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Addendum

Mitochondrial DNA Deletions and Chloramphenicol Treatment Stimulate the Autophagic Transcript ATG12

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Addendum to:

Mitochondrial DNA Deletions Inhibit Proteasomal Activity and Stimulate an Autophagic Transcript

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ABSTRACT

Deletion mutations of mitochondrial DNA (mtDNA) accumulate somatically on a cell-by-cell basis with age, resulting in decreased cell function in muscle and substantia nigra. In osteosarcoma cells deletions incapacitate mitochondria and induce the autophagic transcript ATG12, which is involved in an early step of the mammalian autophagy pathway. We discuss here which consequences of mtDNA deletions could induce ATG12, and provide two new pieces of data. Our previous studies demonstrated that mtDNA deletions decreased mitochondrial ATP production and proteasomal function, induced the AMPK transcript (likely as a consequence of bioenergetic depletion), and decreased the intracellular concentration of 20 amino acids (possibly as a consequence of decreased proteasomal activity). Deletions eliminate essential tRNAs for mitochondrial protein synthesis, as well as essential components of mitochondrial multisubunit enzymes; therefore, the increased level of ATG12 could result from decreased bioenergetic function, increased oxidative damage, or decreased mitochondrial protein synthesis. However, the bioenergetic inhibitor rotenone does not induce ATG12. We show here that chloramphenicol, which inhibits mitochondrial protein synthesis, induces ATG12, and that mtDNA deletions result in an increased burden of oxidatively damaged protein. Thus, mtDNA deletions could induce ATG12 through a mechanism such as the following: deletions > mitochondrial protein synthesis inhibition or ROS > proteasome inhibition > amino acid depletion > ATG12.

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DELETIONS OF mtDNA CAUSE NEUROLOGICAL DISEASE AND AGE-RELATED DYSFUNCTION

Mitochondrial DNA (mtDNA) deletions occur during development and cause neuromuscular disorders, including Kearns-Sayre Syndrome (KSS), Chronic Progressive External Opthalmoplegia (CPEO), and Pearson's Syndrome (PS).[1] Deletions also occur somatically, on a cell-by-cell basis, and cause mitochondrial biochemical defects in aging muscle and substantia nigra neurons.[2,3]

MICROARRAY AND FUNCTIONAL ANALYSIS IDENTIFIES CONSEQUENCES OF mtDNA DELETIONS

To better understand how mtDNA deletions cause disease, we carried out gene expression profiling of nuclear genes by microarray of six patient-derived tissues and cell cybrid (a hybrid cell produced by fusing mitochondria from patient cells with mitochondria-less recipient cells) models bearing mtDNA deletions.[4] Significantly inhibited pathways included transcripts targeted to the mitochondrion and to the Ubiquitin-Proteasome System, UPS. These defects in mitochondrial and UPS transcripts were mirrored by a decreased mitochondrial ATP production, and decreased 20S proteasome activity. Perhaps as a consequence of decreased ATP production, the transcript for energy-responsive AMP kinase (AMPK) was stimulated by deletions.[5] Possibly because of decreased UPS activity and amino acid salvage, the concentration of 20 intracellular amino acids was decreased.[4] Furthermore, the expression of the secretion-related gene ARL2 was decreased, as were the secretion of two vesicularly-secreted proteins, osteoprotegerin and fibronectin.[5]

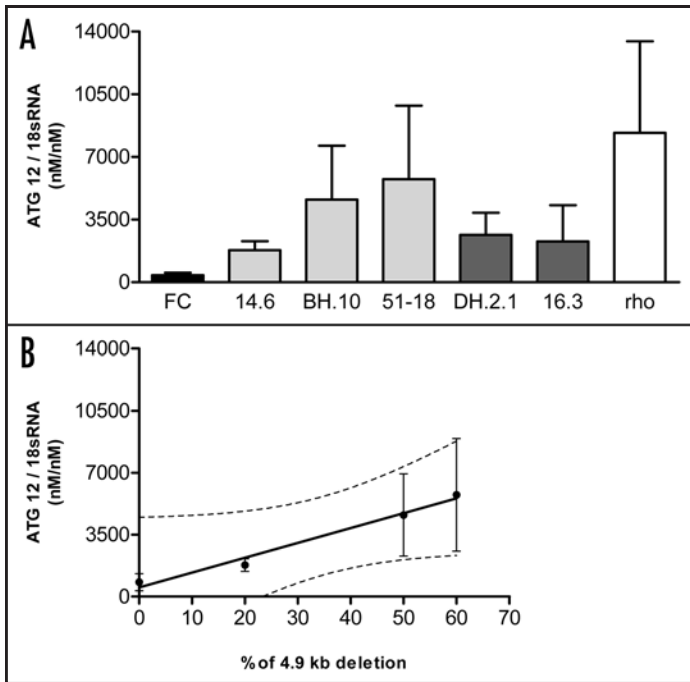

Figure 1. Autophagy-related 12 (ATG12) gene transcript levels are induced in cybrid cells bearing mtDNA deletions. (A) Quantitative RT-PCR analysis of ATG12 RNA expression normalized to 18s RNA expression in trans-mitochondrial cybrids. FC indicates osteosarcoma fusion control cybrids, i.e., cells which had been cybridized with nonpathogenic mtDNAs. Mutant cybrids harboring different levels of the 4.9 kb deletion are indicated in light gray; cybrids bearing different concentrations of the 7.5 kb deletion are represented in dark gray. Rho indicates rho-zero cells, i.e., cells depleted of mtDNA genome. (B) Correlation between 4.9 kb deletion concentration and ATG12 transcript levels (r square: 0.27, p = 0.012).

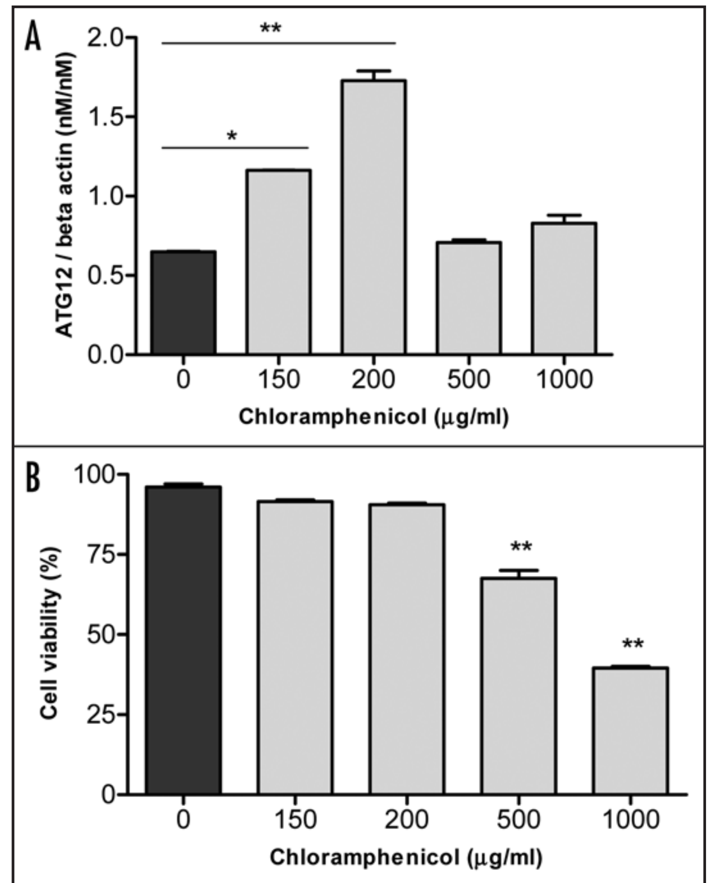

Figure 2. Chloramphenicol treatment induces ATG12 transcript in control cybrids. (A) ATG12 transcript levels were detected by QRT-PCR and normalized over beta actin expression. ATG12 was induced in control cybrids (namely HGA13, HPC7, and HBA2) treated for 24 hours with doses of chloramphenicol higher than 100 μg/ml. For doses higher than 200 μg/ml there was no ATG12 induction but at these doses the drug appeared also toxic to cells as viability was significantly reduced. *Unpaired t test 150 μg/ml vs. untreated, $p < 0.05$; **Unpaired t test 200 μg/ml vs. untreated, $p < 0.01$. (B) Chloramphenicol dose-response curve at 24 hours of cell viability in control cybrids showed that for doses up to 200 μg/ml there is no significant cellular toxicity. **ANOVA analysis with Dunnett's multiple comparison post-test; $p < 0.01$ for 500 μg/ml vs. untreated and for 1000 μg/ml vs. untreated.

mtDNA DELETIONS INDUCE THE ATG12 TRANSCRIPT

mtDNA deletions significantly induced the ATG12 transcript in osteosarcoma cell cybrids (Fig. 1A). ATG12 is known to play an essential role during the activation of mammalian autophagy.[6] A 4.9 kb long deletion is the deletion that occurs most commonly in human mitochondrial genetic disease, and also with aging.[7] Interestingly, the levels of the 4.9 kb 'common' deletion correlated with the increase in the ATG12 transcript (Fig. 1B). The SNARE protein syntaxin 16, which may also participate in autophagy,[8-10] was also significantly induced.

HOW COULD MTDNA DELETIONS INDUCE ATG12?

mtDNA deletions eliminate both tRNAs essential for mitochondrial protein synthesis,[11] and also mitochondrially-encoded subunits of the electron transport chain, which should result in mitochondrial protein misfolding and damage. Thus, conceptually, mtDNA deletions could induce ATG12 through inhibition of protein synthesis, through inhibition of bioenergetic function, or through increased production of mitochondrial Reactive Oxygen Species (ROS).

BIOENERGETIC BLOCK DOES NOT INDUCE ATG12

The mitochondrial inhibitor rotenone blocks electron transport at Complex I. We previously demonstrated that whereas rotenone recapitulated several other consequences of mtDNA deletions, including ATP depletion, induction of AMPK, inhibition of secretory transcript ARL2, and inhibition of osteoprotegerin and fibronectin secretion, rotenone did not recapitulate the induction of ATG12.[5] This suggests that induction of ATG12 by deletions is not the direct consequence of a simple bioenergetic defect.

MITOCHONDRIAL PROTEIN SYNTHETIC BLOCK INDUCES ATG12

To test whether mitochondrial protein synthesis inhibition induced ATG12, fusion control cybrids, i.e., cells which had been cybridized with nonpathogenic mtDNAs, were treated for 24 hours with increasing doses of chloramphenicol, a known inhibitor of mitochondrial protein synthesis. At drug concentrations that had no toxicity on cell viability (150 μg/ml and 200 μg/ml), we observed a dose-response effect on ATG12 transcript induction (Fig. 2). Thus, mitochondrial protein synthesis inhibition is sufficient to induce expression of ATG12.

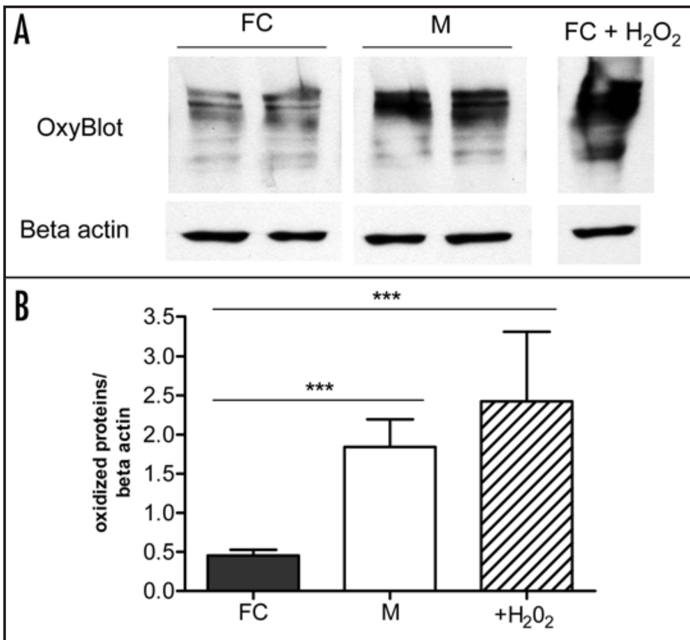

Figure 3. Measurement of total oxidized proteins in mutant and control cybrids. Total oxidized proteins were measured according to OxyBlot manufactor's protocol. (A) Representative immunoblot out of three separate experiments showing oxidized proteins amount in two fusion control (FC) cybrids (namely HGA13 and HPC7), two 143B mutant (M) cybrids (namely 14.6, 16.3), and one FC treated with 500 μM H_2O_2 for 2 hours. The membranes were then stripped and reprobed with anti-beta actin to check the loading amount. (B) Densitometry results of total oxidized proteins over β actin. Error bars represent SEM. ***Unpaired t test; M vs. FC, $p < 0.001$.

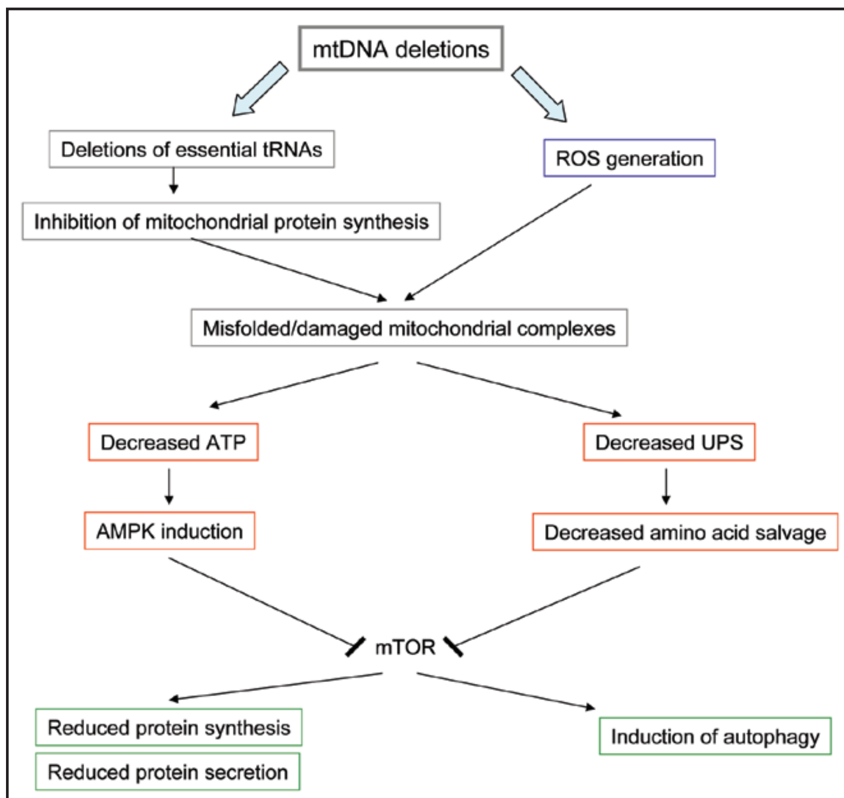

Figure 4. Proposed pathogenetic mechanism for diseases caused by mtDNA deletions.

CELLS BEARING MTDNA DELETIONS HAVE MORE OXIDATIVELY DAMAGED PROTEINS

Deletion of mitochondrially-encoded subunits of multi-protein complexes could cause misfolding of mitochondrial proteins, which could in turn result in increased ROS generation and oxidative protein damage. To test this idea, we measured oxidatively modified proteins in mutant and control cybrids using the Oxyblot method. There were significantly more oxidized proteins in mutant cybrids bearing mtDNA deletions compared to fusion control cybrids (Fig. 3).

AUTOPHAGY INDUCTION COULD TAKE PLACE THROUGH MITOCHONDRIAL PROTEIN SYNTHESIS INHIBITION

Unlike the UPS, which degrades lightly damaged and aged proteins one protein at a time, autophagy is able to degrade entire organelles such as mitochondria.[12] Thus, the induction of ATG12 in cells bearing mtDNA deletions may represent the cellular attempt to eliminate nonfunctional mitochondria through autophagy. Because rotenone does not reconstruct the ATG12 response, but chloramphenicol does, the ATG12 response in the context of deletions is more likely to be the result of decreased mitochondrial protein synthesis than decreased bioenergetics. These observations are also consistent with recent findings that autophagy is triggered in response to impaired UPS activity,[13] and also as a consequence of low amino acid concentration, through inhibition of the mTOR complex, which is a target of AMPK.[14] These data suggest the following pathway: mtDNA deletions → impaired mitochondrial protein synthesis → impaired UPS → AA depletion → mTOR inhibition → ATG12 (Fig. 4).

On the other hand, mtDNA deletions also cause an increase in free radical production[15] and, as we have shown, an increase in oxidative damage to proteins in cells bearing mtDNA deletions. Heavily damaged mitochondrial proteins have been recently demonstrated to be sufficient to reduce UPS activity.[16,17] Accordingly, mtDNA deletions → ROS → oxidative damage → impaired UPS → AA depletion → mTOR inhibition → ATG12 is another possibility (Fig. 4). Thus, we could summarize by suggesting the possibility that mtDNA deletions → ROS or inhibition of protein synthesis → impaired UPS → AA depletion → mTOR inhibition → ATG12.

A MITOCHONDRIAL PARKINSON'S DISEASE MECHANISM COULD REQUIRE AUTOPHAGY

Mitochondrial DNA deletions rise to their highest level in the brain in the substantia nigra,[18,19] and cause cell defects there on a cell-by-cell basis.[2,3] There is substantial support for a role for mitochondria and autophagy in Parkinson's disease. Mitochondrial inhibitors MPTP and rotenone produce Parkinsonism in animal and human models,[20] and induce α-synuclein, which is thought to inhibit the ubiquitin proteasome system to trigger dopaminergic cell death.[21] Furthermore, mutations in PINK1, a mitochondrial stress kinase, and Parkin and UCHL1, components of the ubiquitin-proteasome system, cause Parkinsonism.[22] Finally, there is evidence for an active

autophagic process in dopaminergic neurons of Parkinsonian brains but not controls,[23] and the mitochondrial Complex I inhibitor and Parkinsonogenic drug MPTP induces autophagy in dopaminergic cell models.[24] Thus, mitochondrial stress could trigger an autophagic process in dopaminergic neurons that results in cell death.

PROSPECTS

If the proposed pathogenetic mechanism is correct, therapeutic strategies acting on these routes might benefit patients affected by disease caused by the rise of mtDNA deletions. If autophagy is being induced as a cytoprotective mechanism, then treatments known to further up-regulate the process may represent an interesting possibility. Accordingly, it has been recently suggested that stimulation of autophagy (through rapamycin or other mTOR-indipendent autophagy inducers) could be therapeutic for diseases characterized by accumulation of intracellular aggregates, such as Huntington's disease or Parkinson's disease.[12]

On the other hand, if autophagy is causative of disease, possible therapies may include supplementation with free amino acids, since amino acids in general, and leucine in particular, are known to stimulate the mTOR pathway and inhibit autophagy.[25,26] Or, if the trigger for UPS inhibition is ROS-damaged protein, perhaps antioxidant supplementation would be beneficial. However, this is an early time for understanding the mechanisms by which mtDNA deletions induce autophagy, and thus treatment is still speculative.

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