Brief Communication

Quantification of Mitochondrial Toxicity in HIV-Infected Individuals by Quantitative PCR Compared to Flow Cytometry

Thor A. Wagner,[1,2*] Chen-Han Lin,[3] Nicole H. Tobin,[1] Hélène C. F. Côté,[4] Derek D. Sloan,[5] Keith R. Jerome,[6,7] and Lisa M. Frenkel[1,2,6,8]

[1]Seattle Children's Research Institute, Seattle, Washington
[2]Department of Pediatrics, University of Washington, Seattle, Washington
[3]Division of Research and Development Business, United Biomedical, Inc., Shin Chu, Taiwan
[4]Department of Pathology & Laboratory Medicine, University of British Columbia, Vancouver, Canada
[5]Vaccine Research Department, MedImmune, Mountain View, California
[6]Department of Laboratory Medicine, University of Washington, Seattle, Washington
[7]Vaccine and Infectious Disease Institute, Fred Hutchinson Cancer Research Center, Seattle, Washington
[8]Department of Global Health, University of Washington, Seattle, Washington

Background: Non-invasive diagnostic assays to evaluate mitochondrial toxicity could have significant clinical utility for HIV-infected individuals on antiretroviral therapy (ART).

Methods: This study compared the ratio of mitochondrial to nuclear DNA determined by quantitative polymerase chain reaction (qPCR) to the ratio of mitochondrial to nuclear-encoded proteins by flow cytometry, in peripheral blood mononuclear cells from 73 HIV-infected individuals with and without risk factors for mitochondrial toxicity.

Results: PCR detected similar mitochondrial/nuclear DNA in HIV-infected individuals without a history of ART, and those receiving ART with lipodystrophy, lipoatrophy, or a history of suspected lactic acidosis. However, the ratio was significantly greater in ART-untreated compared to those receiving either stavudine or didanosine. In contrast, flow cytometry did not detect any differences in mitochondrial/nuclear protein (Lin et al., Cytometry B 2009;76B:181–190). There was no correlation between the assays (rho = −0.05, $P = 0.65$).

Conclusions: Assessment of the mitochondrial/nuclear DNA ratio by qPCR performed better than the mitochondrial/nuclear-encoded protein ratio by flow cytometry to detect adverse effects of nucleoside analogs on mitochondria. © **2012 International Clinical Cytometry Society**

Key terms: HIV; antiretroviral therapy; mitochondrial toxicity; flow cytometry; quantitative PCR; diagnostics

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Nucleosides/-tide reverse transcriptase inhibitors (NRTI) are included in most antiretroviral treatment (ART) regimens. Exposure to NRTI is associated with a diffuse spectrum of metabolic adverse reactions consistent with mitochondrial toxicity (1). These side effects were originally thought to be due primarily to the inhibition of mitochondrial DNA (mtDNA) polymerase gamma (γ) (2). Subsequent work suggests that NRTI may cause mitochondrial dysfunction through a variety of mecha-

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*Correspondence to: Thor A. Wagner, Seattle Children's Research Institute, 1900 Ninth Ave, 8th Floor, Seattle, WA 98101.
E-mail: thor.wagner@seattlechildrens.org

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nisms, which may vary depending on the specific NRTI and cell type (3,4). Because the clinical symptoms associated with mitochondrial toxicity are variable and range in severity, there would be substantial clinical utility to a non-invasive diagnostic assay to evaluate mitochondrial toxicity in HIV-infected individuals on ART.

Muscle biopsy is commonly used to diagnose genetic mitochondrial disorders (5), and adipose biopsies appear sensitive to evaluate antiretroviral (ARV) associated lipodystrophy (6,7), but tissue biopsies are not practical for routine monitoring during ART. A variety of non-invasive methods have been developed in an effort to assess mitochondrial toxicity using peripheral blood (8,9). The most established approach has been to monitor changes in the amount of mtDNA in peripheral blood as measured by quantitative polymerase chain reaction (qPCR) (10), although these results have been inconsistently associated with metabolic symptoms and/or exposure to NRTI (7,10–15). qPCR amplifies nucleic acid exponentially, which can result in significant inter-assay variability. Despite extensive assay optimization and rigorous standardization, assay variability may be too great for monitoring mitochondria within an individual over time, given that relatively minor changes in the number of mitochondria may influence mitochondrial function. In an effort to improve assessment of mitochondrial toxicity, we developed a method to assess mitochondrial toxicity by quantifying the ratio of mitochondrial to nuclear encoded proteins using flow cytometry (16). This method was reproducible and potentially measures mitochondria function on a single cell level. However, when we explored the potential utility of the assay the ratio of mitochondrial/nuclear proteins was not associated with exposure to stavudine (d4T) or didanosine (ddI) or symptoms of mitochondrial toxicity such as lipodystrophy. We speculated that the lack of correlation could be because the ratio of nuclear- to mitochondrial-encoded proteins is under homeostatic control such that transcription and translation are regulated in order to maintain a relatively constant ratio of mitochondrial proteins in each cell.

In this study, we compared mitochondrial status in subjects' peripheral blood mononuclear cells (PBMC) as determined by our flow cytometry-based assay to qPCR, in order to further evaluate the utility of our flow cytometric assay in the detection of NRTI-associated mitochondrial toxicity and help guide future development of diagnostic assays.

METHODS

Study Population

The Institutional Review Board at Seattle Children's Hospital approved the project. All participants gave written informed consent to donate clinical specimens to the Center for AIDS Research Repository at the University of Washington. Two groups of participants were selected: (1) HIV-infected without exposure to ARV or symptoms of mitochondrial toxicity; and (2) HIV-infected with history of signs or symptoms consistent with mitochondrial toxicity (lactic acidosis, lipoatrophy, or lipodystrophy). Separate aliquots of cryopreserved PBMC from the same specimen were assayed blindly for evidence of mitochondrial toxicity by flow cytometry and qPCR. The flow cytometry data were previously published (16).

Methods of Quantifying Mitochondrial Toxicity

Flow cytometry was performed as previously described (16); the PBMC were permeabilized and stained with anti-cytochrome c oxidase subunit I (COX-I) (Clone 1D6, Molecular Probes, Invitrogen, Eugene, OR), anti-ATP synthase subunit D (Sub-D) (Clone 7F9, Molecular Probes, Invitrogen, Eugene, OR), anti-CD3, anti-CD56, secondary antibodies, and isotype controls. COX-I and Sub-D are both mitochondrial proteins that function in oxidative phosphorylation. COX-I is encoded by mtDNA and Sub-D is encoded by nuclear DNA. The mean fluorescence intensity of lymphocytes (CD3+ gate) for COX-I and Sub-D was analyzed on a FACSCalibur instrument using CELLQuest software (BD Biosciences, Immunocytometry Systems, San Jose, CA). qPCR was performed as previously published (10,11); a mitochondrial gene (cytochrome C oxidase subunit I (CCOI)) and a nuclear gene (accessory subunit of the polymerase γ [ASPG]) were quantified by real-time polymerase chain reaction (PCR) on a LightCycler 480 (Roche, Roche Applied Sciences, Foster City, CA), using Faststart probe master mix (Roche Diagnostics, Indianapolis, IN). qPCR results were expressed as the ratio of the CCOI copy number to the ASPG copy number.

Statistics

Mann–Whitney test was used to compare results of participants grouped by ARV use and symptoms. Spearman's rank correlation (rho) was used to evaluate the strength of the relationship between the two assays. Statistics were performed using VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html).

RESULTS

Participants

Specimens from 73 HIV-infected study participants were successfully tested for mitochondrial toxicity using both methods (flow cytometry and qPCR). Forty-eight participants had a history of signs or symptoms consistent with mitochondrial toxicity (lactic acidosis, lipoatrophy, or lipodystrophy), of whom six had laboratory documented lactic acidosis, and nine were on d4T and/or ddI at the time of specimen collection. Twenty-five participants were ART-naïve and asymptomatic. The median age of the participants with a history of signs or symptoms consistent with mitochondrial toxicity was 42 years old (range 28–60), and they were 83% male, 81% Caucasian, 10% African American, and 8% Latino/a. The median age of the ART-naïve and asymptomatic participants was 37 years old (range 21–56), and they were

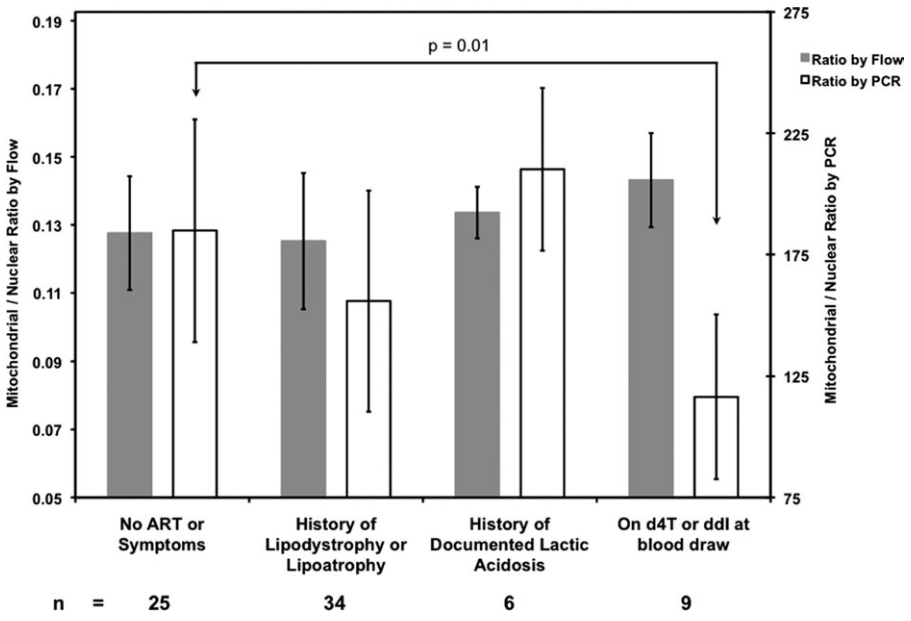

FIG. 1. Comparison of qPCR and flow cytometry to assess mitochondria toxicity in HIV-infected subjects with different risks for NRTI-associated mitochondrial toxicity. DNA ratios by qPCR are shown on the right-side Y-axis and protein ratios by flow cytometry are shown on the left-side Y-axis. The scale and X-intercept of the axes have been adjusted to equalize the ratios in specimens from the HIV-infected individuals without ART exposure or symptoms consistent with mitochondrial toxicity. Error bars reflect one standard deviation. Only the difference between mitochondrial/nuclear DNA qPCR ratio in the d4T or ddI group versus the no ART or symptom group was statistically different, indicated with an arrow above the graph. Number of specimens in each category indicated below the X-axis.

84% male, 68% Caucasian, 28% African American, and 4% American Indian.

Comparison of qPCR to Flow Cytometry

Across all participants, the median mtDNA/nDNA ratio by qPCR was 168 ($n = 73$, range 57–263, interquartile range (IQR) 131–194). The quantities of mtDNA and nDNA in each specimen assayed were modestly correlated (rho = 0.40, $P < 0.001$). In the flow cytometric analysis, the amount of mitochondrial and nuclear protein per lymphocyte were strongly correlated (rho = 0.72, $P < 0.001$). When the two methods were compared, there was no correlation between the ratio of mtDNA/nDNA and that of their respectively encoded proteins as assessed by qPCR and flow cytometry respectively (rho = −0.05, $P = 0.65$).

Relationship Between Results and Clinical Characteristics

Among individuals without a history of ART exposure, the median mtDNA/nDNA ratio was 185 ($n = 25$, range 99–263, IQR 140–199), which was not statistically different from those receiving ART who had a history of signs or symptoms consistent with mitochondrial toxicity (lactic acidosis, lipoatrophy, or lipodystrophy) ($n = 48$, median ratio 166, range 57–251, IQR 130–188, $P = 0.35$). However, the mtDNA/nDNA ratio of the untreated participants was significantly greater than individuals receiving either d4T or ddI whose median ratio was 116 ($n = 9$, range 99–194, IQR 102–148), ($P = 0.01$). There were no statistically significant differences between the three groups by flow cytometry (Fig. 1).

DISCUSSION

A robust non-invasive approach to monitor for mitochondrial toxicity would simplify the management of ARV used to treat HIV infection. Côté et al. pioneered a qPCR-based approach to quantify the ratio of mtDNA to nDNA in the peripheral blood cells, which has been observed to correlate with severe symptoms of mitochondrial toxicity related to NRTI exposure (10). In an effort to develop a sensitive method suitable for monitoring small changes in mitochondria number within individuals during ART, we previously developed a flow cytometry-based method of quantifying the ratio of mitochondrial-encoded versus nuclear-encoded proteins per lymphocyte (16). In the current study, we evaluated separate aliquots of the same samples by qPCR to compare to our flow methods. The goal was to better quantify NRTI-associated changes in mitochondrial concentrations and ultimately to improve the diagnosis and management of ARV-associated mitochondrial toxicity.

Our analysis of repository samples selected based on risk factors for mitochondrial toxicity found that the qPCR but not flow cytometry detected a relative decrease in mtDNA among subjects receiving d4T or ddI at the time the specimen was collected, compared to control HIV-infected subjects. This is consistent with the recognized association of d4T and ddI and mitochondrial toxicity (17). Neither assay detected differences in mitochondrial concentrations between the groups with a history of symptoms consistent with mitochondrial toxicity and controls. Importantly, our population with past "mitochondrial toxicity" had relatively heterogeneous symptoms which may have substantially resolved by the time the specimens were collected. A longitudinal study of individuals with more strictly defined evidence of active mitochondrial toxicity at the time of blood collection may be more informative.

Interestingly, there was no correlation between the two methods. This lack of relationship may reflect differences in the cell populations assayed by the two methods. The flow-cytometry method specifically targets viable lymphocytes, whereas the qPCR-method assays all PBMC-derived DNA in a given sample. It is conceivable that variability in cell viability, and/or the number of lymphocytes relative to other mononuclear cells (e.g., B-cells, monocytes) existed between specimens. We speculate that viable cells have the ability to regulate the transcription, translation, and degradation of essential mitochondrial proteins. If cells with depleted mitochondria or changes in the ratio of mitochondrial proteins are less viable, they may have been excluded from our flow cytometry gating strategy. This suggests that future studies should be done using flow cytometry with less restrictive gating strategies to determine whether mitochondrial toxicity is detectable in other cells types or in cells that appear less viable. This may help explain why the qPCR protocol, that assesses all PBMC, was able to detect mitochondrial changes (diminished mt/nDNA) in participants taking d4T or ddI. However, it is also possible that ARV-exposure reduces mtDNA concentration, but that individuals only begin to develop symptoms of mitochondrial toxicity when cells no longer have enough mtDNA to produce sufficient mitochondrial-encoded proteins. To test these hypotheses, future prospective studies of individuals who develop more severe mitochondrial toxicity would be helpful.

CONCLUSION

In summary, our comparison of qPCR-based quantification of mtDNA/nDNA and flow cytometric detection of mitochondrial and nuclear encoded proteins suggests that the two assays perform differently. As currently designed, the flow method does not appear to detect ARV-associated decreases in mtDNA. Further research is warranted to improve the flow assay, and to understand the limits of each assay in patient management, including the concentrations of mitochondria that correlate with clinical signs and symptoms of mitochondrial toxicity.

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