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Introduction

The mitochondria are the power plants of the cell; when they are unable to meet the brain's substantial energy demands, neurodegeneration occurs. Leigh syndrome (LS) and Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP) are two such neurodegenerative diseases, and are caused by dysfunctional ATP synthase. This enzyme is composed of fifteen subunits that work together to couple the flow of protons into the mitochondrial matrix to a rotating complex that catalyzes the ADP + $P_i \rightarrow$ ATP reaction (Baracca et al., 2000; Elston et al., 1998). Specifically, the ATP6 gene that is encoded in the mitochondrial genome forms the proton channel subunit of ATP synthase (Baracca et al., 2000; Elston et al., 1998). In LS and NARP patients, the ATP6 gene contains either a T8993G (Holt et al., 1990) or much less common T8993C (deVries et al., 1993) nucleotide mutation. Consequently, proton conductance is reduced and ATP synthesis as well as holoenzyme assembly is decreased (Trounce et al., 1994; Garcia et al., 2000; Baracca et al., 2000). Because ATP6 is a mitochondrial gene, LS and NARP syndromes are maternally inherited and result when the cellular heteroplasmy (mutant dosage) is 90-95% and 70-90%, respectively (Tautch et al., 1992).

My research interests focus on the development of a gene therapy solution for LS and NARP syndromes that increases ATP synthesis. In addition to ATP synthase dysfunction, the T8993G mutation results in increased levels of reactive oxygen species (ROS), which induce an increase in the expression of the free-radical scavenging superoxide dismutases (SOD) and trigger apoptosis (Geromel et al., 2001). Furthermore, oxidative stress damages mitochondrial lipids and impairs function of the election transport chain, consequently decreasing ATP synthesis (Mattiazzi et al., 2004). ATP synthesis increases after antioxidant treatment of 100% (homoplasmic) T8993G cybrids synthesized from enucleated plate cells and osteosarcoma cells (Mattiazzi et al, 2004).

The most promising possibility for increasing ATP synthesis and ATP synthase function in LS and NARP cells arises from the success of allotropic expression of wild-type (WT) ATP6 within homoplasmic T8993G Human Embryonic Kidney (HEK) cells (Manfredi et al., 2002). This is achieved through the use of a recombinant Adeno Associated Virus (AAV) to deliver WT-ATP6 into the nuclear genome (i.e. Allotopic Expression; Manfredi et al., 2002). ATP synthesis is significantly increased in HEK cells allotopically expressing WT-ATP6 compared to non-treated mutants; however, it remains to be discovered whether the increase in WT-ATP6 expression will increase ATP synthesis above the disease-causing threshold (Manfredie et al., 2002).

Because allotopic expression of WT-ATP6 and treatment of T8993G homoplasmic cells with antioxidants both increase ATP synthesis, I propose combining these two approaches into one that employs the AAV vector developed by Manfredi et al. (2002), modified to contain the nuclear encoded mitochondrial manganese superoxide dismutase gene (MnSOD) and WT-ATP6 (Geromel et al., 2001). MnSOD is a free-radical scavenging enzyme that has increased expression in T8993G cells (Mattiazzi et al., 2004; Geromel et al., 2001). I believe overexpression of MnSOD in combination with allotopically expressed WT-ATP6 will increase ATP synthesis compared to primary T8993G neurons that allotopically express WT-ATP6 only (Geromel et al., 2001; Mattiazzi et al., 2004; Manfredi et al., 2002). Thus, the combined ATP synthesis increasing effects of replenishing WT-ATP6 and reducing oxidative stress may increase respiratory efficiency enough to prevent neuronal atrophy.

Specific Aims

Determine if ATP synthesis is increased in LS and NARP patient brainstem neurons by treatment with either the WT-ATP6-AAV vector developed by Manfredi et al. (2002) or the antioxidant DHLPA (Mattiazzi et al., 2004).

Increase ATP synthesis in neurons from Aim 1 by reducing the concentration of ROS and dysfunctional ATP synthase via the Manfredi et al. WT-ATP6 AAV vector modified to include MnSOD (2002).

Experimental Proposal

In order to accomplish Aim 1, brainstem neurons will be harvested upon autopsy from LS and NARP patients as well as decreased neurologically intact individuals. I hypothesize that allotopic expression of WT-ATP6 using the AAV vector developed by Manfredi et al. (2002) will significantly increase ATP synthesis in LS and NARP patient neurons based on the Manfredi et al. findings in T8993G homoplasmic HEK cells (2002). Furthermore, I hypothesize that treatment of LS and NARP patient neurons with the antioxidant dihydrolipoic acid (DHLPA) will increase ATP synthesis based on the Mattiazzi et al. results in T8993G homoplasmic cybrids (2004). This first experiment is essentially a repeat of the Mattiazzi et al. (2004) and Manfredi et al. (2002) studies because neither have been performed on primary neuron cultures and further support of their conclusions is needed to pursue either as a future treatment strategy.

The Manfredi et al. AAV vector containing WT-ATP6 tagged with a mitochondrial localization signal (MLS) will be incubated with mutant neuronal cultures (2002). Experimental controls will consist of mutant and WT neurons treated with an empty AAV vector, and WT neurons with WT-ATP6-AAV as well. These controls allow us to determine if any changes in ATP synthesis in the mutants are the result of WT-ATP6 expression only. ATP synthesis will be measured using the method described by Manfredi et al. (2001).

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If ATP synthesis in mutants increases, then we conclude that it resulted from competition between WT and mutant ATP6 during ATP synthase assembly, and that the concentration of WT-ATP synthase had increased in the mitochondria. However, if ATP synthesis does not increase, it is possible that the increase in ATP synthesis observed by Manfredi et al. in HEK cells was cell-type specific (2002). An identical experimental setup will be performed for cells treated with the antioxidant DHLPA as described by Mattiazzi et al. (2004), with controls consisting of 1) treatment or 2) no treatment of WT and mutant cells. If ROS particles are causing electron transport chain dysfunction, then we expect to see an increase in ATP synthesis for both LS and NARP cells treated with DHLPA. If we do not observe a significant increase in ATP synthesis, we *cannot* conclude that ROS are not involved because the types of ROS generated in neurons and consumed by DHLPA may differ from those in the Mattiazzi et al. cybrids (2004).

Increased MnSOD activity is associated with cells homoplasmic for the T8993G-ATP6 mutation (Geromel et al., 2001; Mattiazzi et al., 2004). This enzyme is critical to reducing the presence of ROS within the mitochondria and protecting the cell from oxidative stress. The MnSOD gene is encoded in the nuclear genome and contains a MLS; thus, there is no need for genetic modification before it is cloned into the AAV genome containing WT-ATP6 (Manfredi et al., 2002). I hypothesize that overexpression of MnSOD in combination with allotopic WT-ATP6 expression will significantly increase ATP synthesis compared to only allotopic WT-ATP6 expression in mutant neurons. The experimental setup will consist of WT and mutant neurons receiving 1) empty AAV vector or 2) the WT-ATP6+MnSOD-AAV construct. LS and NARP cells will be infected with the virus containing both WT-ATP6 and MnSOD in its genome, and ATP synthesis measured as described by Manfredi et al. (2001). If T8993G mutant cells overexpressing MnSOD and allotopically expressing WT-ATP6 have increased ATP synthesis compared to those treated with the WT-ATP6-AAV construct from Aim 1, we can conclude that reduction of ROS within the mitochondria by MnSOD overexpression increases ATP synthesis. In contrast, if we observe identical ATP synthesis between both AAV constructs, then MnSOD overexpression has no effect on ATP synthesis.

Conclusion

The proposed experiments will contribute significantly to the development of a gene therapy approach to increasing ATP synthesis in LS and NARP patients. Specifically, combining two previously established methods for increasing ATP synthesis in homoplasmic T8993G-ATP6 cells into one viral expression system may provide the critical increase in ATP synthesis necessary to prevent LS and NARP manifestation (Manfredi et al., 2002; Geromel et al., 2001; Mattiazzi et al., 2004).

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