differential viral progression, immunological and drug toxicity mechanisms, with respect to human models.

Within this framework, we aimed to create an in vitro culture model using a Jurkat T cell line incubated with plasma of different HIV progressors, in order to: (i) reproduce differential mitochondrial and apoptotic status in distinct progressions of the infection, according to the findings observed in a previously described ex vivo study; (ii) demonstrate whether mitochondria are involved in differential HIV progression; and (iii) establish an in vitro platform to test the mitochondrial and apoptotic toxicities of ARV schedules containing non-thymidine NRTIs. The establishment of an in vitro model of differential HIV progression would allow the development of strategies for the prevention of cell impairment and for the use of therapeutic assays in a standardized way.

Even though most of the differences in the mitochondrial and apoptotic parameters characterized in the cell model used in the present study did not reach the level of significance, we found a consistent trend towards a common pattern of gradual increase in mitochondrial lesions and apoptotic rates related to faster progression of HIV infection. The proposed culture model using Jurkat cells with plasma from patients partially reproduces the findings observed in ex vivo studies, as it consistently shows intermediate mitochondrial lesion and apoptosis rates that are higher in naive Sp patients than in LTNP patients.

The present work advocates a feasible and simple cell culture model in which it is possible to evaluate the mitochondrial and apoptotic toxicity and/or safety of one of the most frequent current ARV combinations used as an initial treatment, consisting of tenofovir + emtricitabine + efavirenz. This model made it possible to detect differential trends of mitochondrial function in the populations studied. In the model, mitochondrial lesions and apoptotic injury promoted by the virus and enhanced in typical progressors versus LTNP, occurred in Sp2 patients after >12 months of receiving an ARV schedule including tenofovir + emtricitabine + efavirenz. A recent study described depletion in mtDNA and mitochondrial dysfunction in CI and CIV enzyme activities in adipose tissue of HIV-infected patients receiving tenofovir + emtricitabine plus efavirenz or atazanavir/ritonavir, although there was no evidence of any mitochondrial and apoptotic T cell effect associated with >12 months of treatment with tenofovir + emtricitabine + efavirenz. In the proposed in vitro study model, the fact that the Sp2 group tended to partially recover its mitochondrial parameters and show a decrease in all apoptotic events supports the concept that this specific ARV schedule seems to be safe with respect to cell viability. A common pattern such as this (observed in mitochondrial genetic and functional parameters as well as in apoptotic events) may point out the association between mitochondrial status and HIV progression, placing mitochondria as a key orchestrator, and the main source of cell apoptotic events, within the T cell population (Jurkat cells in our model).

**Discussion**

Since the explosion of interest in HIV and ARV toxicity, there has been increasing demand for the establishment of animal or cell

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**Figure 5.** Apoptosis initiation. VDAC-1 content expressed as the VDAC-1/β-actin ratio, as an indicator of mitochondrial transition pore formation for apoptosis initiation. The normal range of non-infected controls is shown as a box (mean ± SD).

**Figure 6.** Early apoptosis. Mitochondrial depolarization analysis using the MMP sensor JC1, expressed as the percentage of cells with depolarized mitochondria with respect to the total number of cells, an indicator of early apoptosis. The normal range of non-infected controls is shown as a box (mean ± SD).

**Figure 7.** Advanced apoptosis. Apoptosis and cell viability measurement using the markers annexin V and propidium iodide, respectively, expressed as the percentage of cells with double fluorescence for both markers with respect to the total number of cells, an indicator of late apoptosis. The normal range of non-infected controls is shown as a box (mean ± SD).
Secondary conclusions can be extracted from the present study. Interestingly, we noticed in this in vitro model that coordination between the nuclear and mitochondrial genomes remained unaltered in all groups despite differential HIV progression or treatment intervention, as our findings showed a significant and positive correlation among the contents of structural protein subunits of the COX enzymes of the MRC, encoded by the different genomes. Thus, this intergenomic interaction is not compromised within different types of HIV progression or an ARV consisting of tenofovir + emtricitabine + efavirenz. Additionally, mitochondrial dynamics, which encompass the processes of mitochondrial fusion and fission for organelle renewal and recycling, recently associated with diverse pathologies, have also been studied in the proposed model of HIV infection by MFN2 measurement (mitochondrial fusion). In our study model, MFN2 gradually decreased in faster HIV progressors with respect to the LTNP group. Although these findings are consistent with previously reported associations between Vpr viral protein and decreased MFN2, the lower levels of MFN2 found in treated Sp2 patients require an alternative hypothesis. Notably, experimental data are lacking in the field of mitochondrial dynamics in HIV infection.

Even though the findings derived from the proposed model led to a homogeneous pattern for all the mitochondrial and apoptotic parameters, comparable to in vivo data, this in vitro model presents some limitations. First, the fact that most of the parameter differences did not achieve statistical significance could be due to the small sample size. However, some patients, such as those in the LTNP group, are uncommon due to the very low prevalence of the LTNP status. Second, despite their analogy to T lymphocytes, Jurkat cells belong to a cancerous cell line and present a high glycolytic metabolism rather than an oxidative and mitochondrial metabolism. These cell features, associated with increased proliferative ratios, confer on these cells a reduced mitochondrial function. However, there have been a remarkably wide range of scientific studies using Jurkat cells as a model to investigate the roles of HIV and ARV molecular mechanisms. Third, differential HIV progression is a multifactorial trait comprising at least two variables: the virus and its host. The proposed study model discards the endogenous cells of the host, and thus their mitochondria, replacing them with Jurkat cells, in order to elucidate whether the influence of HIV progression and ARV toxicity rely on a factor in the plasma. The replacement of endogenous T cell lymphocytes with a Jurkat cell line avoids the requirement of a huge amount of blood from patients. Furthermore, the use of Jurkat cells makes it possible to keep the cells alive in a cell culture system and to avoid dealing with activation of the coagulation cascade. Their use reduces host differences, but discards one of the main factors promoting slow HIV progression or ARV toxicity. Finally, other factors have been described as contributing resistance to HIV- or ARV- secondary effects, in addition to mitochondria. The hypothesis to explain differential HIV progression in the present model considering the mitochondrion as a target organelle within a target cell is not detrimental to other reportedly protective factors, such as HLA, co-receptor polymorphisms, antibodies, cytokines and antimicrobial peptides (α/β defense). When considering ARV toxicity, pharmacogenetic variability among individuals in drug processing should also be considered. Lastly, another potential limitation of this study is the possibility that the experimental conditions, such as temperature and concentrations, were not optimal for the reproduction of in vivo conditions.

From a translational point of view, if mitochondrial and apoptotic cell lesions are the basis of future clinical onset, the improvement of such lesions would minimize the adverse effects of medication and the sociosanitary costs associated with the management of infected individuals, potentially improving the quality of life and lifespan of the patient.

Our in vitro model partially reproduced the differential mitochondrial and apoptotic lesions characteristic of HIV progression previously described in patients and indicated potential pathways for slowing viral progression by reducing mitochondrial impairment and apoptotic events. Moreover, in this cell model the therapeutic tenofovir + emtricitabine + efavirenz combination was confirmed as a first-line therapy that is safe with respect to mitochondrial function.

In summary, we have proposed a simple and feasible study model that makes it possible to evaluate the effects of HIV infection and its therapy on mitochondrial function. This model could be useful in clinical research. Further studies will be needed to confirm our observations, to determine their clinical relevance and to test the potential use of serum and/or fresh plasma in the incubation process. The main limitation of this study is that the method did not reveal any significant differences among different groups, and thus it needs further validation, including a proof of concept with statistically significant results.

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Transparency declarations
None to declare.

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