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Low Intracellular Proline: A Cause of Toxicity in Human RPE Cells?

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Introduction

Gyrate atrophy of the choroid and retina (GA) is an autosomal recessive disease caused by mutations in the nuclear gene coding for the mitochondrial enzyme ornithine aminotransferase (OAT; Valle & Simell, 2001). The primary symptoms for this congenital metabolic disease include night blindness, myopia, cataracts, and progressive reduction in peripheral vision culminating in blindness by the age of forty. The loss of OAT leads to high plasma levels of the amino acid ornithine and low levels of proline and creatine (Valle & Simell, 2001).

Our knowledge about the pathophysiology of GA is very limited: we do not know the mechanism for toxicity or the primary toxic species. However, we do know the following: first, the initial cells affected are the retinal pigmented epithelial (RPE) cells (Wang et al., 1996); second, ornithine enters the cells primarily through the cationic amino acid transporter (CAT)-1 (Kaneko et al., 2007); third, lowering plasma levels of ornithine prevents the progression of the disease (Wang et al., 2000).

Since proper CAT-1 functioning is essential for the survival of the organism, this channel cannot be systemically blocked as a treatment for GA (Kaneko et al., 2007). The most efficient treatment has been reducing the plasma ornithine level by restricting the intake of its precursor arginine (Wang et al., 2000). This treatment is not without its problems. Only about 20% of the patients are able to follow this diet (Wang et al., 2000), as it is highly restrictive and requires the patient to take many dietary supplements supplying the essential amino acids, vitamins, and minerals (Valle & Simell, 2001).

Ueda et al. (1998) showed that treatment with proline prevented toxicity caused by high concentrations of ornithine in RPE cells lacking OAT. The authors suggest that low proline levels might be the primary cause of toxicity in GA. The study, however, provides inconclusive evidence to support the hypothesis. The levels of proline in the cell are not measured in the study, and toxicity to RPE cells by low levels of proline alone is not measured. The prospect that low proline levels cause GA cytotoxicity is an exciting one since dietary supplementation of proline is much easier than restriction of arginine. The cheaper and easier treatment that could result from this possibility necessitates an investigation into the role of proline in GA.

Specific Aims

Based on the research by Ueda and colleagues (1998), I will investigate the toxic effect of low proline levels in human RPE cells. I will render the cells incapable of biosynthesizing proline, which will enable me to control

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the level of proline in the cell by varying its concentration in the serum.

Secondly, I will vary the intracellular proline levels and correlate any toxicity observed with the amount of proline inside the cell. If low levels of intracellular proline cause toxicity, cell death should be independent of OAT activity but inversely proportional to the level of proline in the cell. It is therefore unnecessary to mimic the Ueda et al study and inhibit OAT in the cells (1998).

Experimental Proposal

The study conducted by Ueda et al. (1998) suggests that proline starvation might be the cause of toxicity in GA. However, the evidence for this is indirect and based on the cytoprotective role of proline in the experiment. To support the hypothesis that low proline levels cause RPE cell atrophy in GA, I will carry out experiments to show the correlation of low proline levels to cell death.

Silencing the P5C Reductase Gene
P5C reductase is the final enzyme required for the biosynthesis of proline (Ueda et al., 1998). To fully control the amount of proline in the cell, I shall silence the P5C reductase gene in the telomerase-immortalized hTERT-RPE cells purchased from Clontech. This will be done by stable retroviral transfection of a short interfering dsRNA coding region complementary to a region of the P5C reductase mRNA using the Knockout™ RNAi-Ready pSIREN-RetroQ vector from Clontech. The resulting RPE cells (ΔP5Cr) will be incapable of biosynthesizing proline and will thus be maintained in proline rich media. Scrambled siRNA with no homology to any known human gene will be used as a control and shall be transfected the same way to give rise to scP5Cr cells. Northern blotting, using ^{32}P labeled P5C reductase and GAPDH will be used to determine the effect of RNA interference on P5C reductase expression. If the RNAi treatment is successful we should not detect any P5C reductase RNA in the ΔP5Cr cells, but should see it in the scP5Cr cells. The amount of GAPDH RNA produced will act to control the amount of cells.

Toxicity Assay
Toxicity will be evaluated using the [^{3}H]Thymidine incorporation assay described by Ueda et al. (1998). The tritium labeling will enable us to detect the amount of DNA in the culture, which corresponds to the amount of cells present. The data will be normalized by comparing it to the amount of tritium in scP5Cr cell culture. Morphological changes in the cell will be evaluated by microscopy.

Intracellular Proline Concentration
The amount of proline in the cell shall be detected by high performance liquid chromatography after washing and lysing the cells. To account for the difference in the number of cells in each culture, the proline level will be reported as a ratio of the amount of proline to the amount of tritium in the cell culture detected by the [^{3}H]Thymidine incorporation assay.

Effect of Different Proline Levels

Since the ΔP5Cr cells get all their proline from the media, their intracellular proline level can be varied by treating them with different concentrations of proline, starting from well below the physiological concentration of 150μM (Ueda et al., 1998) and working up to it. The scP5Cr cells shall be used as the control since they should contain physiological levels of proline. Intracellular proline concentration and toxicity will be measured using the assays described above.

I believe that intracellular proline levels below the physiological level will be toxic to the ΔP5Cr cells, leading to cell atrophy by a pathway other than apoptosis as reported by Ueda et al. (1998). If we observe no toxicity, it indicates that the cytoprotection due to proline observed by Ueda and colleagues was due to an unknown reason, which would need to be studied, and low proline levels were not the cause of toxicity. This is a probable outcome of this experiment, since the amount of proline needed to prevent toxicity (10mM) was much greater than the normal physiological concentration of proline (Ueda et al. 1998).

This experiment cannot tell us if toxicity due to proline starvation is caused by the same unknown pathway as in GA, but will provide useful evidence for the viability of proline supplementation as a treatment for GA.

Conclusion

This experiment will help determine if low proline levels lead to RPE cell death in GA. This knowledge will further our understanding of the pathophysiology in GA and prompt more research exploring it as a proline deficiency disorder. This study will also lead to informed trials attempting to prevent GA progression by proline supplementation, which is an easier and cheaper treatment than arginine restriction.

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