Fluorescence of OGG1 knock-out and wild types as indicators of oxidative stress on living systems

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Abstract

Base excision repair (BER) is the primary method through which damage caused by Reactive Oxygen Species (ROS) is mended. Repair of the DNA base guanine, the most readily oxidized base, is performed by 8-oxoguanine-DNA glycosylase-1 (Ogg1), a DNA repair enzyme. We investigated the behavior of BER under oxidative stress using a fluorescent- quencher model. Fluorescence is emitted when the modified oxidized oligonucleotide is cleaved from the fluorescent strand, casting it away from the Quencher. This allows it to fluoresce, and enables us to quantify the kinetic activity of the OGG1 enzyme in cells undergoing induced oxidative stress. While the fluorescence is detectable in OGG1 knock-out cells it emits less fluorescence than wild type mice. Wild type mice exhibited variable fluorescence, following a general trend of increasing fluorescence according to incubation time. There was a substantial increase after incubation with formamide. This fluorescent-quencher system allows for a high-throughput quantitation of OGG1 activity, permitting a comparison of fluorescent results with chromatograms (using Reverse Phase HPLC) of six wild-type and six knockout mice.

Introduction

"Aging is a disease," an illustration offered by Jaques Cohen in Johnathan Weiner's novel *His Brother's Keeper*. However, aging has developed into a euphemism for a symptom of metabolic processes whose collection in post-mitotic cells generates oxidative stress, disrupting the balance between metabolic wastes and repair enzymes. These wastes often cause skewed helixes that interfere with genetic code. Overtime, the build up of these damaged sites contributes to the development of neurodegenerative disorders. Investigating the activity of OGG1 using a fluorescent quencher model and HPLC analysis we may distinguish differences between wild type and knock out mice.

Experimental Design

Establishing fluorescent standards for two models, Working A – composed of a serial dilution of 6-FAM stock solution and TE Buffer – and Working B – another serial dilution of 6-FAM stock solution, Quencher oligo and TE Buffer. Reading the fluorescence of the emission wavelength (518 nm) and excitation wavelength (494 nm) we determine which concentration is the most appropriate for our investigation.

Isolating the caudate putamen, the cerebellum, the midbrain and the cortex from one knockout and one wild type mouse, place samples in 1.5 mL eppi-tubes on ice until they are ready to be sonicated before siphoning a homogenous mixture into a separate 1.5 mL eppi-tube. After determining the protein concentration of each sample using Bradford Protein Assays, adjust the concentration of each to 250 ug/uL.

Preparing duplicate 1.5 uL tubes both with oligo solution and glycosylase extract, inserting Quencher in only one of the duplicates, heat tubes in 100oC boiling water and allow samples to anneal and cool to room temperature. Transfer samples into 96 well-plate and incubate at 37oC for 5 minutes then measure fluorescence. Incubate again for 30 minutes and measure fluorescence, then incubate for 60 minutes and measure fluorescence. Finally, incubate samples with additional 30 uL of formamide and incubate at 37oC for 30 minutes and read/record fluorescence.

Table 1

	Control Solution	Fluorescent Solution	Fluorescent + Quencher Solution
Nicking Solution	49.0 uL	48.5 uL	48.0 uL
Oligo Solution	0.5 uL	0.5 uL	0.5 uL
Glycosylase extract	0 uL	1 uL	1 uL
Quencher Solution	0.5 uL	0 uL	0.5 uL
Total	**50.0 uL**	**50.0 uL**	**50.0 uL**

Using HPLC to analyze three brain regions (hippocampus, cortex and caudate) of six wild type and six knockout subjects for a more comprehensive comparison between the behavior of oxidative stress in OGG1 knockout and wild types. Samples must be purified using phenol and chloroform, then de-phosphorylated, cleaving a phosphoryl group from the objective base cytidine, allowing the column to appropriately process the sample and the detector to distinguish peaks for 2dC and 5mdC. A calibration curve of heights and areas reported in the chromatogram, then determines the quantity of 2dC and 5mdC in the sample. Essentially, samples with damaged cytosine will have significantly smaller 2dC and 5mdC peaks.

Base Excision Repai

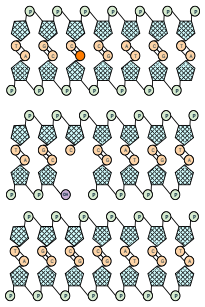


Figure 1

In this figure, an oxidized guanine, oxo^{8}dG, is recognized and excised by 8-oxoguanine glycosylase-1.

Detecting Kinetic Activity of OGG1

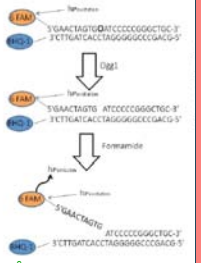


Figure 2

Figure 2 elucidates the molecular processes of our florescent-quencher model. 6-FAM represents the modified oligonucleotide and BHQ-1 represents the quencher introduced into the system.

Image 1

The yellow-ish fluid in the first tube is an image of 6-FAM – a modified oxidized oligonucleotide that is indicated by the broad O in Figure 2

The second tube is BHQ-1.

Reverse Phase Liquid Chromatography

Reverse Phase Chromatography generates chromatograms of various samples. Using this branch of High Performance Liquid Chromatography (HPLC), DNA damage may be detectable within the localized peaks at given time-markers.

During HPLC, the detector may identify several different substances in a single sample. However, those compounds are distinguishable from one another, because each is analyzed according to solubility. For example, 5mdC possesses a methyl group that permits it to be more soluble in water, therefore it is detected much later than 2dC, whose peak may be observed 4 ½ minutes after the initial injection of the sample.

Image 2

KO and WT Chromatograms

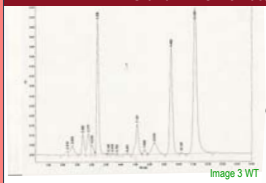


Image 3 WT

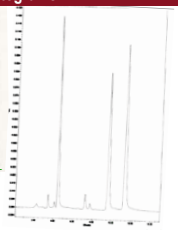
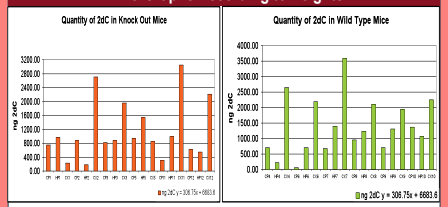


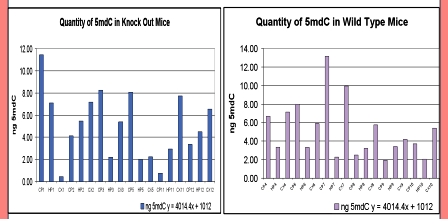
Image 4 KO

These chromatograms are an analysis of Wild Type and Knock Out mice and where peaks at 4.396 min and 7.638 min describe the quantity of 2dC and 5mdC detected in the sample.

HPLC Graphs According to Heights

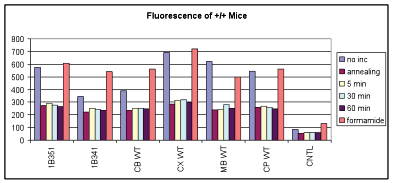


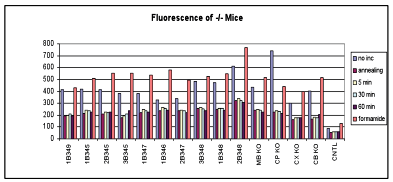
The data above indicates the amount of 2dC in wild type and knock out mice. Averaging 2450.7 ng in the Cerebral Cortex, wild type mice show significantly more OGG1 activity than knock-out mice whose average ng of 2dC within the Cerebral Cortex is 1831.0 ng



Comparing the quantity of 5mdC, there is a collective average of 5.1 ng in wild type mice and 4.979 ng in knock-out mice.

Figure 3





Both graphs indicate that before incubation, the fluorophore and the quencher are not appropriately aligned, therefore a significant amount of fluorescence is emitted. After annealing, however, the oligo-nucleotides adjust allowing the quencher to substantially reduce emission until formamide cleaves the fluorophore (6-FAM) through a phosphodiester linkage removing the fluorophore from the duplex – refer to Figure 2 – causing the fluorescence detected within the system to be more than or equal to the initial emission.

Summary of Results

Essentially, we notice that wild types generally exhibit mildly more OGG1 activity, demonstrated by our fluorescent-quencher model designed to measure the kinetic activity of the OGG1 enzyme in mouse tissues. Furthermore, the superiority of OGG1 activity in wild type samples is corroborated through HPLC analysis whose data report quantities of 2dC measured in each sample. Overall, wild type mice possessed little more 2dC and more 5mdC than knock out mice.

Conclusion

In conclusion, fluorescent-quencher coupling has offered an accurate and reliable method of detecting OGG1 activity. As expected knock out samples did not perform as actively as wild type mice. However, this difference is not statistically significant so more research will need to be performed to accurately determine OGG1 repair activity level.

Future Directions

There are multiple techniques that may be exploited to examine the activity of DNA repair enzymes. For example, a P^{32} DNA repair assay accurately quantifies OGG1, however, the assay itself is consumes much more time than the fluorescent-quencher model and is hazardous because of its radioactive substrate.

In future experiments we may use the P^{32} DNA repair assay to further compare activity. Additionally, including a larger sample size may more clearly identify the OGG1 activity trends between knock out and wild type samples.

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