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Intrinsic mitochondrial DNA repair defects in Ataxia Telangiectasia

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ABSTRACT

Ataxia Telangiectasia (A-T) is a progressive childhood disorder characterized most notably by cerebellar degeneration and predisposition to cancer. A-T is caused by mutations in the kinase ATM, a master regulator of the DNA double-strand break response. In addition to DNA-damage signaling defects, A-T cells display mitochondrial dysfunction that is thought to contribute to A-T pathogenesis. However, the molecular mechanism leading to mitochondrial dysfunction in A-T remains unclear. Here, we show that lack of ATM leads to reduced mitochondrial DNA (mtDNA) integrity and mitochondrial dysfunction, which are associated to defective mtDNA repair. While protein levels of mtDNA repair proteins are essentially normal, in the absence of ATM levels specifically of DNA ligase III (Lig3), the only DNA ligase working in mitochondria is reduced. The reduction of Lig3 is observed in different A-T patient cells, in brain and pre-B cells derived from ATM knockout mice as well as upon transient or stable knockdown of ATM. Furthermore, pharmacological inhibition of Lig3 in wild type cells phenocopies the mtDNA repair defects observed in A-T patient cells. As targeted deletion of LIG3 in the central nervous system causes debilitating ataxia in mice, reduced Lig3 protein levels and the consequent mtDNA repair defect may contribute to A-T neurodegeneration. A-T is thus the first disease characterized by diminished Lig3.

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1. Introduction

Ataxia Telangiectasia (A-T) is a neurodegenerative disorder that leads primarily to cerebellar degeneration and predisposes patients to cancer [1]. A-T patients present with a plethora of additional symptoms such as immunodeficiency, growth retardation, premature aging and insulin resistance [2]. While the molecular mechanisms associated with A-T neurodegeneration are unknown, mitochondrial dysfunction occurs in A-T patients [3,7] and, like in many neurodegenerative disorders [8], may play an important role in disease pathogenesis. A-T is caused by mutations in the gene encoding the DNA-damage response kinase ATM, absence of which is associated not only with nuclear genomic instability but also altered mitochondrial metabolism, including increased reactive oxygen species (ROS) production, decreased electron transport chain activity, and altered mitochondrial membrane polarization [3,7]. However, the molecular cause of mitochondrial dysfunction in A-T remains unknown and may represent a key feature for fully understanding A-T etiology and disease progression, and finding new therapeutic targets for the disease.

In trying to understand the role of mitochondria in A-T pathogenesis we turned to analysis of mtDNA integrity, a key feature required to maintain mitochondrial function but still largely overlooked in the context of A-T. Here we show that lack of ATM leads to decreased mtDNA integrity and impaired mitochondrial function in patient-derived cells and in tissues from an ATM knockout (KO) mouse model. We also identified that in the absence of ATM mitochondrial base excision repair (BER) is impaired specifically due to decreased protein levels of DNA ligase III (Lig3), which is the only known DNA ligase operating in mitochondria. Treatment of cells with MG132, a proteasome inhibitor, increased levels of Lig3 and pharmacological inhibition of Lig3 in ATM-proficient cells phenocopied the mtDNA repair defects detected in A-T. Our data

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confirm previous work showing that Lig3 is required to maintain mtDNA integrity and function, and highlight a new function of ATM in regulating DNA Lig3 stability and consequently mtDNA repair. Our results also indicate that targeting mitochondrial dysfunction, including mtDNA instability, may help alleviate some symptoms associated with A-T. Our findings can help explain the complex and variable phenotype of the disease, which is reminiscent of mitochondrial disorders, and support the notion that A-T may be a mitochondrial disease. Finally, A-T becomes the first example of a human disease characterized by diminished DNA Lig3 levels and function.

2. Materials and methods

2.1. Cell culture and animal samples

AG01522 (WT), AG04405 fibroblasts (A-T), GM07532 and GM02052 (A-T) were grown in MEM medium supplemented with 15% FBS and 1% penicillin/streptomycin. These fibroblasts are available at the Coriell Repository (NJ). Non-immortalized A-T fibroblasts were consistently used in lower passage (usually 2–4 from the passage obtained by Coriell) as compared to the WT counterpart (up to 10 passages) so as to avoid potential confounding effects associated to telomere shortening. We also routinely monitored changes in cell cycle regulation, cell morphology and doubling time as these are typically altered when cells are senescent or approaching senescence. Cerebrum and cerebellum from 3 ATM null mice and 3 WT littermates were analyzed (details on the animals can be found in D'Souza et al. [7]). Animals were maintained at the Yale Medical School vivarium and handled according to IACUC protocol. All experiments presented were performed at least 3 times using cultures grown at different times.

2.2. H_2O_2 treatment and mtDNA integrity analysis

Protocols for H_2O_2 exposure and QPCR analysis of mtDNA integrity were followed as previously described [10,12]. Briefly, cells were exposed to 200 μM H_2O_2 for 60 min. Following treatment genomic DNA was isolated and a large (8.9 for human and 10 kb for mouse) and a small fragment of the mtDNA were amplified using specific primers. Amplification of sample of interest was compared to that of control and these numbers were used to estimate the amount of lesions present on the mtDNA. Each experiment was independently reproduced at least 3 times; each DNA sample was submitted to 2 QPCR reactions; the average of the 2 reactions was then considered as $N = 1$. For more details see Refs. [11,12]. Repair kinetics were performed both under normal growth conditions (MEM) and with supplemented medium (see doubling time below), and showed similar results ruling out confounding effects associated to cell cycle.

2.3. H_2O_2 exposure and kinetics of H_2O_2 decomposition

The same number of WT and A-T-patient derived cells were plated and 16 h later cells were exposed to 200 μM of H_2O_2 for 60 min. Immediately after the treatment cells were washed, harvested and the different mitochondrial parameters analyzed (see below). To measure kinetics of H_2O_2 decomposition (Fig. S3), amounts of H_2O_2 were followed over time using Amplex red and a H_2O_2 standard curve as reported earlier by us [10]. Each experiment was repeated at least 3 independent times.

2.4. Mitochondrial ROS, membrane potential and ATP measurements

For mitochondrial superoxide measurements, after exposure to H_2O_2 (as above) cells were loaded with Mitosox fluorescent probe 10 μM (Invitrogen) for 10 min at 37 °C. The data were collected by FACS. FCCP was employed to completely uncouple mitochondria and as such abolish the mitochondrial-generated superoxide signal. The signal from FCCP-treated samples was then subtracted from the samples and these final values were used for the comparisons. Mitochondrial membrane polarization was determined after incubation of cells for 10 min with JC-1 (10 μM) (Invitrogen, T3168) by FACS. Total ATP production was estimated using a Luciferase based assay (Invitrogen, A22060) and an ATP standard curve; the data were normalized to cell numbers.

2.5. Doubling time

Cells were counted and 300,000 cells of each cell type were plated in either regular medium (MEM + 15% FBS) or supplemented medium (MEM + 15% FBS + pyruvate + 50 μg/ml uridine + 3.5 mM glucose) to support glycolytic growth. Upon reaching confluency, cells were trypsinized, counted and replated (300,000). Cell growth was followed using this protocol for 3 weeks; error bars represent SEM for each cell type from 2 independently grown cultures (stock frozen at independent times).

2.6. Mitochondrial content estimation using Mitotracker green

Cells were treated as above and immediately after the exposure to 200 μM H_2O_2 as well as 24 h later, cells were incubated with 200 nm Mitotracker green (Invitrogen) for 30 min before submitting for FACS analysis. ImageStream was used to assure the signals were solely mitochondrial. Fluorescence values were compared between H_2O_2-treated samples and non-H_2O_2-treated control at both time points. Experiments were reproduced 3 independent times.

2.7. Isolation of mitochondrial from fibroblast and mouse tissue

Mitochondrial isolations from human fibroblast were performed as recently reported by us [44]. The preparation of mitochondria from the different mouse tissues was performed following published protocols [45] with minor modifications. Briefly frozen cerebrum and cerebellum were washed free of blood with homogenization buffer (0.250 sucrose, 10 mM HEPES pH 7.4, 1 mM EGTA, 1 mM DTT) supplemented with protease inhibitors (Sigma). The ratio of homogenization buffer to tissue was 1:10. The minced tissue was then transferred to a Teflon/glass homogenizer and homogenized at 2000 rpm for 5 min. The homogenate was differentially centrifuged at 600 × *g* for 10 min and 10,000 × *g* for 10 min. The supernatant was saved as cytosolic fraction and the pellet was resuspended in mitochondria suspension buffer (0.250 M sucrose, 3 mM EGTA/Tris and 10 mM Tris/HCl, pH 7.4) and washed three times using the same buffer. The washed mitochondrial pellet was stored at −80 °C for further experimental use. Protein concentrations were determined using the Lowry method, with BSA as standard.

2.8. Preparation of nuclear and mitochondrial extracts for ligase assay

All steps were performed at 4 °C. The pelleted nuclei from cells were resuspended in the hypotonic nuclei extraction buffer containing 10 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$, 10 mM KCl 1 mM DTT and 350 mM NaCl and a cocktail of protease inhibitors. After 1 h