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Chemotoxicity Recovery of Mitochondria in Non-Hodgkin Lymphoma Resulting in Minimal Residual Disease

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Abstract

Background—The mechanisms responsible for resistant disease or recurrence of non-Hodgkin lymphoma (NHL) in children cover a wide spectrum from drug resistance to genetic mutations. A unique mechanism suggesting the role of mitochondria as the key energy source is studied following a clinical observation where pediatric Burkitt lymphoma (BL) specimens from patients on therapy were found to have increased copies of mitochondria DNA (mtDNA) in specimens which were shown to be positive for minimal residual disease and/or persistent disease (MRD/PD). This study hypothesized that the mitochondria play an important role in a cell's recovery from toxicity via a compensatory increase in mtDNA.

Procedure—BL specimens with MRD/PD were assayed for mtDNA. An in vitro model was then designed using Ramos cell lines by exposing the lymphoma cells to varying concentrations of doxorubicin and vincristine for 1 hr; and allowing for recovery in culture over 7 days. DNA was extracted from aliquots over several days to determine mtDNA copy numbers by real-time polymerase chain reaction (PCR).

Results—Increased mtDNA copy numbers were found in clinical specimens with MRD/PD as well as in recovering Ramos cells from chemotoxicity.

Conclusions—The recovering lymphoma cells from the chemotoxic effects appeared to compensate by increasing mtDNA content, which may contribute to the clinical residual or resistant disease in some cases of childhood BL.

Keywords

minimal residual disease; mitochondrial DNA; non-Hodgkin lymphoma

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INTRODUCTION

Mitochondria are metabolic energy sources vital to cell survival and function, as well as a compensatory energy supply to overcome cellular damage. These inherent properties of the mitochondria, however, can also have adverse effects in diseases such as cancer. When assessing the relationship between cancer and mitochondria, the mitochondrial DNA (mtDNA), an important component of the organelle, could provide clues and insight into pathogenesis [1]. Specifically, the presence and quantitation of mtDNA by assessing mtDNA copy number is frequently assayed to determine a cell's response to external factors [2]. Quantifying mtDNA in malignant cells could possibly be linked to tumor progression, chemotherapy response, or lead to understanding mechanisms of disease recurrence.

In specific malignancies, such as ovarian cancer, mtDNA content was significantly higher in tumor cells when compared to cells from normal ovaries [3]. Additionally, others have found increasing mtDNA content as histopathology grade worsens in head and neck cancers [4]. The study hypothesized that increase in mtDNA in these head and neck malignancies may reflect a compensatory mechanism from decline in respiratory chain function due to oxidative damage [4]. While cancer chemotherapy eradicates malignant cells, we hypothesize that an additional affect on the mitochondria may also be occurring. Initially, chemotherapeutic agents, as they affect malignant cell, also affect mtDNA [5]. The impact seen with decrease in mtDNA content is accompanied by mtDNA mutations from the drugs as reactive oxygen species (ROS) are generated [6]. ROS in turn is detrimental to mtDNA and is not able to repair mtDNA in response to the stress factor [7]. The oxidative stress stimulates synthesis of mtDNA, thus increasing mtDNA copy number in recovering cells [4,8]. We therefore hypothesize that minimal residual disease or persistent disease (MRD/PD) is partially explained through emergence and recovery of malignant cells. The gold standard of assessing residual disease in pediatric non-Hodgkin lymphoma (NHL) is through standard light microscopy (1/100 cells) while MRD is typically measured using polymerase chain reaction (PCR) [9,10]. As in other tumors, the presence of MRD/PD in NHL could be a more aggressive disease that possibly leads to recurrence and overall poor prognosis. Since the mitochondria in the tumor cells are a source of energy, we hypothesized that these residual cells might survive from the compensatory energy provided by the mitochondria. In the current study, we hypothesized that pediatric NHL specimens from patients on therapy that have MRD/PD would have higher mtDNA copies; and an in vitro model would demonstrate that residual cells have similar increases in mtDNA.

MATERIALS AND METHODS

Patient Specimens and MRD Assessment

The study was reviewed and approved by the University of Hawaii Institutional Review Board. DNA was extracted from paraffin-embedded tissue, unstained blood, and bone marrow (BM) slides, which were previously obtained and analyzed from children diagnosed with Burkitt lymphoma, BL. DNA was extracted as per the QIAamp DNA Micro protocol: Isolation of Genomic DNA from Small Volumes of Blood (Qiagen; Valencia, CA) with no additional sample preparation. Quality of DNA was assessed by analysis of the 260/280 absorbance ratio using the ND-1000 Spectrophotometer (NanoDrop Technologies; Wilmington, DE) with average total DNA yield approximately 100 ng. Specimens were available at diagnosis and at follow-up time points prior to the next cycle of chemotherapy. Patients were treated on COMP-based chemotherapy regimens that included vincristine and doxorubicin [11]. Blood and/or BM obtained at diagnosis and follow-up were assessed for the presence of residual disease by standard microscopy pathological criteria and for MRD/PD as previously reported and confirmed with immunoglobulin primer pools; with a sensitivity up to 1/10⁶ cells [12]. An improved sensitivity and approach to real-time PCR was utilized to confirm previous findings of MRD/PD. Briefly, primer pool sets from the FR1 region [V region (VH)] of the variable

IgH region (Operon Biotechnologies, Inc, Huntsville, AL) were combined to form two unique primer pools from VH3 and VH4 families: VH3a GA(AG) GTG CAG CTG GTG GAG; VH3b CAG GTG CAG CTG GTG GAG, VH3c GAG GTG CAG CTG TTG GAG; and VH4a CAG (CG)TG CAG CTG CAG GAG and VH4b CAG GTG CAG CTA CAG CAG [13]; and paired with two consensus 3' primers (LJH and VLJH) [12]. Semi-nested PCR was performed in duplicate using an iCycler (BioRad Laboratories, Hercules, CA) with appropriate negative and positive controls followed by second-round PCR.

mtDNA Assessment

DNA samples were assayed for mtDNA copies using real-time PCR with an iCycler instrument (BioRad Laboratories) as previously described with minor modifications [14]. The mitochondrial primers (forward: CAC AGA AGC TGC CAT CAA GTA; reverse: CCG GAG AGT ATA TTG TTG AAG AG) amplified the mtDNA region encoding the NADH dehydrogenase subunit 2, which has been used in similar studies and shown to be appropriately quantified in malignant cells [4,15]. The genomic primers (forward: GGC TCT GTG AGG GAT ATA AAG ACA; reverse: CAA ACC ACC CGA GCA ACTAAT CT) amplified the genomic DNA region encoding for Fas Ligand. A control plasmid with the mitochondrial and genomic regions was used to create a standard curve with serial dilutions ranging from 10^6 copies to 10^2 copies. Standards, samples, and appropriate positive/negative controls were set up in triplicate with each reaction containing iQ SYBR Green Supermix, 10 pmol forward primer and 10 pmol reverse primer in a final volume of 25 μ l. The PCR cycling parameters were: 95°C for 1 min; 40 cycles of 95°C for 30 sec; 58.9°C for 40 sec; 72°C for 4 min; and 50°C for 30 sec. At the conclusion of the PCR, melt curve data were collected by the iCycler software using a start temperature of 65°C, with 0.5° incremental increases every 5 sec for duration of 70 cycles. Copy numbers of mtDNA were obtained as previously reported [14].

Cell Lines and Chemotherapy Exposure

Ramos cells, a human BL cell line, were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 1X (Mediatech Incorporated; Herndon, VA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 2% sodium bicarbonate, 1% glucose, 1% sodium pyruvate, 1% HEPES, and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Cells were exposed to vincristine sulfate (MP Biomedicals; Solon, OH) in increasing concentrations of 0, 3, 6, and 12 nM; and doxorubicin-HCL (Biomol Internationals; Plymouth Meeting, PA) in increasing concentrations of 0, 100, 300, and 500 nM; diluted in culture media. Drug concentrations used in the cultures were similar to clinically relevant plasma levels [16]. Prior to drug exposure, Ramos cells were centrifuged at 1,200 RPM for 10 min with the supernatant decanted and followed by treatment with 10 ml Accumax (Innovative Cell Technologies; San Diego, CA) and incubated for 10 min at 37°C. Cells (1.33×10^6 cells/ml) were then suspended in 1 ml of Ramos culture media including 250 μ l of the chemotherapy agent; in duplicate. After 1 hr drug exposure, cells were washed twice with fresh media; and resuspended in 1 ml of fresh media (5×10^5 cells/ml); remained in culture for 7 days. Cell viability was estimated daily by Trypan blue exclusion method. Non-viable cells were removed from culture on days 2, 4, and 6 by centrifugation at 500 RPM for 7 min and resuspended in fresh media. Aliquots of the cultures were obtained on days 1, 3, 5, and 7 to assess mtDNA copies. To determine the contribution of non-viable cells to mtDNA copy number, 100 μ l aliquots were removed from a Ramos cell control plate where cells were not spun down or resuspended in fresh media for the 7-day period. Cell proliferation assays (Biovision; Mountain View, CA) were performed on days 2, 4, and 6 to assess the expansion of viable cells by measuring activity of mitochondrial dehydrogenase in response to growth factors, cytokines, mitogens, and nutrients.

RESULTS

Patient Samples and mtDNA Copy Number

Specimens were obtained from pediatric cases of tumor and follow-up BM and/or blood of children diagnosed with BL. mtDNA copies were assessed from seven patients in which diagnostic and follow-up specimens were available regardless of MRD/PD status. A statistically significant decrease in mtDNA copies per cell was found in follow-up specimens, (Patients 1, 2, 3, 4, and 6) compared to the normal range of mtDNA found in PBMC, Table I. These same specimens were also negative for MRD/PD.

In contrast, two patients (Patients 5 and 7) demonstrated unique mtDNA findings. Patient 5 showed an initial decline in mtDNA copy numbers (3 months after diagnosis) in which the same specimen was negative for MRD/PD. Three months later, the BM became positive for MRD/PD and was also found to have increased mtDNA copies; Patient 5 had a clinical relapse 2 months later. From Patient 7, an MRD/PD-positive specimen (6 months after diagnosis) had mtDNA copy numbers which were lower than the diagnostic specimen, but significantly higher than control. Three months later, Patient 7 had a clinical relapse.

Chemotherapy-Treated Ramos Cells: mtDNA Copy Number

Since we hypothesized that MRD/PD in pediatric BL cases is due to BL cells recovering from chemotoxicity, in vitro experiments were designed to examine the effect on mtDNA of BL cells following exposure to chemotherapy. mtDNA copy numbers were assessed in Ramos cells exposed to various concentrations of doxorubicin and vincristine. Following 1 hr exposure of the drug, Ramos cells recovered from the acute toxicity and mtDNA from the recovered cells was assessed. As expected, the number of viable cells decreased with increasing drug concentrations following chemotherapy exposure, as assessed by Trypan blue staining. As the drug-exposed cells recovered, mtDNA copies gradually increased over time, even though the total number of cells decreased or remained stable (Fig. 1). For both the doxorubicin- and vincristine-exposed Ramos cells, there was a statistical increase in mtDNA copy number when cells remained in culture every day for 7 days. There was minimal contribution from non-viable cells in mtDNA copy number over the 7 days that the cells were in culture (Fig. 2). The cell proliferation assays showed an overall decrease proliferation after exposure to vincristine and doxorubicin (Table II).

DISCUSSION

The aim of this study was to investigate the effect on mitochondria following chemotherapy as mechanism leading to MRD/PD. Following the clinical observation that positive MRD/ PD status was associated with increased mtDNA copy number in children treated for BL, in vitro experiments were designed to assess the direct effect of the drug on mtDNA in surviving lymphoma cells. The results show that cells recovering from chemotherapy were associated with increasing mtDNA numbers. The data are consistent with the hypothesis that mitochondria may play a role in residual malignant cells recovering from chemotherapy suggesting that an increase in mtDNA copy number is consistent with a survival cellular phenotype [2]. As suggested by Mambo et al., increased mtDNA copies could be a response to enhanced mtDNA repair and increased survival from oxidative damage due to chemoexposure. While mitochondria have been linked to both apoptosis and tumor progression, the role of that these organelles play during recovery from chemotoxicity may provide clues on how mitochondrial pathways contribute to mechanisms of MRD/PD in childhood BL. The mitochondria's contribution to MRD/PD should be seen in the context of other mechanisms leading to residual disease [17]. Drug-resistant genes or proteins, such as P-glycoprotein, multidrug resistance-associated protein 1, breast cancer resistance protein, and lung resistance protein are well

recognized as mechanisms of resistance to chemotherapy. Some of these proteins have relationships with MRD/PD as multi-drug resistance efflux pumps by predicting relapse. Additionally, other genes/proteins, such as TOP2A and BUB3, are functionally related in terms of their role in cellular proliferation. Relative levels of expression of TOP2A and BUB3, which are cell proliferating genes, compared to normalized housekeeping genes, were decreased and found to be associated with doxorubicin and vincristine resistance, respectively [18]. Thus, the role of mitochondria in MRD/PD could hypothetically complement the role of drug-resistant genes as an explanation for MRD/PD in some patients who experience a relapse of their disease.

Understanding mtDNA content in cancer progression or resistance to therapy may provide clues to mechanism of disease pathogenesis. Increases in mtDNA copy number have been associated with other types of malignancies such as endometrial cancer as an example of mtDNA involvement in aggressive disease [19]. Others have shown that increase in mtDNA could be associated with chemotherapy resistance in surviving cancerous cells through reduction of ROS generation [20]. Furthermore, it has also been found that ROS play a regulatory role in the mitochondrial controlled pathway of vincristine-induced apoptosis in Jurkatt cells [21].

The two chemotherapy agents utilized in the current study, vincristine and doxorubicin, have different mechanisms of action. Vincristine binds to tubulin dimers leading to inhibition of microtubule assembly, while doxorubicin damages DNA [16,21,22]. In contrast, increased mtDNA copy number has been observed in cells where the overall synthesis of ATP has been disrupted [23]. In an animal model, recovering cells exposed to gamma-irradiation had an increase in mtDNA copy numbers, which was thought to be secondary to induction of new mtDNA synthesis to compensate for damaged mtDNA [24]. Thus, when cells recover from the toxic effects either from chemotherapy or radiation and which inhibit ATP synthesis, mtDNA copy numbers may increase as a compensatory mechanism. To elucidate mechanisms responsible for increase in mtDNA, future studies could be designed to directly inhibit ATP with inhibitors or to use vincristine- or doxorubicin-resistant cell lines [25]. Similar types of chemo-resistant cell lines are available (ATCC, Manassas, VA) or can be established in vitro [25].

The study provides new data on the effect that vincristine and doxorubicin have on mitochondrial numbers following chemotherapy exposure and the possible association with resistance to treatment. After finding increased mtDNA copy numbers in MRD/ PD-positive BL specimens from pediatric patients exposed to vincristine and doxorubicin, we hypothesized that the mitochondria may be an important factor in the persistence or emergence of residual cells. We described similar increase in mtDNA in BL cells after in vitro exposure to both chemotherapy agents individually. The recovering BL cells in culture were not found to be actively proliferating, but rather the cells were found to have increased mtDNA per cell. The study is limited by the number of clinical specimens analyzed. A larger study including clinical outcomes is needed to determine the significance of mtDNA in MRD/PD. Future studies are planned to identify mechanisms and pathways that lead to increased mtDNA in malignant cells which may provide insight into mechanisms of chemotherapy resistance.

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Fig. 1.

Mitochondrial DNA copies following exposure to drug. **A:** mtDNA copy number of vincristine-treated cells exposed at various concentrations for a duration of 1 hr. Viable cells were maintained in standardized culture conditions for a 7-day period with mtDNA copy number assessed on days 1, 3, 5, and 7 of the protocol. Drug exposure was performed at four concentrations in duplicate: 0, 3, 6, and 12 nM. **B:** mtDNA copy number of doxorubicin-treated cells exposed at various concentrations for a duration of 1 hr. Viable cells were maintained in standardized culture conditions for a 7-day period with mtDNA copy number assessed on days 1, 3, 5, and 7 of the protocol. Drug exposure was performed at four concentrations in duplicate: 0, 100, 300, and 500 nM. *Denotes Significance, $P < 0.01$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 2. Mitochondrial DNA copies in control plate: untreated Ramos cells. In order to assess dead cell contribution to mtDNA copy numbers, a control plate was run in parallel with the drug-treated Ramos cells where media was left unchanged for the 7-day period resulting in complete cell death. mtDNA copy number of these dead, untreated Ramos cells were then assessed on the same days as the drug-treated Ramos cells.

TABLE I**Mitochondrial DNA Copy Number and MRD/PD Status**

Patient	MRD/mtDNA	Entry	Visit 1	Visit 2
1	MRD <i>mtDNA</i>	Tissue (+) 105	Blood (–) 77	
2	MRD <i>mtDNA</i>	Tissue (+) 107	Blood (+) 299	Marrow (–) 46
3	MRD <i>mtDNA</i>	Tissue (+) 104		Marrow (–) 72
4	MRD <i>mtDNA</i>	Tissue (+) 100	Blood (–) 56	
5	MRD <i>mtDNA</i>	Tissue (+) 113	Blood (–) 65	Marrow (+) 149
6	MRD <i>mtDNA</i>	Tissue (+) 109		Marrow (–) 53
7	MRD <i>mtDNA</i>	Tissue (+) 454		Marrow (+) 172

Numbers are mtDNA copies per cell. (+) indicates positive MRD/PD status; (–) indicates negative MRD/PD status. Range of mtDNA from PBMCs from normal healthy individual is 40–99 (s.d. 14).

TABLE II

Cell Proliferation Assay Measuring Mitochondrial Dehydrogenase Activity

	Day 2	Day 4	Day 6
Dox 0	0.16	0.40	0.48
Dox 100	0.31	0.10	0.65
Dox 300	0.30	0.09	0.22
Dox 500	0.26	0.07	0.19
Vin 0	0.23	0.65	0.22
Vin 3	0.38	0.85	0.43
Vin 6	0.34	0.48	0.55
Vin 12	0.25	0.05	0.22

Numbers are absorbance based on 480 nm wavelength with 650 nm used as reference.