EFFECTS OF LOW DOSE IONIZING RADIATION ON TRANSCRIPTIONAL EXPRESSION OF DNA REPAIR AND REACTIVE OXYGEN SPECIES SCAVENGING GENES: STUDIES AT CHORNOBYL

by

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INTRODUCTION

1.1 Background and Significance

1.1.1 Ionizing Radiation

From the time that Wilhelm Konrad Roentgen discovered X-rays in 1895, ionizing radiation (IR) has been a part of everyday life. This natural and anthropogenic product has given rise to better medicine and scientific research, as well as, the facilitation of an alternative energy source. However, IR's negative attributes (i.e. human and environmental health risks) are what the public has focused on. For these reasons, it is of utmost importance to inform the public as to the true effects of IR exposure. Early work documenting the effects of ionizing radiation on organisms and their subcellular components dealt with high doses administered acutely [1,2]. The results of these experiments were alarming. Ionizing radiation, when administered in high doses, can be extremely dangerous, causing mutations or death in exposed individual. Radiation biology is increasingly concerned with sub-acute and chronic, low dose exposure [3].

1.1.2 Low Dose Ionizing Radiation and the Chornobyl Environment

The United States Department of Energy uses two standards to define low dose IR. The first is no detection of adverse health effects below a certain level, and is expressed numerical as ~0.2 Gray (Gy). The second is the level of radiation exposure in a natural background setting (~3.7mGy) [4]. This movement to define the effects of low dose IR

has been facilitated by the advancements in molecular and radiation biology. Conclusions drawn from such studies can have direct applications to the possible affects of human exposure to ionizing radiation (e.g. nuclear power plant workers, x-ray technicians, and exposure to cosmic radiation as a consequence of ozone reduction). Studies using low dose and low dose rate exposure are difficult for two reasons. First is the inability to assimilate absorbed dose from a given exposure environment [5,6], and second is the extrapolation of low dose effects from knowledge of high dose impacts. Most scientists assume that the relationship between exposure to IR and health risk (i.e. cancer rate) is linear [7]. Even though the linear-no threshold model currently (and historically) is the model used to implement governmental regulations concerning exposure and dose, a growing mass of research suggests alternative models should be adopted [8-32]. While acute exposure studies are routine in a laboratory setting, accurate assessment of the effects of sub-acute to chronic low level exposure are extremely rare. One opportunity to study chronic low level IR is the natural laboratory environment left as a result of the Chornobyl Nuclear Power Plant (ChNPP) accident.

In April of 1986, the world experienced its worst nuclear power plant disaster. While conducting a series of safety checks, Reactor IV at the ChNPP exploded. This led to the release of between 100 and 200 million Curies of numerous radionuclides into the environment [8]. Today, the primary radionuclides present within the 10-km and 30-km zones surrounding Reactor IV include Strontium-90 (^{90}Sr), a biological analog of calcium, Cesium-137, a biological analog of potassium. This environment presents researchers with a natural laboratory to conduct studies involving sub-acute to chronic

exposures to low linear energy transfer (LET), low doses of IR. To date, a number of controversial conclusions from studies conducted at Chornobyl are found in the literature. While a number of studies have been from previous lab members [5-6,8,11-12,17-19,21-22,24,26-27,29,31-32] the desire to accurately assess the effects of IR found in the Chornobyl environment encompasses the entire scientific community. For a review of research using the Chornobyl environment see the following [8-32].

1.1.3 Radiation Hormesis and the Radioadaptive Response

The radioadaptive response is a specific form of hormesis. Southam and Ehrlich [33] coined the term "hormesis" in their work studying the effects of natural antibiotics in cedar wood that inhibits the growth of wood-decaying fungi. Their findings suggested that subinhibitory concentrations of the antibiotic had the reverse effect and stimulated fungal growth. Hormesis has been used to describe human responses to antibiotics, alcohol, nicotine, and other toxicant agents. The adaptive response (radiation hormesis) mechanism is described as a sublethal exposure (priming dose) to a toxic agent leading to some form of resistance when followed by a subsequent higher (challenge) dose of the same toxic agent. The concept of a beneficial dose of IR was suggested by the early work of T. D. Luckey [review 34]. Following Luckey, Wolff and collaborators [35-38] laid the ground work for future studies in low dose radiation biology. Jaworowski [39-41] cites unexpected results in Chornobyl studies as evidence for hormesis. As technology evolved, assays gained sensitivity and different endpoints were used to measure and assess the radioadaptive response. Such endpoints included cytogenetic

work with human lymphocytes [42], apoptosis [43], chromosomal damage [44,45], and oxidative stress [46,47]. The focus has now turned to trying to understand the underlying mechanisms (either cellular or molecular) responsible for such a response. Researchers have suggested two different mechanisms to mediate radioadaption. One mechanism involves DNA repair proteins and enzymes [48,49], and the second suggests that antioxidants are the key to protection under oxidative stress [50,46,51,47].

1.2 Experimental Justification

Because there are conflicting data sets about the consequences of exposure to IR studies must begin with a systematic experimental design. The ability governmental agencies have for accurately implementing legislation regarding human safety depends on scientific studies with a sound experimental design. Such a design includes the appropriate model system, pertinent biomarkers, accurate assessment of exposure regimes, and unbiased usage of samples [52]. For example, tissues from each individual used in this study have been archived in an accredited collection (Natural Science Research Laboratory [NSRL] at the Museum of Texas Tech University) (http://www.nsrl.ttu.edu/), so that this study, and others, can be performed in the future. This is extremely important because of the technological advances in the field of molecular biology and conflicting results for studies investigating the effects of radiation, particularly low dose, chronic exposure. The debate over whether or not a threshold exists has developed into a dichotomy in radiation biology. The first hypothesis is the linear, no-threshold (LNT) model in which there is a linear relationship between exposure

and consequence (i.e. cumulative effects model). This model assumes that no threshold exists below which deleterious consequences can be measured [53-56]. The second hypothesis is the linear, threshold (LT) model in which there are no adverse effects detectable below a certain threshold level of exposure. The LT model then, includes the dose range where the radioadaptive response has been observed. This model implies that below a defined threshold, no toxicity is evident [57-61]. With advancing technology, a third response has also been hypothesized. This model includes a threshold but is concerned with ultra-low dose rate. Hooker et al. [62] did not use the Chornobyl environment, but their findings suggest that exposure to ultra-low dose rates can induce damage well above endogenous levels. These authors outline a new set of responses to such an exposure level and conclude that ultra-low dose IR should be managed separately from low and high dose exposure.

1.2.1 Molecular Mechanisms

In order to maintain genome integrity from negative effects resulting from exposure to mutagenic agents, either endogenous or exogenous, organisms have developed an array of detoxification and repair systems. For example, a human cell is estimated to undergo 10,000-150,000 oxidative damaged DNA sites per day [63,64]. If not repaired, the fate of these (and other) lesions would be replicated through subsequent rounds of DNA replication and become fixed in somatic tissues or the germ line. To better facilitate accurate repair, these defense mechanisms have a degree of redundancy. Complexity of these systems is illustrated in the three forms of excision repair (i.e.

Mismatch Repair [MR], Base Excision Repair [BER] and Nucleotide Excision Repair [NER]).

Oxygen is a necessity for aerobic organisms to live. The paradox is that the requirement for an aerobic life is also extremely toxic. Oxidative damage to cells and macromolecules is thought to play a large role in such disease states as cancer, immune deficiency, neurodegenerative diseases and aging. Oxygen is considered to be a major electron sink for biological systems and that leads to the formation of intermediate molecules referred to as reduced or reactive oxygen species (ROS) [65]. The most highly reactive and consequently dangerous ROS is the hydroxyl radical ($OH^{\cdot}$). Other biologically important ROS are the superoxide ($O_2^{\cdot-}$) free radical and hydrogen peroxide (H_2O_2). The latter two are much more stable relative to the hydroxyl radical, but their potential to generate further production of hydroxyl radicals increases their importance as damaging agents to organisms. Organisms have evolved mechanisms to localize and reduce the damaging effects of these ROS. Examples of these include the compartmentalization of redox reactions involved in the electron transport chain (ETC) to the mitochondria, antioxidant compounds (ascorbate) and detoxifying enzyme systems (superoxide dismutase family of enzymes) to reduce the levels of ROS present.

My thesis focuses on the transcriptional response of genes encoding molecules involved in genome integrity following exposure to genotoxic agents such as ionizing radiation to determine if any of these pathways are associated with induction of the radioadaptive response. I assayed the transcriptional levels of two genes involved in BER (*Apex1* and *Polb*), NER (*Ercc1* and *Ercc2*), and ROS scavenging pathways (*Sod1*

and *Gpx1*) using QRT-PCR. Below, I have outlined each pathway in greater detail, as well as described the chemistry and methodology of QRT-PCR.

1.2.1.1 Base Excision Repair

Base Excision Repair is considered the primary defense against lesions derived from endogenous sources such as ROS and oxidative stress [66]. Reactive oxygen species are a main source of spontaneous damage to DNA and are produced as either by-product of oxidative metabolism or through the radiolysis of water following exposure to IR. The distinguishable component of BER from other excision pathways is that damage-specific DNA glycosylases are used to identify damaged or inappropriate bases (Fig 1.1). There is some degree of redundancy among the DNA glycosylases even though a number of them require a specific substrate. For monoadduct base damage, the key function of any DNA glycosylase is the excision and release of the damaged base [67]. A unique DNA N-glycosylase hydrolyzes the N-glycosylic bond between the base and the deoxyribose leaving an apurinic/pyrimidinic or abasic site (AP sites). This AP site is identical to a spontaneous DNA depurination or depyrimidination. A number of DNA glycosylases posses an AP lyase activity. The reaction of an AP lyase activity is the cleavage of the DNA backbone on the 3' side of the AP site resulting in a 5'-phosphate end [66]. The next step is the cleavage of the DNA backbone by AP endonuclease (APEX) on the 5' side of the AP site resulting in a 3' OH end [68,69]. For short patch BER, DNA polymerase beta (POLB) incorporates a single, correct base, and DNA ligase III in partnership with the XRCC1 protein seals the backbone [70,71].

1.2.1.2 Nucleotide Excision Repair

Nucleotide Excision Repair is mechanistically similar to BER, but is a much more complex pathway involved in repairing different types of lesions. There are two subpathways of NER that are characterized by which portion of the genome they maintain (Fig 1.2). The first subpathway is the global genome NER (GG-NER). This includes the non-coding portion of the genome and the untranscribed strand of coding portions actively being transcribed. The other subpathway is referred to as transcription coupled NER (TC-NER) and is responsible for the repair of only the actively transcribed strand [72]. The NER pathway primarily corrects crosslink or bulky DNA adducts, such as UV-photoproducts, including cyclobutane pyrimidine dimers [73] and secondarily a small portion of oxidative damage [74]. Defects in a number of NER-deficient organisms have led to the discovery of many proteins involved in these pathways. Defects include Xeroderma Pigmentosum (XP), Cockayne syndrome(CS) and Trichothiodystrophy (TTD). Clinical characteristics include photosensitivity, increased risk for skin cancer, neurological defects, premature aging features, icthyosis, and CS-like brittle-hair syndrome [75-78].

The key step of NER is the excision of a stretch of oligonucleotides approximately 24-32 nucleotides long. A simplistic overview of this pathway is the recognition of the lesion, unwinding of the DNA duplex surrounding the damaged site, dual incision both 5' and 3' of the damaged site, gap filling by DNA repair synthesis, and strand ligation. As outlined by Wood [79], the RPA, XPA, and XPC-hHR23B (specific to GG-NER) proteins recognize a distortion within the helix caused by a bulky adduct.

This complex of proteins causes local unwinding around the lesion. This region is further opened by an ATP-dependent process involving the TFIIH complex of proteins (including ERCC2). The damaged strand is then cleaved by the XPG protein 3' and by the ERCC1-XPF protein complex 5' releasing the 24-32 nucleotide (nt) stretch of damaged DNA. DNA repair synthesis is carried out by the PCNA-dependent DNA polymerase δ and ε holoenzyme. The final step is ligation of the strands by DNA ligase.

1.2.1.3 Reactive Oxygen Species (ROS) Scavenging

IR can directly or indirectly interact with biological material. Production of free radicals followed by molecular attack is one result of an indirect interaction. Approximately 60-70% of the damage caused by IR is thought to be through the production of free radicals, primarily the hydroxyl radical, from the radiolysis of water [80]. The majority of products resulting from water radiolysis (Fig 1.3) are ionized molecules and unstable ROS [81]. Such species include ionized water (H_2O^+), hydrogen radicals ($H^{\cdot}$), hydrogen ions (H^+), hydroxide ions (OH^-), hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), and superoxide ($O_2^{\cdot-}$). A number of these radicals are also produced endogenously as by-products of normal cell metabolism. One such example occurs within the mitochondria and involves the production of adenine triphosphates (ATPs) by the ETC [82]. Because oxygen is used as the final electron acceptor, it is available to react with free electrons that have escaped from complex I (NADH-dehydrogenase), III (ubiquinone-cytochrome b) and IV (cytochrome oxidase) of the ETC resulting in the production of $O_2^{\cdot-}$ [83]. Superoxide can either undergo subsequent

reactions (ultimately forming water) or react with other components of the ETC, lipids membranes, DNA and other macromolecules.

In order to maintain levels of homeostasis, the cell uses a number of preventative mechanisms including compartmentalization of redox reactions and ROS scavenging enzymes to reduce the levels of ROS present (Fig 1.4). I chose to assay the transcriptional responses of two enzymatic antioxidant defense mechanisms. These include cytosolic Cu/Zn superoxide dismutase (Sod1) and Se glutathione peroxidase (Gpx1).

The SOD superfamily includes Cu/Zn SOD (Sod1), Mn SOD (Sod2) and EC-SOD (Sod3). Each member is localized to a specific cellular compartment. Sod1 is primarily active in the cytosol and nucleus and is thought to comprise a significant portion of total Sod enzyme activity [84]. As its name implies Sod1 utilizes both copper and zinc to function properly. Experiments aimed at defining the mechanism of SOD1 revealed that the copper metalloid participates in the catalytic reaction and that zinc exists as a structural entity [85]. McCord and Fridovich [86] first described the catalytic action of Sod1 as the dismutation of superoxide radicals into H_2O_2 and O_2. Crapo and Tierney [87] discussed Sod1 activity as one of the initial defense mechanisms when cells or organisms are under oxidative stress.

Gpx1 is one of four antioxidant enzymes belonging to the class of selenoproteins. The remaining three other glutathione peroxidases include Gpx2 (gastrointestinal), Gpx3 (plasma), and Gpx4 (phospholipid hydroperoxide). The majority of Gpx1 activity is localized to the cytoplasm, but activity has also been detected in mitochondrial extracts

[88]. Gpx1 is one of two enzymes responsible for reducing cellular levels of H_2O_2. The other significant reducer of H_2O_2 is catalase (CAT). The major end product of H_2O_2 reduction is the production of H_2O.

Radiolysis of water by IR produces ionized water (H_2O^+) and free electrons (e^-), and the subsequent reactions with surrounding water produce hydrated electrons (e_{aq}^-), $H^\cdot$, OH^-, $OH^\cdot$, and H_2O_2. The hydrated electrons react with O_2 to produce superoxide radicals. Molecular oxygen can also give rise to superoxide radicals by reacting with hydrogen atoms. As mentioned above, the dismutation of two molecules of superoxide react with two molecules of hydrogen ions to produce molecular oxygen and H_2O_2. Hydrogen peroxide is either indirectly produced through radiolysis of water or reducing superoxide by Sod1. Gpx1 enzymatically reduces H_2O_2 to water and O_2. Hydroxyl radicals are also indirectly produced using H_2O_2 as an intermediate. Two deleterious events can occur when Sod1 produces an abundant amount of H_2O_2 and Gpx1 (or Cat) can not maintain homeostasis. The first is an increase of the Haber-Weiss reaction (i.e. superoxide plus hydrogen peroxide produces hydroxyl radicals) [89]. The second is the possibility for superoxide to react with iron leading to the production of hydroxyl radicals by the Fenton reaction [review 90]. Therefore, the interactions between SOD and GPX that maintain a balance between $O_2^{\cdot-}$ and H_2O_2 are critical in maintaining the oxidative environment of the cell.

1.2.2 Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)
(TaqMan® Methodology and Chemistry)

Quantitative real-time polymerase chain reaction is a powerful and sensitive means of quantifying messenger RNA (mRNA) by combining reverse transcription PCR (RT-PCR) with dual-labeled fluorogenic hybridization probes [91-97]. Ever since the development of PCR by Mullis et al. [98] and Saiki et al. [99], nucleic acid quantification has been a rapidly evolving field. Other examples of quantification include the use of fluorescence labeling and polyacrylamide gels [100], and radioactive labeling and blotting with phosphorimaging [101]. When PCR is converted to a log base scale, the three phases that comprise the reaction are exponential, linear, and plateau. The ability to detect fluorescence in "real time" during the exponential phase of PCR is what allows QRT-PCR superiority in comparison to the post-PCR processing involved in the earlier techniques.

There are a number of different detection chemistries available for QRT-PCR. These include hydrolysis probes (TaqMan®), intercalating dyes (SYBR green), molecular beacons, scorpions and hybridization probes. For my thesis I used the hydrolysis TaqMan® probe. The TaqMan® chemistry uses the 5′-3′ exonuclease activity of *Taq* polymerase in combination with fluorescent resonance energy transfer (FRET). A simple overview of FRET is shown in Fig 1.5. A reporter and nonfluorescent quencher dye are in close proximity so that the light emitted from the reporter dye is quenched (absorbed) by the nonfluorescent quencher dye. When these two fluorophores (dyes) are separated, the reporter dye signal is no longer quenched and can be detected and measured. In QRT-PCR, a gene-specific probe is synthesized with a 5′-reporter and

3′-quencher molecule in close proximity to one another. Following denaturation of cDNA produced from RT-PCR of a sample (e.g. control or treatment), the probe anneals to the target sequence in the gene-of-interest (goi), along with gene-specific flanking primers. As primer extension occurs, the *Taq* exonuclease activity degrades the probe, separating the reporter and quencher, as well as, amplifying the goi. Each round of amplification (and probe degradation), results in measurable increases in energy emission from the reporter. Because the cDNA template is generated from sample-specific mRNA, the number of cycles required to reach the plateau phase is directly related to the amount of template present. Raw data is expressed in threshold values (C_T) over time, and is equivocal to the cycle where PCR reaches the linear phase. This equates to a low C_T value indicating a larger amount of starting material (i.e. amount of mRNA molecules). In other words, as starting material increases, C_T value decreases [review 102]. The TaqMan® probe and chemistry allows for an increase level of specificity and detection of low abundant transcript template [103].

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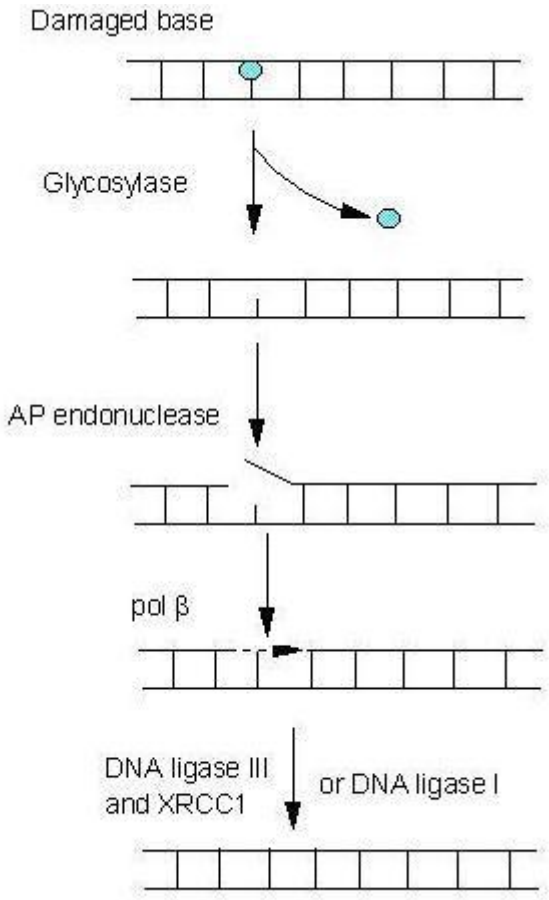

Fig 1.1. Schematic of the Base Excision Repair pathway. Here a DNA-N-glycosylase removes a damaged base (black circle) away from the sugar-phosphate backbone creating abasic site. AP endonuclease cleaves the phosphate backbone 5′ of the damaged base. Subsequent exonuclease activity removes the helix completely, and Polb and DNA ligase complete DNA synthesis. Image taken from http://www.murray.francis.com/repair/7-BER.htm.

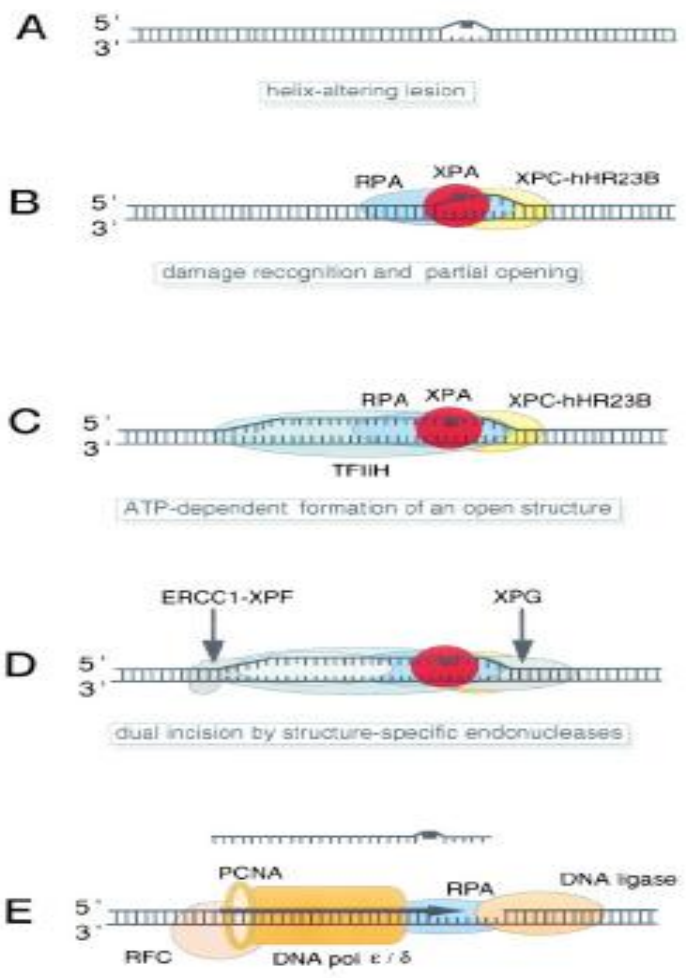

Fig. 1.2. Schematic of the Nucleotide Excision Repair (NER) pathway. Local distortion of the helix indicated by the black square in part A is recognized by a complex of NER proteins including XPA, RPA, and XPC-hHR23B (TC-NER). The TFIIH complex of proteins (including ERCC2) opens the helix around the distortion. The damaged strand is cleaved 3′ and 5′ by XPG and ERCC1-XPF nucleases respectively. DNA synthesis is carried out by the PCNA-dependent DNA polymerase δ and ε holoenzyme, and DNA ligase fills the gaps. Image taken from Wood 1997.

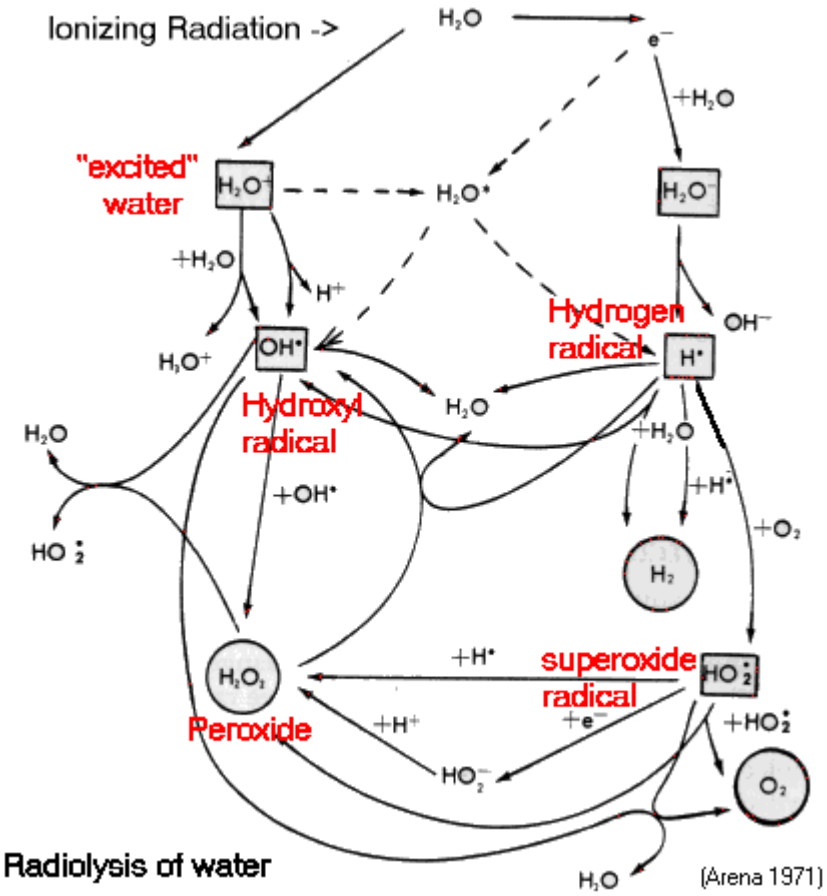

Fig. 1.3. Cartoon of the Radiolysis of water by Ionizing Radiation (IR). This cartoon outlines the production of reactive species after radiolysis of water by IR. The initial event is the production of ionized water and free electrons. The second phase depicts the multiple interactions that the different reactive oxygen species have with one another. Image taken from *Ionizing Radiation and Life* 1971.

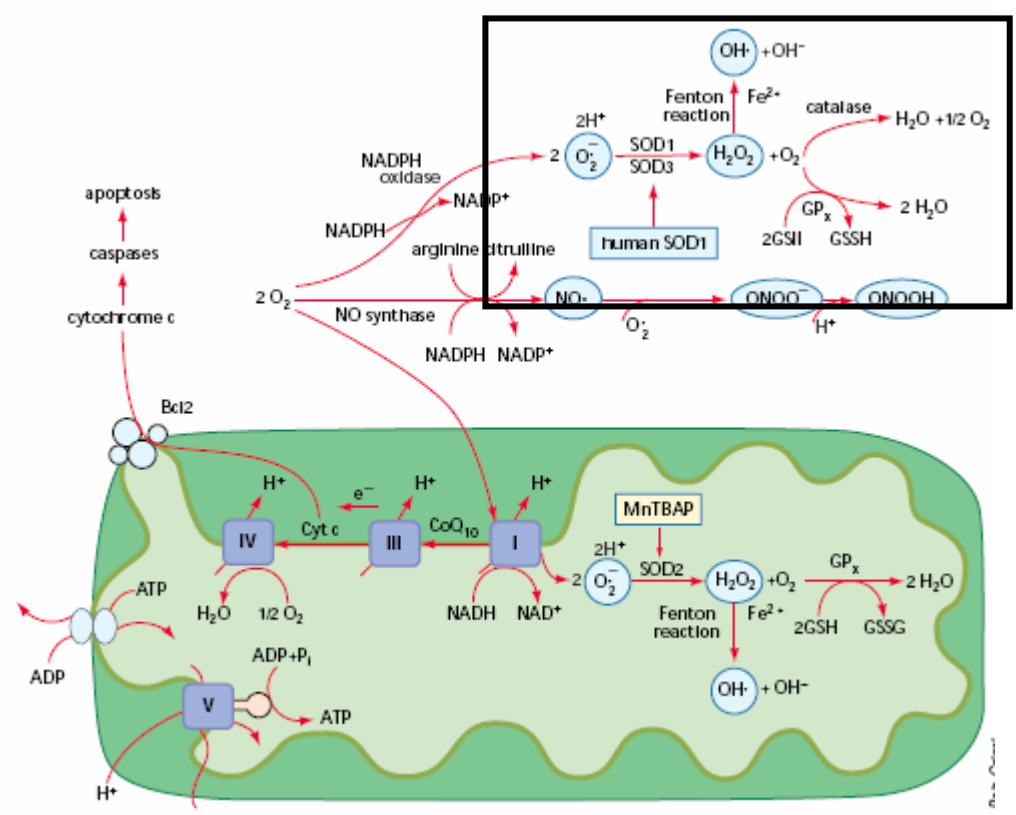

Fig 1.4. Schematic outlining the different production pathways for endogenously derived ROS and the various enzymes used to maintain cellular homeostasis. Important for my work is the redox reactions subsequent to electron leakage produced throughout the electron transport chain in the mitochondria outlined by the square. Images taken from Wallace and Melov 1998.

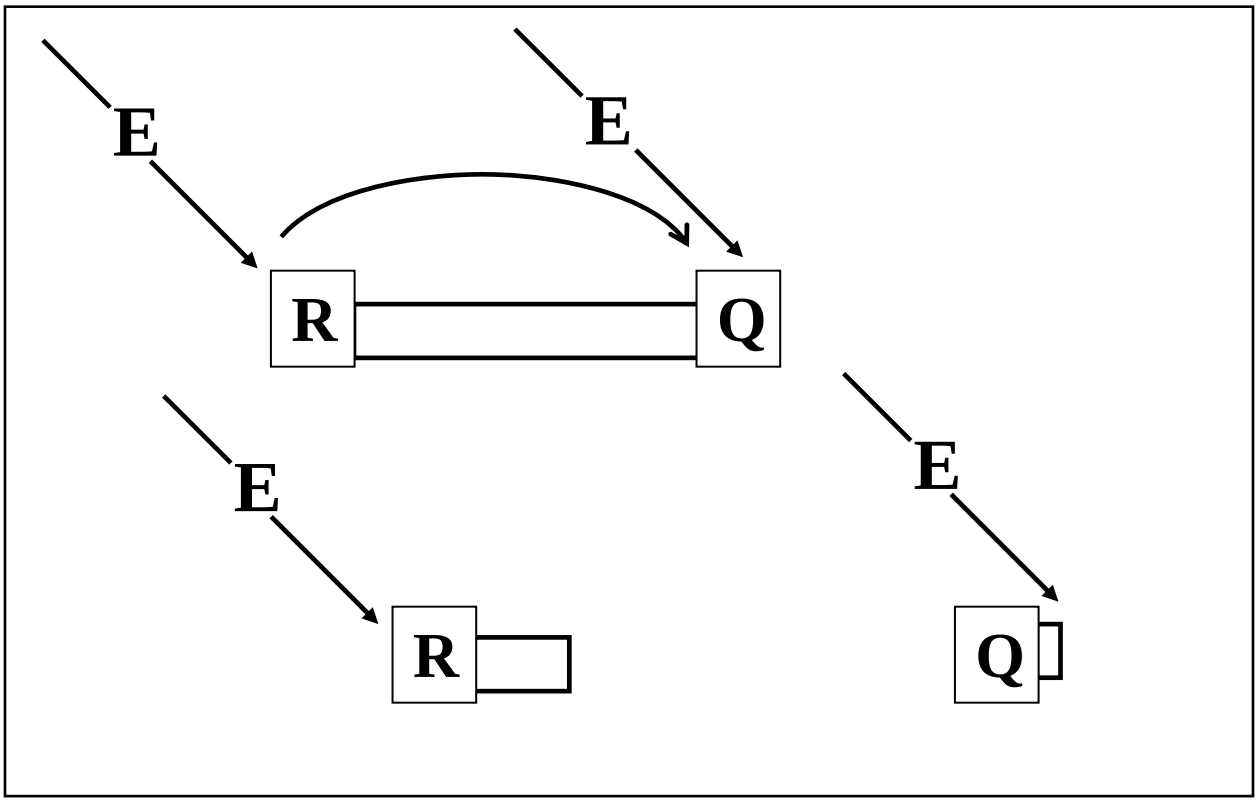

Fig 1.5. Cartoon outlining fluorescent resonance energy transfer (FRET). Light energy (E) excites a reporter molecule (square with R) and a quenching molecule (square with Q) quenches the signal (i.e. FRET is occurring). When the reporter and quencher are separated FRET does not occur and reporter fluorescence is detected.

CHAPTER II

STUDIES OF THE RADIOADAPTIVE RESPONSE AT CHORNOBYL AND CU/ZN SUPEROXIDE DISMUTASE (*Sod1*)

2.1 Abstract

Ionizing radiation (IR) can initiate a large spectrum of damage which leads to the induction of DNA protective and repair pathways. Historically, IR studies have assessed the effects of high dose, acute exposures; however a recent shift in radiation biology has focused its efforts to cellular responses of low dose IR exposure. We used the radioactive environment surrounding the Chornobyl Nuclear Power Plant to assess the potential for the development of radioresistance in exposed animals and the possible molecular mechanisms that maybe responsible. To better understand the phenomenon of radioresistance, we exposed male BALB/c mice externally to a priming (pr) dose of 10cGy at two dose rates ($6.94x10^{-4}$cGy min^{-1} and 0.5cGy min^{-1}) followed by a subsequent 1.5Gy challenge (ch) dose ($6.25x10^{-3}$Gy min^{-1}). We measured absolute mRNA levels using quantitative real-time polymerase chain reaction (QRT-PCR) of enzymes and proteins believed to maintain genome integrity under increased levels of oxidative stress. Twenty four hours post exposure there was no induction of *Apex1* and *Polb* (Base Excision Repair [BER]), *Ercc1* and *Ercc2* (Nucleotide Excision Repair [NER]) and *Gpx1* (Reactive Oxygen Species [ROS] scavenging). We were able to detect a significant amount of differential expression in two of our exposure groups for the *Sod1*

(ROS scavenging) gene. When compared to micronucleus (MN) data as a measurement of radioadaption of these same mice, the high expression levels of *Sod1* suggest a temporal-dependent component. Therefore, we conclude that *Sod1* expression levels suggest a role in radioadaption at ultra-low dose rates.

2.2 Introduction

Exposure to IR produces a diverse set of responses in life forms. The earliest studies on IR used acute, high doses. These studies demonstrated that IR, when administered in this fashion was deleterious and potentially lethal. Muller's [1] seminal work describing the mutagenic effects and damaging capabilities of IR resulted in fear of exposure. Cytogenetic and molecular genetic analyses determined acute exposures could alter chromosome structure [2] and specific gene sequences [3]. Lower level exposures used medically or as tools for examining tissues were later determined to be causally related to cancer [4]. As new techniques were developed, researchers demonstrated other detrimental effects as a consequence of exposure to IR. These include induction of apoptosis, elevated levels of exogenously derived free radicals, and damage to macromolecules other than DNA [5]. As the ability to deliver controlled, lower exposures developed, unexpected responses to IR were encountered. This is also true of responses associated with exposure to environmental IR in conjunction with improved methods of detection and dose estimation. These responses to low level exposure include an adaptive response [6-9], bystander effect [10,11], and genomic instability [12-14]. One explanation for these responses has been hypothesized to be the differential gene and

protein expression in cellular and molecular pathways responsible for DNA repair, free radical scavenging, cell cycle regulation/apoptosis, and cell to cell communication. This paradigm shift in radiation biology has been outlined by Brooks [15].

The adaptive response is the phenomenon where a sublethal exposure (priming dose) to a toxic agent leads to some form of resistance when followed by a subsequent higher (challenge) dose of the same toxic agent [16,17,9]. The early work of Olivieri et al. [18] noted a radioadaptive response in their cytogenetic work with human lymphocytes exposed to IR. In subsequent studies this phenomenon produced different responses such as apoptosis [19], chromosomal damage [20,21] and oxidative stress [22,23]. Sasaki et al., [24] report that mammalian cells exhibit the radioadaptive response at an optimal dose range of ≤ 0.1Gy. These levels fall under the definition of low dose exposure set by the International Commission on Radiological Protection (ICRP) during the 1987 meeting of the National Council on Radiation Protection and Measurements (NCRP) stating that a low dose exposure is ≤ 0.2 Gy [25]. These levels are currently recognized by the United States Department of Energy (http://lowdose.tricity.wsu.edu/). Studies have also noted that a transcriptional response is seen at low dose exposures [26-28]. In other studies though, certain transcripts are altered at lower exposure but not at higher exposure [29].

Studies have established how rodents [30-32] and cell lines [33,34] respond to high doses of acute radiation. Recent research working to define the response to chronic low dose IR has produced a number of studies using cell lines [35], but little research has been done documenting an organismal level response. Insights gained from such

research will aid in the understanding of and more accurately predict the biochemical and genetic effects in humans and wildlife chronically exposed to IR. There are two primary competing hypotheses concerning chronic exposure to low dose IR. The first hypothesis is the linear, no-threshold (LNT) model in which there is a linear relationship between exposure and consequence. This model implies that the consequences to any level of exposure to IR, no matter how low, are negative (i.e., as levels of exposure increase the frequency of cancer, mutations, and other detrimental effects increase) [36]. The second hypothesis is the linear, threshold (LT) model in which there are no adverse effects detectable below a certain threshold level of exposure [37]. This model implies that below a defined threshold, no toxicity is evident. Even though the concept of a threshold model is simplistic, the biological complexities involved in achieving this state of homeostasis have been, and are currently under intensive investigation. Furthermore, this model includes the dose range where the radioadaptive response has been observed in which exposure to certain levels may actually be protective or beneficial. This beneficial effect is defined as hormesis [38]. Identifying the correct model and its regulation at the molecular level is critical because most areas and organisms are chronically exposed to low levels of radiation.

In order to better understand the radioadaptive response on the organismal level, we designed an experiment to profile transcriptional responses of genes encoding proteins thought to play a role in this response. We exposed male BALB/c mice to ultra-low dose rate, low linear energy transfer (LET) IR and performed QRT-PCR on genes in pathways involved in free radical scavenging and DNA repair to test for a transcriptional

response following exposure to the Chornobyl environment. The Chornobyl environment was used as a model area because a continuous, chronic dose of low LET IR can be delivered. Two major radionuclides can still be found within the Red Forest region at Chornobyl. These are strontium-90 (^{90}Sr) and cesium-137 (^{137}Cs). Studies estimating concentrations and dose rates [39] and radioresistance of naïve bank voles [40] estimated that the mean total (external and internal) daily dose rate to mammals in this area for ^{90}Sr and ^{134,137}Cs to be 10.0 and 24.0 mGy/d, respectively. This exposure environment is similar to naturally occurring or anthropogenically contaminated areas with high levels of IR. The dose and dose rate within the Chornobyl environment allows a unique opportunity to truly assess the effects of exposure to continuous, chronic, low dose IR that are almost impossible to replicate in a laboratory environment.

Biodiversity (defined as both the number of species and density of species) in the radioactive regions contaminated by the Chornobyl accident has actually increased in comparison to relatively uncontaminated reference sites [41]. This increased biodiversity associated with radioactive contamination is probably the result of several factors. The most important factor could be that the habitat is now protected as human activity in those areas is severely restricted. Another potential reason, not exclusive from the first, could be that the species there experience no ill effects resulting from the IR dose rates that they are encounting.

Approximately 60-70% of the damage caused by IR is thought to be through the production of free radicals, primarily the hydroxyl radical, from the radiolysis of water [42]. Additional reactive oxygen species (ROS) may also include superoxide and organic

radicals that can potentially induce other ROS (hydrogen peroxide and organic hydroperoxides) [43]. The accumulation of these ROS and their reactions with reactive redox metallic ions (e.g. Fe(II)) can induce an increase in oxidative stress that will likely produce DNA damage in the form of base modification, base loss, sugar damage, and single strand breaks (ssb) [44-46]. If this ultra-low dose rate of IR increases ROS derived DNA damage, then an expected molecular defense would be an induction of pathways involved in radical scavenging, base excision repair (BER), nucleotide excision repair (NER), strand break repair, and/or apoptosis. For the above mentioned reasons, we chose, in this initial experiment, to measure absolute transcript copy number for *Apex1* and *Polb* (BER), *Ercc1* and *Ercc2* (NER), and *Sod1* and *Gpx1* (ROS scavenging).

Based on a subset of data investigating micronucleus formation following exposure to IR in the Chornobyl environment, male BALB/c mice exhibited aspects of a radioadaptive response to a challenge dose of 1.5Gy following either a subacute or an acute priming dose of 10cGy (Holmes et al. unpublished). We profiled transcriptional responses in these same mice studied by Holmes et al. (unpublished) to determine if altered gene expression correlates with this radioadaptive response. The transcriptional profiles of these mice for DNA repair and ROS scavenging genes suggest that the antioxidant enzyme copper/zinc superoxide dismutase (Sod1) could play an active role in the radioadaptive response. These results correspond with other studies suggesting that: 1) low dose (~1Gy) IR can induce differential expression of mRNA [47,48]; 2) exposure to low dose radiation can increase the production of superoxide radicals [43,49]; 3) the increase in radical production can induce the enzyme activity of Sod1 [50]; 4) Sod1

enzyme activity has been linked to cell survival and adaptive responses associated with oxidative stress [22].

2.3 Materials and Methods

2.3.1 Animals and Irradiation

Male BALB/c mice matched in age and mass were purchased from a research breeding colony in the city of Chornobyl, Ukraine. Before being placed within enclosures in the Red Forest, each animal was assigned a unique number and toe-clipped for identification. The locations of the areas within the Red Forest where the animals were placed are outlined in Holmes et al. (unpublished). Mice were either administered a subacute priming dose (10 days) or an acute priming dose (20 min). The Chornobyl environment was used to administer the continuous subacute priming dose. The acute priming dose and subsequent acute challenge doses were administered using a ^{137}Cs source in a laboratory in the city of Chornobyl, Ukraine. The subacutely primed animals received a dose of 10cGy administered at a rate of $6.94x10^{-4}$cGy per minute (~10 days). The acutely primed animals received a dose of 10cGy administered at a rate of 0.5 cGy per minute (~20 minutes). The acute challenge dose was 1.5Gy administered at a rate of $6.25x10^{-3}$Gy per minute (~4 hours). The environmental dose rates were estimated by the dose calculated by Chesser et al. (2000) for the Chornobyl environment. Total dose accumulation for each endpoint was verified using lithium fluoride (LiFl) thermoluminescent dosimeters (TLDs) inserted into soy-paraffin (density ~0.98) mouse-model phantoms. TLD fluorescence was measured with a Harshaw 3600 TLD reader

and raw scores were converted to total dose (Grays; Gy) and dose rates (cGy h^{-1}) for each chip.

Mice were euthanized twenty-four hours after completion of exposure. Each mouse was immediately weighed and processed. For this study, liver tissue (approximately 3 mm^3) was excised and placed into 1.8ml of RNA*later*™ in a 2ml polypropylene tube (Ambion Inc). The remaining liver tissue was flash frozen in liquid nitrogen and archived in the Natural Science Research Laboratory (NSRL) (http://www.nsrl.ttu.edu/) at the Museum of Texas Tech University. To verify the inbred strain of *Mus* used, DNA from four randomly chosen animals were genotyped by Charles Rivers Laboratory to verify that the model system was BALB/c. All four samples were found to be 99% BALB/c (data not shown). Husbandry of animals was in accordance with the Texas Tech University Animal Care and Use Committee (Lubbock, TX USA) guidelines and protocols (ACUC# 04009-03).

2.3.2 RNA isolation and cDNA synthesis

Total RNA was isolated from RNA*later*™-stabilized liver for exposed and control animals using the RNeasy® Mini Kit (QIAGEN) according to the manufacturer's protocol. Briefly, 30mg of stabilized liver was disrupted and homogenized using a PowerGen 125 (Fisher Scientific) rotor-stator style homogenizer in the presence of the lysis buffer provided by the manufacturer. Before final elution, all samples were treated with an on-column RNase-free DNase digestion (QIAGEN) according to the manufacturer's protocol to remove any trace amounts of DNA. Total RNA was eluted

into 75µl of RNase-free water. The quantity and purity of total RNA isolated was measured using UV spectrophotometric analysis of A260 and A260/A280 ratio, respectively on an ND-1000 spectrophotometer (NanoDrop Technologies).

cDNA synthesis was carried out using the TaqMan® Reverse Transcription Kit supplied by Applied Biosystems, Inc. (ABI). All reverse transcription (RT) reactions called for a starting amount of 1µg of total RNA and were performed in a final volume of 50µl according to the manufacturer's protocol for a two-step reverse transcription-PCR (RT-PCR) reaction.

2.3.3 Quantitative Real-Time PCR (TaqMan®)

QRT-PCR measurements for transcripts from *Apex1*, *Polb*, *Ercc1*, *Ercc2*, *Sod1*, *Gpx1*, and *Cdkn1a* were performed using an ABI Prism 7700 Sequence Detection System (ABI). Each gene and its associated accession number are listed in Table 2.1. Primer and probe sets were designed for optimal melting temperature (T_m) values and secondary binding properties using PrimerExpress® software v2.0 (ABI). The sequence of primers and probe combination are in Table 2.1. Sequence analyses were performed to verify that each amplicon was mouse-specific, and to eliminate possible DNA carryover as a confounder. Briefly, virtual PCR analysis of the designed amplicon was analyzed using the NCBI program, BLAST (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/), and to ensure transcript specificity, the location of intron-exon junctions were located using the University of California Santa Cruz program BLAT (BLAST-Like Alignment Tool)

(http://www.genome.ucsc.edu/goldenPath/help/hgTracksHelp.html). Before RT-qPCR assays were performed, each amplicon was sequenced on an ABI 3100 Genetic Analyzer (ABI) to verify target amplification.

2.3.4 Standard Curve Generation and Absolute Quantification

Standard curves for each gene-of-interest were determined using a recombinant DNA method generated from cDNA synthesized from total RNA isolated from a control animal. Primers flanking the TaqMan® primer/probe sites were designed with PrimerExpress and used to amplify and clone each gene of interest (goi). Following RT-PCR amplification, each amplified product was visualized by electrophoresis on a 3% agarose gel stained with ethidium bromide. All amplified products, except *Ercc1*, were purified using the QIAquick® Spin Kit (QIAGEN). For *Ercc1*, the 226bp target-sized band was excised and purified using the QIAquick® Gel Extraction Kit (QIAGEN). Each RT-PCR amplicon was sequenced on an ABI 3100 Genetic Analyzer (ABI) to verify target amplification. All RT-PCR products were then subcloned using the Promega pGEM®-T Vector Systems (Promega Corp.). Colonies were screened using the blue/white method, and positive transformations were then cultured overnight. Recombinant plasmid DNA was isolated and purified using the QIAprep® Miniprep Kit (QIAGEN) according to the manufacturer's protocol. A restriction enzyme digest of each purified miniprep using *BsfZ I* (Promega Corp.) according to the manufacturer's protocol, followed by visualization by electrophoresis on a 3% agarose gel stained with ethidium bromide, was used to verify that each goi had successfully been subcloned. The

quantity and purity of all minipreps containing the appropriate goi was measured using UV spectrophotometric analysis of A260 and A260/A280 ratio, respectively on an ND-1000 spectrophotometer (NanoDrop Technologies). Finally, all purified minipreps were sequenced on an ABI 3100 Genetic Analyzer (ABI) to verify target amplification using the SP6 and T7 promoter plasmid specific primers (Promega Corp.; Madison, WI).

Each standard curve was derived from purified plasmid DNA for each goi over a 4-log serial dilution (10^4 to 10^8). The reaction for each standard curve was run on the same plate as its control and experimental reactions. Equation 1 was used to determine the number of copies of each goi, i.e.,

Number of copies per µl = goi quantity (pg/µl) / number of pg of P+I per P+I,

where: P stands for Plasmid and I stands for Insert. Equation 2, i.e.,

> Initial amount (100 million copies) of template (µl) added to 1ml of nuclease-free water for standard curve construction: $2x10^{10}$ number of copies / number of copies per µl,

determined the initial amount of starting template for construction of the standard curve. The linear regression equation, i.e.,

> Mean absolute value of each exposure treatment for each goi / mean absolute value of each goi control group,

and its respective correlation coefficient are in Table 2.2.

2.3.5 QRT-PCR for Genes-of-Interest

QRT-PCR for each sample was performed in triplicate. To avoid plate to plate variation among individual genes of interest, each gene was assayed on a single 96-well plate. All reactions had a final volume of 50µl and contained: 100ng of cDNA, 1X

primer/probe mix (final concentration of 900nM each and 250nM, respectively), 1X TaqMan® Universal Master Mix (ABI), and nuclease-free water. Reaction conditions were as follows: 2 minute incubation at 50ºC for uracil cleavage; 10 minute denaturation at 95ºC, and 40 cycles for amplification (two steps: 95ºC for 15 seconds and 60ºC for 1 minute). The number of molecules was calculated from the linear regression of the standard curve generated found in Table 2.2.

2.3.6 Statistical Analysis

All treatment groups consisted of a sample size of n = 4 animals except for the 1.5Gy acute group. For analysis, one animal was withdrawn reducing this group's sample size to n = 3 animals. We tested for transcriptional variation within each goi across all exposure groups using a one-way analysis of variance (ANOVA) with ranked relative transcript number for each goi. Those genes for which the ANOVA was significant at the $p<0.05$ level were further analyzed to test for significant pairwise differences between exposure groups (within each goi) using a Bonferroni-corrected post-hoc analysis at the $p<0.05$ level.

2.4 Results

Using absolute QRT-PCR, we measured the number of mRNA molecules in pathways thought to participate in maintaining genomic integrity following exposure to genotoxic agents such as ionizing radiation to determine if any of these pathways are associated with an induction of the radioadaptive response. Based on absolute mRNA copy number of the control animals for the three pathways, the two genes involved in free

radical scavenging were constitutively expressed at high levels compared to both sets of DNA repair genes (Table 2.3.1). The following are the results of the normalized ANOVA analysis categorized by pathway and can also be found in table 2.3.2 and figure 2.1.

2.4.1 NER pathway

The ANOVA found no significant difference among any of the exposure groups for either of the nucleotide excision repair genes (*Ercc1* and *Ercc2*) we assayed.

2.4.2 BER pathway

There was no significant difference among any of the exposure groups for the *Apex1* gene. A significant difference among exposure groups for *Polb* was detected. The test for pairwise comparison found two groups with means significantly different from one another. Gene expression in both the 10cGy acute pr (1.31 ±0.03) and the 10cGy subacute pr (1.16 ±0.11) groups were significantly higher compared to the 1.5Gy acute (0.59 ±0.14) group. Values are arithmetic means ± the standard error of the mean. None of the exposed groups were found to be significantly different from control samples. Therefore, we are hesitant as to what the biological significance of these intragroup differences could be.

2.4.3 Reactive Oxygen Species scavenging

For both genes measured in this study significant differences among the exposure groups were detected. Pairwise comparisons found three groups differed significantly from one another for *Gpx1*. Expression of *Gpx1* in the 10cGy subacute pr/ch group (1.21 ±0.22) was significantly higher compared to the 10cGy acute pr/ch group (0.65 ±0.1) and the 1.5Gy acute group (0.53 ±0.07), and expression in the 10cGy subacute pr group (1.21 ±0.22) was significantly higher compared to the 1.5Gy acute group (0.53 ±0.07). Again though, none of the exposed groups were found to be different from control samples. Pairwise comparisons found two groups differed significantly from one another for *Sod1*. Unlike the other genes, significant difference was detected between the control and the two subacute exposure groups. The expression values for the control group (1.00 ±0.20) was found to be significantly lower when compared to the 10cGy subacute pr group (10.06 ±3.97) and the 10cGy subacute pr/ch group (10.44 ±1.38). Both of the subacute groups were also significantly higher than all other exposure groups (Fig. 2.2).

2.5 Discussion

Ionizing radiation induces cellular stress including genetic damage that can lead to mutations and genome instability [42]. Without an effective cellular defense system, an organism exposed to IR may suffer from radiation sickness, cancer, or possibly death. IR's ability to both directly and indirectly interact with DNA attributes to it being an important genotoxicological agent. Traditionally, the deleterious effects of IR have been based on studies using cell lines exposed to acute, high doses. The uniqueness of this

study is twofold. First, we accurately administered a subacute (10d) dose (1.0cGy/d) of low level IR continuously. Secondly, the Chornobyl environment allowed us to profile the cellular response in different tissues after exposure of a whole organism (BALB/c *Mus*) versus exposure of specific cell lines. This exposure regime permits for a better estimate of how organisms, specifically humans, respond to low level IR.

Modern molecular biology has made possible a paradigm shift in many divisions of biology. Relevant to this study is the shift in radiation biology [15]. Prior to the development of technological advancements, phenomena like radiation hormesis [16] could not be mechanistically investigated at the cellular and molecular levels. We now have the ability to test the radioadaptive response hypothesis, as well as begin to uncover the cellular and molecular processes controlling this unique phenotype.

We chose to use the BALB/c inbred strain of *Mus* in this study for two reasons. The first is its increased level of radiosensitivity relative to other inbred mouse strains [51,52]. The second reason stems from the findings of Rodgers et al. [53]. Using MN formation as a genotoxicity measurement following exposure to Chornobyl IR, Rodgers et al. [53] suggest that laboratory mice that are chronically exposed exhibit some degree of radioadaption. In fact, Holmes et al. (unpublished) found that when the same mice used in this study were administered an acute or subacute priming dose of 10cGy, they exhibited decreased MN formation subsequent to a challenge dose of 1.5Gy. This result suggested that these primed animals exhibited a radioadaptive response and leads us to question what physiological responses, if any, would elicit such a response. Exposure to a genotoxicant such as IR should increase the levels of DNA damage (via direct or

indirect interactions). These increases would suggest an induction of those enzymes and proteins responsible for DNA protection and damage reversal. Therefore, we hypothesized that exposure of whole organisms to such an agent would elevate the transcriptional products that would be responsible for enzyme and protein production. In addition to the increase of mRNA levels, we hypothesized that those pathways important to survive oxidative damage would be responsible for induction of the radioadaptive response. In order to test these ideas, we measured the absolute number of transcripts from genes coding for proteins or enzymes involved in ROS scavenging, BER, and NER. QRT-PCR was used to measure transcript number for the following genes: *Sod1* and *Gpx1* (ROS scavenging), *Apex1* and *Polb* (BER), and *Ercc1* and *Ercc2* (NER). The genes were chosen because they represent three different levels of DNA protection.

2.5.1 Molecular Responses

At the levels of exposure in this study, genes involved in the NER pathway were not found to be differentially expressed. Our results agree with the concept that the primary function of NER is the correction of bulky DNA adducts such as large molecule adducts and UV-photoproduct crosslinks [54]. Inoue et al. [33] found similar expression patterns for *Ercc1* in TK6 human lymphoblastoid cell after exposure to IR. Therefore, we conclude that the long patch repair mechanism does not participate in DNA repair at low level exposure to IR and that NER likely does not participate in the radioadaptive response.

Significant intragroup differences in *Polb* expression were found; however none of the groups were different from the control. This lack of difference of *Polb* expression agrees with the findings of Inoue et al. [33] where they found no transcriptional response for *Polb* in TK6 human lymphoblastoid cells exposed to IR. These results differ from those by Chen et al. [55], who found an increase in *Polb* after mouse monocytes and fibroblasts were exposed to oxidative stress-inducing agents. Taken together, *Polb* may respond differently, at the transcriptional level, to stress induced by chemicals compared with IR. Our results for *Apex1* expression levels are similar to those in Inoue et al. [33], but contradict the findings of Raman et al. [56] using HeLa cells. We suggest that BER might not be the primary pathway for the repair of DNA upon exposure to low level chronic IR, as well as, it does not participate in the radioadaptive response. Another possibility for the lack of induction of the BER and NER pathways is the type of damage (i.e. small amounts of base damage versus increase amounts of strand breakage) induced by low levels of IR. The inconsistency of results for different cell lines and organismal studies warrants further investigation into why discrepancies exist for the various models and exposure regimens.

Contrasting with the DNA repair pathways is the differential expression of the two genes (*Sod*1 and *Gpx1*) involved in ROS scavenging. Although the transcript levels of *Gpx1* were the highest for any of the genes assayed, we found that no groups differed from controls. Conversely, two exposed groups (both subacute exposed) were found to be different from controls and all other groups for *Sod1*. These results suggest that the level of *Sod1* mRNA in relation to low dose IR exposure is not dose dependent, but rather

dose rate dependent. Furthermore, transcriptional activation of *Sod1* might be playing a role in a radioadaptive response.

ROS are endogenously produced as a result of aerobic respiration, but exposure to IR and alkylating agents increases oxidative stress through the increased production of ROS [44]. ROS that are produced include the superoxide ($O_2^{\cdot-}$) radical, hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$). Sod1 catalyzes the dismutation of $O_2^{\cdot-}$ into molecular oxygen and H_2O_2. When H_2O_2 is in high concentrations, the enzyme catalase (Cat) converts H_2O_2 into water and O_2, but at low concentrations the selenium-containing glutathione peroxidase (Gpx1) converts H_2O_2 to water and O_2 [57]. Hydrogen peroxide has a number of unique properties. One being its ability to serve as an intermediate for the production of more ROS, and a second that in the presence of transition metals (e.g. Fe (II)) H_2O_2 can give rise to the $^{\cdot}OH$ radical via the Fenton reaction [58]. The hydroxyl radical is considered one of the most dangerous and highly reactive ROS. Therefore, an increase in $O_2^{\cdot-}$ may increase the need for Sod1 and result in an increase in the transcriptional level of *Sod1*. The abundant amounts of H_2O_2 would form molecular oxygen and water through the reduction of H_2O_2 by Gpx1 and Cat and could be accompanied by increased transcription of *Gpx1* and possibly *Cat*. However, increased *Gpx1* expression was not observed in this study. It is possible that either increased *Sod1* expression leading to increased enzyme production is not appreciably increasing the intracelluar levels of H_2O_2 or constitutive levels of Gpx1 and possibly Cat are sufficient to detoxify increased levels of H_2O_2 produced by elevated Sod1 activity.

This study documents that the gene expression patterns of *Sod1* exhibited differential expression in a dose rate (i.e. temporal) dependent manner. Subacute (10d) exposures to a total dose of 10cGy induced the transcription of *Sod1*. However, both primed (subacute and acute) and challenged groups exhibited a radioadaptive response (Holmes et al. unpublished.). Using the MN data from Holmes et al. (unpublished) as a measure of a radioadaptive response, we suggest that this response seems to be controlled by different molecular pathways depending on dose rate. Even though *Sod1* transcription appears to participate in radioadaptation at ultra- low dose rates (i.e. subacute), and it does not appear to respond when the same dose is administered acutely. Rather this response and the one elicited following an acute priming dose appear to be driven by different and unknown mechanisms. Future studies are needed to investigate these differences so radioadaptive responses resulting from various exposures and dose-rates can be understood.

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Table 2.1

Sequence and GenBank accessions of each primer/probe set used for QRT-PCR

Gene Name	Accession No.	Forward Primer (5′ to 3′)	Probe (5′ to 3′)	Reverse Primer (5′ to 3′)
Apex1	NM_009687	TTATGGCATTGGCGAGGAA	CAATCACCCGGCCTTCTTGATCATGTT*	TTGAGTCCTTTGTCCTGGTAACAG
Polb	NM_011130	GCTTCCCAGCGAGAAGGAT	ATCCACACAGGAGAATCGATATCAGGTTGATC	CCAAAGATCAGTACTACTGTGGTGTTCT
Ercc1	NM_007948	CAGACCCTCCTGGCTACATTTG	ATCCCTGGAACAGCTCTTCACCGCA	GATCTAGCCTTATGCCCGGG
Ercc2	NM_007949	CTATGTCTATACCCAGAGCCGAATTC	CAAGGCCCGGCTAGAGTATCTGC	AGTTCCAGATCCGAGAGAACGAC
Gpx1	NM_008160	GACTGGTGGTGCTCGGTTTC	TGCCATTCTCCTGGTGTCCGAACTGAT*	TGAAGAGATTCTGAATTCCCTCAA
Sod1	NM_011434	GGCCCGGCGGATGA	TGCCCAGGTCTCCAACATGCCTCT*	TGTGACTGCTGGAAAGGACG
Cdkn1a	NM_007669	GATCCACAGCGATATCCAGACAT	CAGAGCCACAGGCACCATGTCCAA	TGATGTCCGACCTGTTCCG

* indicate that the sequence used was generated from the antisense strand

Table 2.2

Properties of the standard curves for each gene assayed. Each standard curve was derived from purified plasmid DNA for each goi over a 4-log serial dilution. Both the regression equations and correlation coefficients were determined using the software supplied with the ABI 7700 SDS machine.

Gene Name	Linear regression equation	Correlation coefficient
Apex1	y = -3.64x + 43.28	0.94
Polb	y = -4.06x + 47.26	0.99
Ercc1	y = -3.21x + 41.24	0.99
Ercc2	y = -3.02x + 38.02	0.98
Gpx1	y = -3.52x + 43.23	0.99
Sod1	y = -3.42x + 42.89	0.95

Table 2.3.1

Absolute amount of mRNA molecules per µg of total RNA. Values are expressed as mean ±sem (all values x10^3)

Mechanism	Gene	Exposure Groups					
		control	**10cGy subacute pr**	**10cGy subacute pr/ch**	**10cGy acute pr**	**10cGy acute pr/ch**	**1.5Gy acute**
	******Sod1*	85.76 ±17.40	863.13 ±340.54	895.18 ±117.97	668.06 ±35.08	538.28 ±68.12	317.76 ±50.50
Oxyradical Scavenging							
	******Gpx1*	6632.5 ±1314.40	8070 ±1425.35	8450 ±472.33	5507.5 ±411.47	4322.5 ±949.98	3580 ±450.81
	******Polb*	172.07 ±9.55	201.29 ±9.55	180.55 ±20.10	226.21 ±10.76	165.63 ±33.88	101.53 ±23.83
BER							
	Apex1	430.72 ±22.01	371.78 ±45.80	450.97 ±13.72	427.17 ±13.66	342.38 ±46.68	333.09 ±58.46
	Ercc1	1.31 ±0.32	1.19 ±0.14	1.73 ±0.34	1.3 ±0.25	1.29 ±0.20	1.16 ±0.60
NER							
	Ercc2	3.09 ±0.39	2.91 ±0.36	3.51 ±0.22	4.02 ±0.39	2.65 ±0.31	3.03 ±0.34

10cGy subacute exposure in 10days (6.94x10-4cGy per minute)

10cGy acute exposure in ~20minutes (5.0x10-3cGy per minute.)

1.5Gy acute exposure in ~4hours (6.25x10-3Gy per

Table 2.3.2

Relative expression values of experimental exposure groups normalized to control. Values are express

Mechanism	Gene Name	Exposure Groups					
		mean mRNA ±sem					
		control	10cGy subacute pr	10cGy subacute pr/ch	10cGy acute pr	10cGy acute pr/ch	1.5Gy acute
	**Sod1*	1.00 ±0.20	10.06 ±3.97	10.44 ±1.38	7.79 ±0.41	6.28 ±0.79	3.71 ±0.59
Oxyradical Scavenging							
	**Gpx1*	1.00 ±0.2	1.21 ±0.22	1.27 ±0.07	0.83 ±0.06	0.65 ±0.1	0.53 ±0.07
	Polb	1.00 ±0.06	1.16 ±0.11	1.04 ±0.03	1.31 ±0.03	0.96 ±0.11	0.59 ±0.14
BER							
	Apex	1.00 ±0.05	0.86 ±0.06	1.04 ±0.12	0.99 ±0.07	0.79 ±0.1	0.77 ±0.08
	Ercc1	1.00 ±0.24	0.9 ±0.11	1.31 ±0.26	0.98 ±0.19	0.98 ±0.15	0.88 ±0.46
NER							
	Ercc2	1.00 ±0.13	0.93 ±0.12	1.13 ±0.07	1.29 ±0.13	0.85 ±0.1	0.97 ±0.11
Micronucleus Data[1]		1.00 ±0.13	1.02 ±0.05	9.64 ± 2.26	3.16 ±0.3	5.84 ±1.00	15.28 ±0.47

pr stands for priming dose (10cGy)

ch stands for subsequent challenge dose (1.5Gy)

10cGy subacute exposure in 10days (6.94x10-4cGy per minute)

10cGy acute exposure in ~20minutes (5.0x10-3cGy per minute)

1.5Gy acute exposure in ~4hours (6.25x10-3Gy per minute)

*indicates difference at the 95% confidence level

[1]Holmes et al (unpublished)

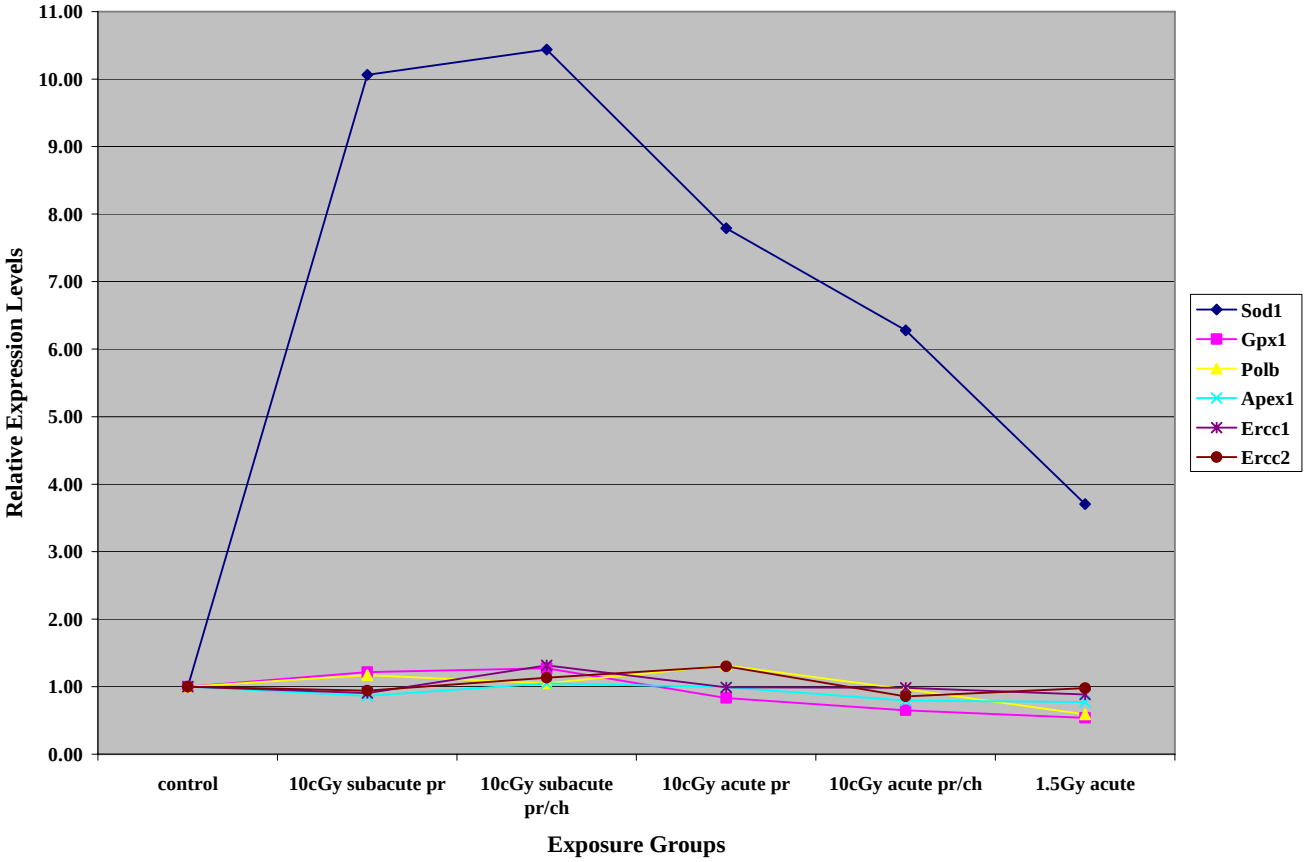

Fig 2.1. Relative expression of all gene assayed in the study. QRT-PCR analysis of mRNA levels of BER, NER, and ROS scavenging genes in mouse liver samples. Expression values were determined from 24 hr post exposure of whole organisms from subacute and acute priming and acute challenge IR. Each point represents the mean of three replicates (error bars not shown).

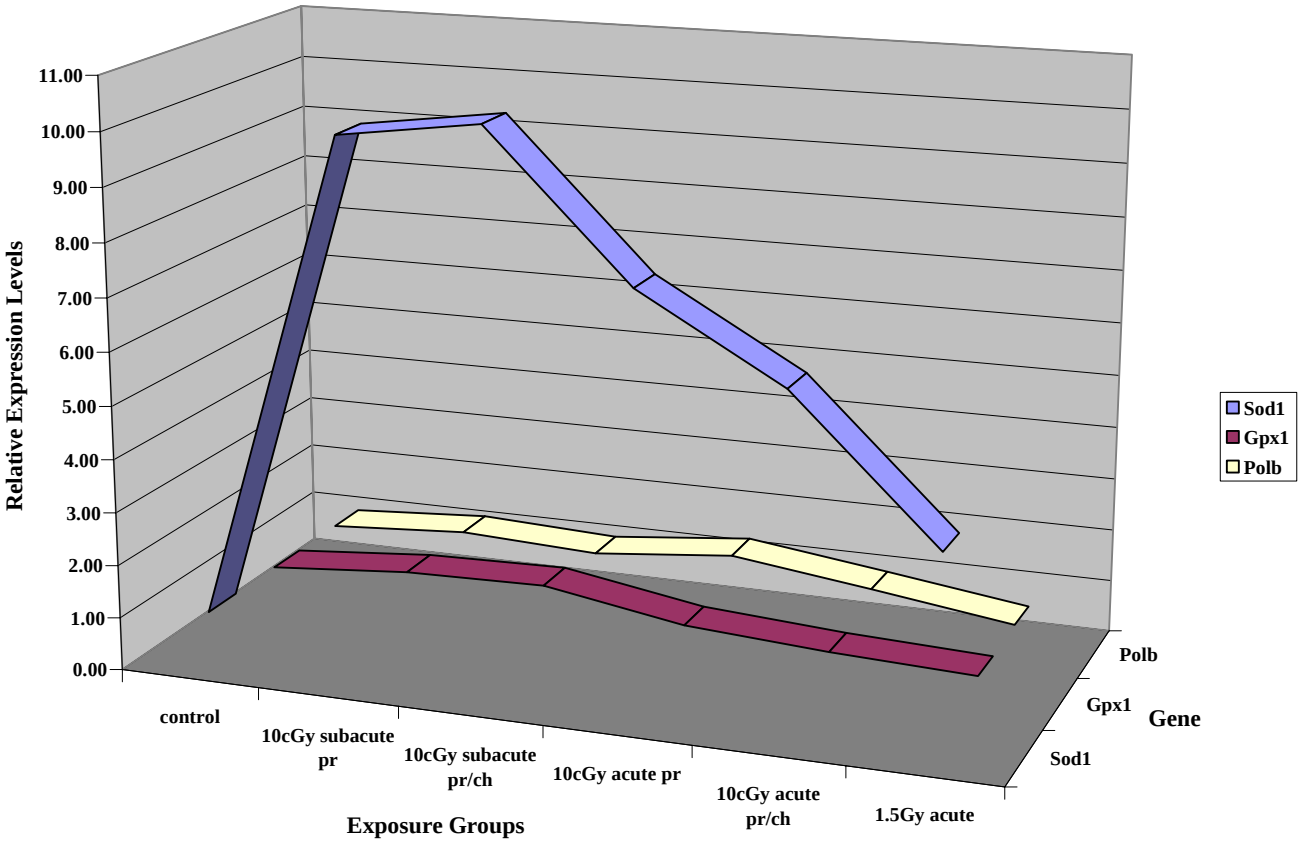

Fig 2.2. Relative expression of three genes found to significant in the study. The results for the Polb, Sod1 and Gpx1 are shown. These genes were found to be significantly expressed using a one-way ANOVA with ranked relative transcript number for each goi at the $p<0.05$ level (error bars not shown).

CHAPTER III

CONCLUSIONS

3.1 Summary and Conclusions

To the authors' knowledge, this thesis represents the first study to measure transcriptional responses after chronic, low linear energy transfer (LET), low dose ionizing radiation (IR) administered to a whole organism. The primary objectives of this study were twofold. The first was to address the effects that chronic, low dose IR has on the transcriptional differences of a subset of genes thought to participate in the integrity of the genome, The second objective encompassed the work in this thesis, as well as the work done by Holmes et al (unpublished). This objective was designed to interrogate, at the molecular level, if any of the genes in these protective pathways had a role in the radioadaptive response (radiation hormesis). The following conclusions are a result of the data presented in Chapter II of this thesis:

1. Chronic, low dose IR found at Chornobyl can induce differential expression of genes involved in various DNA repair and detoxification pathways. These include the genes coding for *Polb* (Base Excision Repair [BER]) and *Gpx1* and *Sod1* (reactive oxygen species [ROS] scavenging) (Table 2.3.1 and Fig. 2.1).

2. Of the differentially expressed genes, *Sod1* was induced at least an order of magnitude greater than the remaining genes relative to control samples (Fig. 2.2).

3. Expression levels of *Sod1* were found to correlate with micronucleus (MN) frequencies used as an end point measurement for DNA damage (Holmes et al. unpublished) (Table 2.3.2).

The results from this study both contradict and support other hypothesizes found in the literature. Inoue et al. [1] used both transcriptional and protein expression levels to study the majority of proteins and enzymes involved in oxidative BER. These authors conclude that acute low levels of IR do not induce any member of this pathway. They further suggest that if the oxidative BER pathway is involved, then they were unable to detect change due to a nuclear localization event or posttranscriptional/translational modification. Likewise, our data suggest that for the time point measured chronic low dose IR, independent of dose rate, does not induce the BER pathway at the transcriptional level. The combination of DNA damage (i.e. chromosomal strand breaks in the form of MN formation) and lack of BER transcription differentiation lends to three hypothesizes. First, the level of change for this pathway is at the protein and/or activity level and not the transcriptional level. Secondly, the window of time (twenty-four hours post exposure) we sampled allowed for the induction and subsequent reduction back to constitutive levels of the BER pathway. Last of all, the production of chromosomal damage might indicate that low level oxidative stress would not induce an excision repair pathway, but a strand repair pathway (Homologous Recombination or Non-Homologous End Joining). Furthermore, this strand damage would have been a result of radicals (produced directly and indirectly) colliding into the phosphate backbone of the helix and not the clustering effect [2].

The most significant results from this thesis suggest that ROS scavenging enzyme genes are transcriptionally induced under this type of treatment. This is apparent by the variable and substantial induction in transcriptional products of *Sod1*. Due to the nature of the stress, we predicted that at least one of the repair pathways and both scavenging genes would be differentially expressed. Instead, only *Sod1* transcription levels were differentially expressed compared to from controls. Furthermore, there is evidence, albeit transcriptional, that *Sod1* plays a role in the radioadaptive response. Holmes et al. (unpublished) measured a radioadaptive response when the priming dose was administered acute, but the response was not measured when the priming dose was subacutely. The highest levels of *Sod1* expression correlate with the subacute prime and prime/challenged MN data. The only inconsistency in the *Sod1* data is that no difference was detected between the prime and prime/challenged subacute groups.

There have been many hypotheses about the roles that ROS scavenging enzymes have in cell protection and survival under oxidative stress [3-6]. A number of studies have measured enzyme activity upon chronic exposure to oxidative stress using cell lines [7,8] and tissue culture [9]. Each of these studies have contradictory results concerning which enzymes, either in cooperation or singly, are acting to protect the cell. The major difference between these studies is the use of various model systems (i.e. cell lines versus tissue types), and this difference is an ongoing struggle within the scientific community. Also, this difference further justifies the work in this thesis, as well as others studies using the Chornobyl environment. This natural laboratory has allowed for most accurate account of how living organisms react to oxidative stress, particularly low dose IR.

For this study, the seminal works found in the literature are those that have specifically looked at Cu/Zn-SOD transcription and activity as it relates to oxidative stress and IR. Because Sod1 has received a vast amount of attention from studying the theory of oxygen toxicity, a number of different conclusions have been made as to how beneficial Sod1 activity is [10]. Such results include elevated levels of Sod1 increase radiation sensitivity in Atm-deficient transgenic mice [11]; a lack of protection from transgenic mice over expressing Sod1 and Cat to gamma irradiation; [Mele et al. unpublished]; the lack of transcriptional expression of the SOD isofamily in both radio-resistant and radio-sensitive mice exposed to acute IR [12]; an increase in Sod1 activity in mice livers exposed to low dose acute [13]; and rats exposed to acute low dose x-rays had an increase in both transcription and activity of Sod1 [14].

The work by Xing et al [15] most likely explains how *Sod1* expression (and subsequent activity) in our experimental design plays a role in the radioadaptive response. Their data suggest that Sod1 can increase cellular susceptibility to oxidative stress by being protective and detrimental, and that Sod1 activity is best represented as a biphasic model. These authors believe that there is a threshold for Sod1 expression in terms of cellular protection, and that below that level Sod1 is beneficial. However, when excess Sod1 is present the cell is destroyed by a high level of H_20_2 and the production of hydroxyl radicals. This model relates to our data by the following hypothesis. Cu/Zn *Sod1* transcription over time leads to an increase amount Sod1 activity and an increase in dismutation byproduct (H_2O_2). Hydrogen peroxide is then reduced either by Gpx1 or Cat, but or data show no differential expression of Gpx1 (Cat unknown). If excess

amounts of H_2O_2 are present and given the opportunity to react by the Fenton reaction with iron (and other transition metals), then the levels of ˙OH radicals increase causing DNA damage and loss of radioadaption. Therefore, *Sod1* transcription appears to participate in radioadaptation at ultra- low dose rates (i.e. subacute), and it does not appear to respond when the same dose administered acutely. Rather this response and the one elicited following an acute priming dose appear to be driven by different and unknown mechanisms.

To better understand the role(s) that Cu/Zn has in the radioadaptive response more work is needed. Assays to measure enzyme levels and activity in liver, as well as, other tissue types would be beneficial. Differentiating between the levels of expression for the other genes used in this thesis among different tissue types is also desirable. This is obvious by the amount of contradictory results found in the literature, and the possible roles that any number of these pathways might have in cancer initiation, propagation and treatment.

3.2 References

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APPENDIX A

List of Chemical Reactions

A.1. Radiolysis of water by IR:

$2H_2O \rightarrow H_2O^+ + e^- + H_2O^*$

$H_2O^+ + H_2O \rightarrow H_3O^+ + OH^\cdot$

A.2. Production of Superoxide radicals ($O_2^{\cdot-}$):

$e_{aq}^- + O_2 \rightarrow O_2^{\cdot-}$

$H + O_2 \rightarrow O_2^{\cdot-} + H^+$

A.3. Dismutation of $O_2^{\cdot-}$ to H_2O_2 by Sod1:

$2O_2^{\cdot-} + 2H^+ \rightarrow O_2 + H_2O_2$

A.4. Reduction of H_2O_2 to H_2O by Gpx1:

$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$

A.5. Fenton reaction:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot-}$

APPENDIX B

Gel Images and Graphs

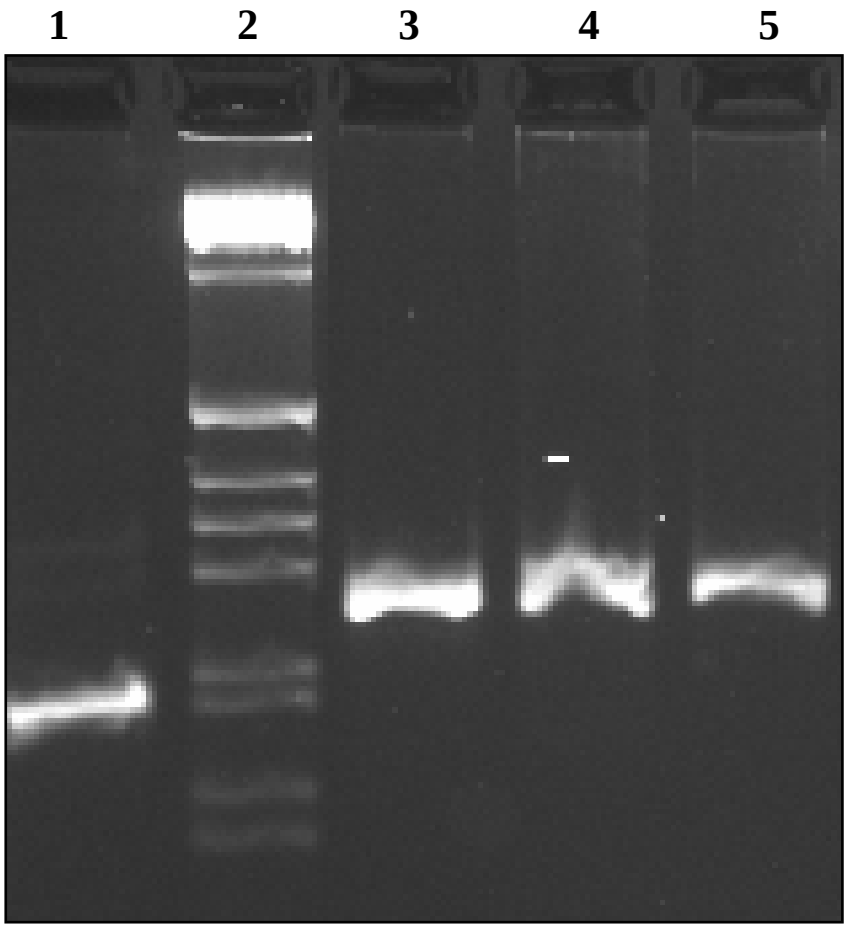

Fig. B.1. Agarose gel (3%, TBE buffer) showing the amplification of four genes amplified out of a plasmid preps using gene-specific primers. Lanes 1,3,4,5 are *Apex1* (201bp), *Polb* (251bp), *Gpx1* (251 bp) and *Sod1* (261bp), respectively. Lane 2 is a 1Kb standard molecular length marker (Invitrogen Corp.).

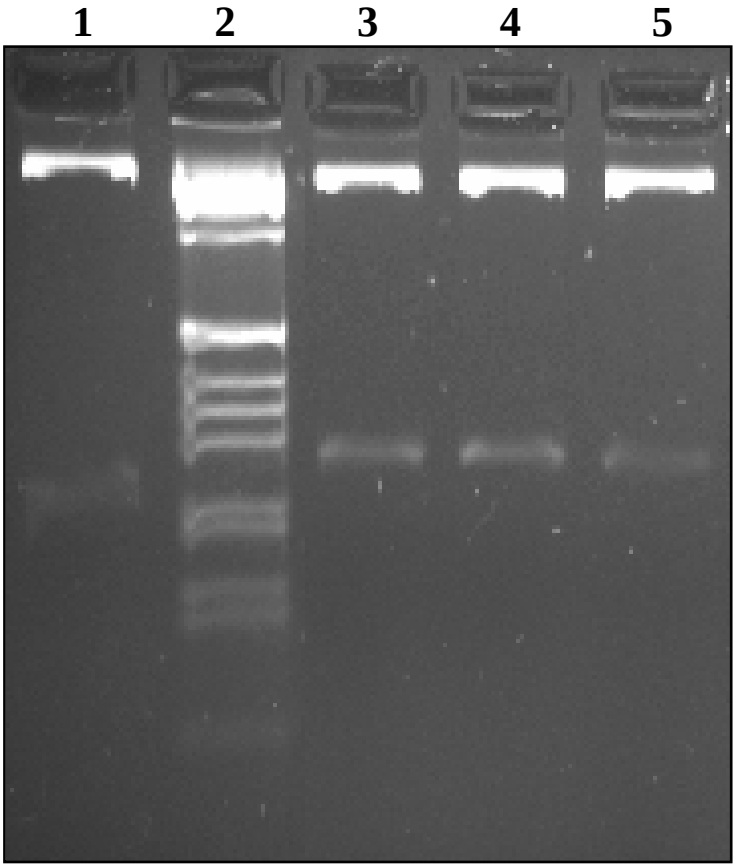

Fig. B.2. Agarose gel (3%, TBE buffer) the showing digested amplicons out of the plasmid using the restriction enzyme *BstZ* I (Promega) after a 3 hour incubation. The top band in each lane is plasmid DNA, and the smaller band in each lane represents cut goi. Notice the uniform increase in size of each band from those in figure B.1. This increase is due to the small amount of plasmid DNA still attached after digestion. Lanes 1,3,4,5 are *Apex1* (201bp), *Polb* (251bp), *Gpx1* (251 bp) and *Sod1* (261bp), respectively. Lane 2 is a 1Kb standard molecular length marker (Invitrogen Corp.).

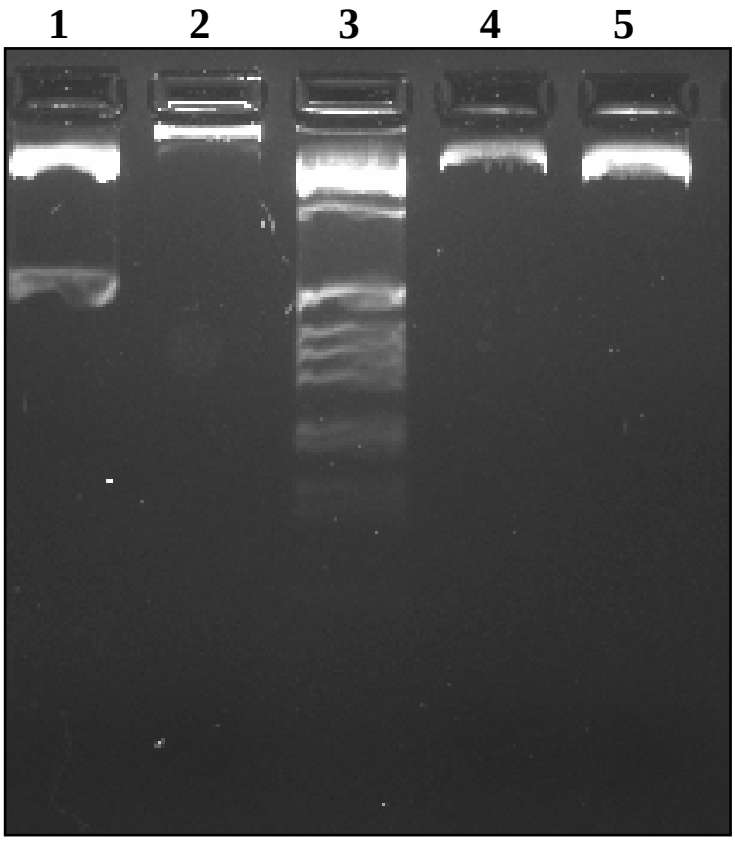

Fig. B.3. Agarose gel (3%, TBE buffer) showing the controls used in the digestion from figure B.2. The restriction enzyme used was *BstZ* I (Promega). The top band in each lane is plasmid DNA. Lanes 1 is cut positive control DNA (256bp) supplied by the manufacturer (Promega), lane 2 is uncut positive control DNA, 4 is cut plasmid DNA without an insert, 5 is uncut plasmid DNA without an insert. Lane 3 is a 1Kb standard molecular length marker (Invitrogen Corp.).

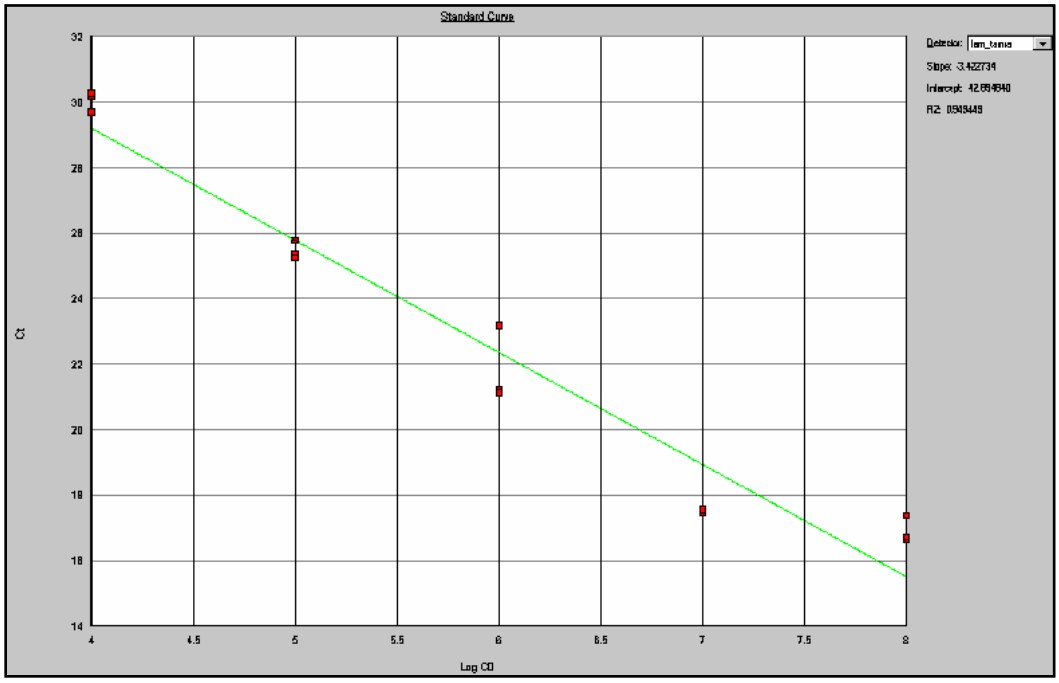

Fig. B.4. The standard curve generated by the software supplied with the ABI 7700 SDS machine for *Sod1*. The y-axis is Ct values, and the x-axis is Log CO values. The slope of this curve is -3.4 and the correlation coefficient is 0.95. This curve was derived from purified plasmid DNA amplified form control samples over a 4-log serial dilution (10^4 to 10^8).

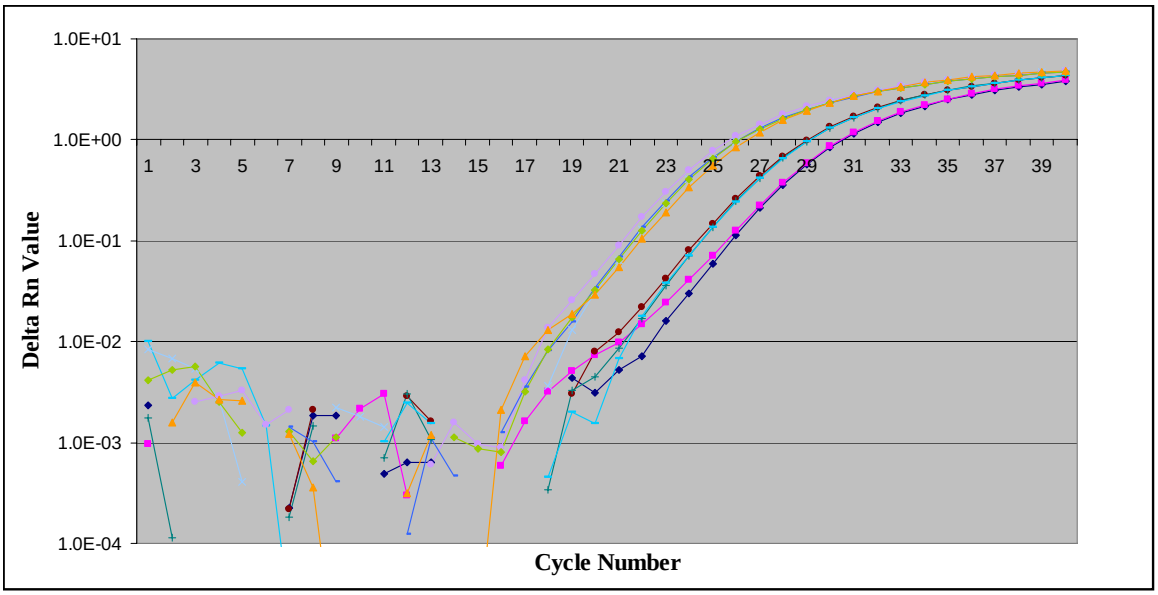

Fig. B.5. The delta Rn versus cycle number plot generated in Excel using values determined by the software supplied with the ABI 7700 SDS machine for *Sod1*. The y-axis is delta Rn values, and the x-axis is cycle number. This plot is on a log scale to represent the 3 phases of PCR (exponential, linear and plateau). The three clusters of lines (from left to right) in the linear phase represent 10cGy subacute prime/challenge, 10cGy prime, and control samples.

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