

**DNA Repair as a Potential Mechanism for Proliferation of
Normal Human Astrocytes after Ionizing Radiation Injury**

by

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Dissertation submitted to the Faculty of the
Neuroscience Graduate Program
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Distribution Statement

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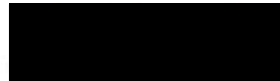
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I would like to thank my friends, family and instructors for their help and support in completing my PhD.

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Michelle Bylicky

October 25th 2018

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ABSTRACT

Mechanisms for Survival and Proliferation of Normal Human Astrocytes after High Dose Ionizing Radiation

Michelle Bylicky, Neuroscience, 2018

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Radiation therapy is commonly used to treat brain malignancies, including primary tumors and metastases. However, due to damage of underlying normal brain tissues, this therapy is linked to long term cognitive impairments, changes in mood, memory and attention. Although neurons have been demonstrated to be sensitive to radiation injury, less research has been done to study normal human astrocytes and their response to radiation injury. Prior research into the ability of astrocytes to respond to various stressors indicates they are capable of utilizing multiple mechanisms to aid in their survival. Research has also demonstrated that astrocytes can provide protection to neurons in their environment. Based on previous studies, we hypothesized that normal human astrocytes would be capable of proliferating after radiation injury, potentially utilizing strong antioxidant responses and DNA repair mechanisms. We chose to compare the survival of normal astrocyte to mesenchymal stem cells (MSC), a well-studied normal human cell type considered to be relatively radiation resistant. We found that astrocytes proliferated after 10 Gy X-ray irradiation, but MSCs underwent accelerated senescence. Neither cell type displayed markers for necrotic cell death or apoptosis. We explored the mechanism of astrocyte radiation resistance. A thorough investigation of antioxidant enzyme responses demonstrated that astrocytes did not activate the redox response transcription factor Nrf2 or significantly upregulate Mn²⁺-superoxide dismutase, catalase, thioredoxin, or cellular glutathione levels. We next investigated DNA repair mechanisms in astrocytes. We found that nonhomologous end joining (NHEJ) and homologous recombination (HR) enzymes were increased in

astrocytes in response to 10 Gy X-ray irradiation. Ku70, a critical NHEJ enzyme was upregulated ~6-fold within 15 min after irradiation. Furthermore, transfecting MSCs with a Ku70 expression vector allowed a transfer of radiation protection of these cells. In conclusion, our findings indicate that astrocytes display extreme radiation resistance, being the only normal mammalian cell type to our knowledge to display proliferative capacity following 10 Gy irradiation. Astrocytes are capable of robust DNA repair responses but without significant changes in antioxidant responses to radiation. Our data suggest that early signal transduction for the activation of DNA repair is a novel mechanism for radiation resistance in astrocytes.

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Chapter 1

I. Introduction: Brain Injury and Cellular Responses

Mechanisms causing damage to the central nervous system (CNS) are numerous and complex, ranging from those associated with age-related neurodegeneration to the acute mechanisms of traumatic brain injury (TBI), ischemic stroke and radiation exposure. In all cases, however, astrocytes play a central role in the compensatory responses that nature has designed to protect against the loss of terminally differentiated, non-replicating neurons.

Like aging, acute injuries can result in a long-term progression of pathogenic changes that alter brain functions for years afterwards (124). Specifically, following an initial TBI, secondary events can occur that extend both the area of as well as the intensity of the injury. Loss of vascular integrity resulting in a breakdown of the blood brain barrier (BBB) causes exposure of the CNS to exogenous immune cell types, abnormal levels of cytokines and other cellular mediators and ionic disruption that can lead to a cascade of pathogenesis (43; 189; 247; 370; 372; 422). Loss of BBB integrity is also observed following ischemic stroke, radiation exposure, and in certain neurodegenerative disorders, due to the loss of neurovascular functions (129; 182; 232; 259). Secondary damage due to vascular and metabolic imbalances leads to increased glutamate release and subsequent excitotoxicity, mitochondrial dysfunction and excessive production of reactive oxygen species (ROS), as well as disruption of glucose metabolism/release, and further alterations of ion concentrations (303; 323; 435). Glutamate is thought to be a central mediator in this constellation of secondary injury events. An increase of extracellular glutamate activates N-methyl-D-aspartate receptors (NMDARs) in neurons, allowing calcium influx (285). The resulting calcium excitotoxicity affects mitochondrial functions, causing a disruption of energy balance and production of excessive ROS, ultimately causing acute necrotic cell death and/or delayed apoptotic cell death (14; 285; 318; 402). Further damage can occur due to prolonged neuroinflammatory and related immune responses that exacerbate the injury (58; 215) and may underlie long-term pathogenesis.

Although the initiating events of CNS damage may differ, similar patterns of secondary injuries are observed (1; 232; 465). This implies that understanding of the mechanisms underlying the CNS response to any injury may allow the development of treatments for other diseases or disorders.

Historically, treatments for acute or chronic damage to the nervous system have focused on neuronal responses and survival. This was due to the neurons' perceived importance in cognition and their post-mitotic status which prevents their replacement when damaged (142; 157). However, more attention is now

being paid to the impact of non-neuronal cell types that function to mitigate damage and promote neuronal function and repair following tissue injury. In recent years, there has been a greater appreciation of the role of astrocytes in brain function and survival. The perceived value of astrocytes has risen from their initially defined role of “brain glue” to current findings that astrocytes are critical for modulating synaptic transmissions, managing energy metabolism, water and ion homeostasis, and protection of neurons from oxidative stress under both mild and catastrophic conditions (30; 192; 309; 315; 386). Here, we review the role of astrocytes in the protection of neurons from the consequences of initial and secondary injury processes (Fig. 1)

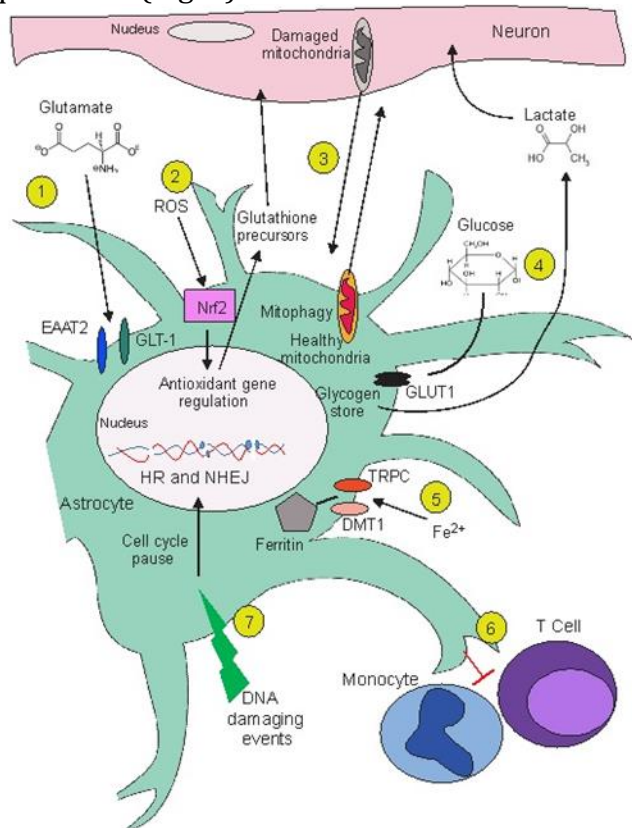


Figure 1. Schematic of mechanisms of neuroprotective effects of astrocytes. There are at least seven distinct mechanisms by which astrocytes protect neurons from damage

1) Protection against glutamate toxicity occurs through astrocyte uptake of extracellular glutamate through the excitatory amino acid transporter 2 (EAAT2) and the glutamate transporter 1 (GLT-1). 2) Protection against redox stress through the activation of Nrf2 and regulation of antioxidant genes; protection of the neurons is also advanced by the export of glutathione precursors to help neurons synthesize glutathione. 3) Mediation of mitochondrial repair mechanisms by which astrocytes received damaged mitochondria from neurons for mitophagy and in return deliver healthy mitochondria to the neurons. 4) Protection against glucose-induced metabolic stress, which required astrocytes to take up extracellular glucose for storage as glycogen; the glycogen can be released to neurons as lactate for their metabolism at a later time. 5) protection against iron toxicity, in which astrocytes take up free iron from the extracellular space via transient receptor potential canonical (TRPC) channels and divalent metal transporter (DMT1); the iron is then stored in complex with ferritin. 6) Modulation of the immune response in the brain occurs by astrocyte inhibition of both T cell and monocyte activation; the mechanisms for these actions are not fully known. 7) Maintenance of tissue homeostasis in the presence of DNA damage, where astrocytes can effectively repair their DNA through both homologous recombination (HR) or non-homologous end joining (NHEJ), following pause of the cell cycle.

II. Astrocytes: Origin, Morphology and Activation

Astrocytes are members of a larger family of non-neural, glial cells which include oligodendrocytes and Schwann cells, both of which form myelin, and microglia, which are specialized macrophages that aid in immunity. Astrocytes, and the other cells of the glial family are defined, in part, by their inability to produce an action potential upon stimulation (293). Astrocytes are embryonically derived from progenitor cells of neuroepithelium which differentiate to function in their traditional roles as support cells. They provide nutrients and remove end products of metabolism (454). Astrocytes exhibit spongiform morphology, with processes in close contact with neuronal synapses and other components of the CNS (456). Recent advances in our understanding of astrocytes, discussed below, reveal the astrocyte to have essential roles in synaptic function, and nervous system repair (260; 291)

Astrocytes, the most abundant nonneuronal cell type in the brain, consist of two main subclasses: protoplasmic and fibrous (311). Protoplasmic astrocytes display a stellate appearance in the grey matter, and fibrous astrocytes primarily exist as long, thin, fibrocyte like-cells in the white matter of the CNS (371). Each subtype has a distinctive profile of gene expression, as reflected in their expression of specific receptors and proteins (258; 270). These two types of astrocytes display differences in their development and their expression of receptors and proteins (258; 270). However both subtypes express glial fibrillary acidic protein (GFAP), the main astrocytic intermediate filament, as well as calcium binding S100B protein (S100B) (48; 359).

Activation of astrocytes can occur in response to a variety of injuries to the brain and in response to inflammation or pathological neurodegeneration (311). The activated state, astrogliosis or reactive astrogliosis, is believed to have multiple functions in the brain and has been the topic of controversy for over 20 years (311; 456; 481). While in some cases, astrocyte activation has been linked to repair and return to homeostasis, in other cases astrocyte activation has been related to the formation of scar tissue and the inhibition of neuronal axon outgrowth (311). Induction of the reactive state of astrocytes can occur through multiple mechanisms including: the presence of amyloid beta peptides (A β peptides) to neuronal damage or neurodegeneration, the release of pro-inflammatory cytokines by microglia and macrophages, or in response to acute injury to cells of the CNS (166; 300; 475). The time course of astrocyte reactivity is heterogenous and may depend on the location and type of injury (407). In certain murine models of mild CNS injury, astrocyte reactivity is transient (408). However, other studies indicate long lasting increases in astrocyte reactivity occur after either moderate or severe CNS injury from TBI or by radiation (70; 437). Mild perturbations of the CNS can be adequately repaired and homeostasis can be maintained with cooperation among glial cells. However,

under more severe conditions astrocytes remain in a state of reactivity indicating an inability to adequately repair. Similarly, astrocytes in post-mortem Alzheimer's patients appear to maintain themselves in a continuous reactive state, consistent with chronic inflammation observed in this disease (369). Thus, astrocyte reactivity persistence may indicate the presence of unresolved dysfunction in the CNS.

The primary alterations in the transformation of normal astrocytes to reactive astrocytes include hypertrophy of their main cellular processes, proliferation and alterations in protein expression (23; 75; 456). Fibrous and protoplasmic astrocytes display differences in the length of their processes following mechanical injury. In a murine model of axonal injury, fibrous astrocytes displayed condensed, retracted processes (408). In contrast, protoplasmic astrocytes displayed increased length and branch complexity of their processes after injury (183; 456). This may be a reflection of their functions within the brain, but more research is required to understand the significance of these changes. Of greater interest is their different sensitivities to damage. Research of brain ischemia and cortical lesions has shown that protoplasmic astrocytes may either die or differentiate into fibrous astrocytes after brain injury caused by ischemia and cortical lesions (183; 251). This suggests that the differences between astrocyte types is fluid and dependent on environmental conditions. Significantly, protoplasmic astrocytes promote the differentiation of neural stem cell (NSC) into neurons via their secretion of brain derived neurotrophic factor (BDNF) secretion (241). Also, while both protoplasmic and fibrous astrocytes aid in motoneuron neurite outgrowth, protoplasmic astrocytes produced factors in the extracellular matrix that aided in axonal growth of V2a interneurons, while extracellular matrix produced by fibrous astrocytes had more factors that inhibited axon growth of V2a interneurons, suggesting that the actions of the protoplasmic and fibrous astrocytes are selective for specific neurons (425). Thus, the differentiation or death of protoplasmic astrocytes may have a significant impact on replenishing neurons and re-growth of neuronal axons in the CNS following injury depending upon the site of injury.

Reactive astrocytes perform a variety of tasks in response to injury which can be beneficial or deleterious to the surrounding neurons, depending on the circumstances of the injury. Reactive astrocytes can form scars after CNS trauma. In some cases scars can be viewed as initially beneficial since they limit immune cell invasion, decrease neuro-inflammation, and maintain ion homeostasis in damaged brain tissue (51; 227). Ablation of proliferating reactive astrocytes after moderate closed cortical impact (CCI) in mice produced increased inflammation and neuronal death, suggesting that the overall value of astrocyte reactivity is for the protection of neurons post-injury (280). Evidence indicates interference in the development of the astroglial scar results in increased neuronal cell death and decreased modulation of inflammation (451). However there is controversy over its long term impact of the scar tissue on repair and functional recovery (290; 336). Prior evidence suggests that glial scar formation prevents or inhibits axonal regrowth of neurons (231). This has been attributed to astrocyte expression of chondroitin

sulfate proteoglycan, a known inhibitor of neuronal axons during embryogenesis (266). However, in murine models where astrocyte scar formation is impaired there was demonstrated to be less neuronal axon regrowth and remodeling (11; 242). Using transgenic murine models, one research group demonstrated that the formation of an astrocytic scar actually improved neuron axonal regrowth, provided that brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) were added (11). Together these studies suggest that, in contrast to initial hypotheses, the presence of astrocytic scars alone do not prevent axonal regrowth, but rather that the lack of adequate growth factors may be the problem.

The beneficial nature of gliosis may become detrimental when damage is too severe for homeostasis to be re-established. For the purposes of this review we will focus mostly on the mechanisms by which astrocytes protect neurons under basal conditions and after injury. We will review the astrocyte's ability to collect and transport vital nutrients, neurotransmitters, and ions in the brain, to release antioxidants during redox stress, to repair mitochondria and DNA after injury.

III. Mechanisms of Astrocyte Protection of Neurons

A. Astrocyte defense against glutamate toxicity

Glutamate is the most abundant excitatory neurotransmitter in the brain, with actions mediated through a diverse family of receptors to modulate synaptic transmission and aid in plasticity (194; 268). In normal synaptic communication, neurons release measured quanta of glutamate into the synaptic cleft. However, following physical trauma, radiation exposure, and in chronic neurodegenerative disorders, including Alzheimer's disease, excessive glutamate is released or fails to be taken up for days after injury (61; 171; 357; 472). The cause of glutamate dysregulation in TBI and neurodegeneration is not completely understood, but elevations in free glutamate are linked to poor clinical outcome (61). Recent evidence indicates that glutamate is released by dying or damaged neurons, possibly via the cystine glutamate antiporter (391; 415). Excessive extracellular glutamate leads to excitatory neuronal cell death attributed to overstimulation of NMDAR and subsequent overproduction of ROS in neurons (2; 301).

Under conditions of normal neuronal activity, astrocytes are responsible for the uptake of excess glutamate from the synaptic cleft. Following uptake, astrocytes process the glutamate into glutamine and return it to neurons for re-use (348). Consistent with this role, astrocytes highly express the excitatory amino acid transporter 2 (EAAT2) and the glutamate transporter 1 (GLT-1) which are responsible for the active uptake of glutamate (123). Glutamate homeostasis is a critical function of astrocytes in the brain, as demonstrated experimentally by the neurotoxicity that results from inhibition of the astrocyte glutamate transporters (344; 421).

Following tissue injury, astrocytes can actively take up excessive glutamate from the extracellular (non-synaptic) space and buffer its potential excitotoxic actions on neurons. The reduction of extracellular glutamate by astrocytes decreases the subsequent lesion size, mitigates neuronal mortality, and improves CNS function post-injury (228).

Under conditions of severe injury, extent of damage and types of injury to the astrocytes themselves can impact the ability of astrocytes to protect neurons from glutamate toxicity (297). For example, astrocytes injured by radiation or more severe forms of TBI display reduced glutamate uptake activity as compared to the uninjured condition, allowing increased neuronal uptake of glutamate, and a greater extent of neuronal cell death and seizure activity (56; 297; 357). The mechanism for radiation inhibition of astrocyte uptake of glutamate is thought to be related to ROS inhibition of the astrocytic glutamate transporter via oxidation of protein sulfhydryl groups critical for function (8; 439). At least three potential mechanisms have been proposed for increased extracellular glutamate expression, and subsequent excitotoxicity in TBI (96; 117). These mechanisms may occur in tandem and are not exclusive. In the first potential mechanism, tumor necrosis factor- α (TNF- α), the pro-inflammatory factor released during brain damage, downregulates glutamate uptake by astrocytes and suppresses conversion of glutamate to glutamine (489). In the second possible mechanism, TBI- and ischemia-induced efflux of glutamate from injured astrocytes may occur in response to thrombin, which is released after BBB disruption (325). In a third potential mechanism, ischemia and glucose deprivation may induce altered glutamate release by astrocytes (60). Under normal circumstances glutamate uptake occurs against its gradient, and must be actively transported into the astrocyte via EAATs. However, under acidic conditions this transporter is reversed and expels glutamate (60). Thus, more severe neuronal injuries and/or chronic disruptions lead to cell death when the astrocytes themselves exacerbate glutamate imbalance as they fail to maintain homeostasis.

B. Redox stress reduction by astrocytes

Basal levels of ROS in the brain can result from normal cellular functions and metabolic activity. While the production of ROS is a natural consequence of mitochondrial respiration, over-production of ROS following injury exceeds the capacity of natural cellular antioxidant mechanisms, resulting in the pathological modification of proteins, lipids, and nucleic acids (144; 185; 381; 403). To combat these processes, the brain utilizes multiple pathways for antioxidant defense including: superoxide dismutases (SOD), catalases and glutathione detoxification pathways, and thioredoxin detoxification pathways (213). These mechanisms are utilized to different degrees by different cell types.

A hallmark of glutamate excitotoxicity is increased intracellular redox stress. Excessive glutamate activation of NMDAR causes Ca^{2+} influx into the cytosol of neurons (73). The excessive intracellular Ca^{2+} can translocate into the mitochondrial matrix where it leads to the collapse of mitochondrial membrane potential with loss

of ATP production, and ultimately, cell death (2; 465). To prevent this, many cell types upregulate uncoupling proteins (UCPs), which aid in removal of intracellular Ca^{2+} and prevention of Ca^{2+} entry into the mitochondria (62; 239). UCPs decrease the levels of hydrogen protons in the mitochondrial intermembrane space, and therefore the mitochondrial electrochemical proton gradient, by leaking them into the mitochondrial matrix (207; 256). Since the electrochemical proton gradient is necessary for ATP synthase function, a decrease in hydrogen protons decreases ATP production (284). The increase of hydrogen protons in the mitochondrial matrix also causes diminished entry of positively charged molecular calcium (164). In the short term, the activity of UCP may benefit the neurons for immediate survival, but in the long term it is detrimental, since this process inhibits ATP production (5; 334). Catastrophic calcium entry due to acute or chronic brain injury can overcome the UCP system, leading to the production of ROS which causes further mitochondrial dysfunction and cell death (40; 210; 405; 465). This mitochondrial membrane depolarization and increase in ROS induced by high Ca^{2+} levels can cause apoptosis by facilitating the release of cytochrome C through the mitochondrial transition pore and activation of caspase 3 (49; 252).

Astrocytes normally display a higher basal level of glutathione (0.91 ± 0.08 mM) as compared to neurons (0.21 ± 0.02 mM), suggesting that under normal conditions, they are capable of detoxification of higher amounts of reactive oxygen and nitrogen species (167; 410). Astrocytes also have a greater inducible expression of glutathione in response to oxidative stress (103; 173). The ROS-inducible transcription factor nuclear factor E2-related factor 2 (Nrf2) regulates the glutathione system, as well as the thioredoxin system and SOD (327; 416; 484). Under basal conditions Nrf2 is constitutively produced and ubiquitinated for degradation by binding to the Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm (438). Under conditions of increased oxidative stress, Keap1 binding to Nrf2 is inhibited (483), allowing Nrf2 to escape degradation and instead to translocate to the nucleus where it interacts with the antioxidant response element (ARE) in gene promoters that activate the expression of oxidative stress response genes. Previous research indicated that astrocytes display higher basal and stimulated levels of ARE binding by Nrf2 as compared to neurons (32).

Interestingly, Nrf2-induced expression and downstream upregulation of antioxidant defenses in astrocytes confers enhanced resistance to oxidative stress for both astrocytes and neurons (179; 373). As stated above, the enhanced Nrf2 within astrocytes effectively upregulates antioxidant genes for the protection of the astrocytes (223). However, Nrf2 expression in astrocytes was also demonstrated to increase neuronal survival in a murine model of Amyotrophic Lateral Sclerosis (ALS) and in vitro in acute hydrogen peroxide exposure (177; 432). The mechanism by which Nrf2 up-regulation in astrocytes allows protection of neurons is complex and further research is required for a full understanding. However, two mechanisms have been proposed for astrocyte protection of neurons in response to ROS. In the first mechanism, Nrf2 induces glutathione secretion from astrocytes into the

extracellular matrix where it is cleaved to one of its precursors (CysGly, γ GluCys or cysteine) which are then taken up and used by neurons for glutathione re-synthesis for their own detoxification (1; 99; 355). In the second mechanism, the increased levels of Nrf2 induces the up-regulation of the EAAT3 in astrocytes. As describe above, this neurotransmitter transporter is critical for the removal of extracellular glutamate which after injury can induce neuronal excitotoxicity. Thus, the removal of extracellular glutamate protects neurons via a second independent mechanism (106). This redox buffering capacity of astrocytes was demonstrated to be necessary for neuronal homeostasis under normal basal conditions (365).

C. Astrocyte defense against mitochondrial dysfunction in neurons

As describe in Section 4, brain injury can lead to Ca^{2+} -induced mitochondrial dysfunction, including over production of ROS, loss of mitochondrial membrane potential and pH gradient, and failure to generate required amounts of ATP (17). Recently, the transfer of mitochondria from one cell type to another has been described as a mechanism for the replacement and repair of damaged mitochondria. The benefits of mitochondrial transfer were initially shown in cell culture studies in which human mesenchymal stem cells (hMSC) repaired the aerobic respiration of A549 transformed lung epithelial cells that contained mutated mitochondria (392). Mutant A549 cells which received mitochondria from hMSCs displayed improved ATP production, increased lactate uptake, and higher levels of oxygen consumption, a marker of electron transport chain activity (392). This study provided compelling evidence for mitochondrial transfer and demonstrating the benefits of this activity as an effective means for protecting vulnerable cell types. The mechanisms by which mitochondria and other organelles are trafficked between different cell types are still not well understood. One proposed mechanism for organelle transfer involves the creation of tunneling nanotubes (TNT)s (445; 448). TNTs are created by a cell after it is subjected to stress and has been demonstrated to occur during neuronal development (411; 448). Of special interest, neurons are capable of guiding the formation of astrocyte TNTs during periods of high synaptic activity and thus, high energy demand (411). Transference of healthy mitochondria from astrocytes to neurons in a murine model of stroke was observed *in vivo* (152). Further, it was noted in this model that astrocytes only transferred healthy mitochondria to damaged neurons in a calcium-dependent manner, suggesting neuronal activity was necessary for transference (152). Conversely, in a separate model, it was demonstrated that retinal ganglion cells are capable of shedding damaged mitochondria and that the shed mitochondria were shown to be taken up by adjacent astrocytes where they underwent mitophagy (84). Thus, evidence suggests that mitochondrial transfer provides a means to deliver healthy mitochondria to injured neurons, and for the elimination of damaged mitochondria involved in the over-production of ROS.

D. Astrocyte protection against glucose-induced metabolic stress

The brain is highly metabolically active, utilizing fully 25% of the body's glucose (30). Accordingly, efficient glucose uptake and distribution throughout the brain is critical for cognition and survival. Disruptions in the delivery of glucose to the brain induces neuronal cell death. Under normal conditions, the BBB acts as a selective barrier to control entry of glucose into the brain; however, this barrier is often disrupted in brain injury (151). Endothelial cells of the BBB and astrocytes express glucose transporter 1 (GLUT1), a facilitated glucose transporter, to aid in glucose entry into the brain (276). Astrocytic endfeet encircle endothelial cells of the blood brain barrier and mediate the uptake of glucose (118; 181; 264). Once past the BBB, glucose is taken up by all cell types of the CNS. In astrocytes, glucose is converted into glycogen and stored (59). In times of need, astrocytes mobilize their glycogen to make lactate available for neuronal use. This is especially important when energy demand is high but neuronal glucose supply is low, such as under hypoglycemic conditions (313; 414; 455). While neurons express glucose transporter 3 (GLUT3), a high affinity glucose transporter, they have been shown to prefer lactate as an energy substrate during times of high synaptic activity (44; 93; 462). Glutamate induces the rapid uptake of glucose in astrocytes. Because extracellular glutamate is released during neurotransmission, this indicates that glutamate-stimulated glycogen production in astrocytes is linked to neuronal activity (156; 243).

Insulin and insulin like growth factor 1 (IGF-1) increase glycogen storage in many cell types of the body but have no impact on astrocytes, since they do not express the insulin/IGF-1 responsive glucose transporter 4 (GLUT4) which is expressed primarily in adipose and striated muscle (277). However, selective ablation of insulin receptors in mouse astrocytes in vivo results in a significantly lower cerebral glucose levels, suggesting that astrocytes are also responsive to metabolic conditions in the rest of the body (128). This indicates a central role for astrocytes in monitoring neuronal metabolic activity and maintaining whole brain energy balance in a manner that is responsive to insulin release in the blood, but in a manner that is different from the regulation that occurs in other tissues.

Acute brain damage, including radiation, TBI, and ischemic stroke can produce sudden damage to the BBB which can lead to a disruption in the supply of glucose as well as imbalances in extracellular ions. Of particular importance in BBB permeability is increased extracellular potassium that must be removed from the extracellular space (195; 286). The increase in extracellular potassium may be due to multiple factors including direct cellular injury and secondary mechanisms that compromise potassium buffering by astrocytes (15; 33; 419). While glial cells are capable of buffering normal increases in extracellular potassium, they become overwhelmed under conditions of more severe injuries and the potassium overload can cause death of neurons (15; 27). Both initial disruption of the BBB and the need to maintain ion homeostasis produce a rapid depletion of glucose and metabolic emergency (195; 286; 477). Hypo- and hyperglycemic conditions both induce greater cell death in neurons than astrocytes (10; 137; 352). Astrocyte survival in

hypoglycemic conditions may rely on several factors including glycogen storage within the astrocytes, alternative energy metabolism of fatty acids, and utilization of antioxidant systems to manage increased oxidative stress (102; 139; 307). In vitro research also demonstrates that astrocytes can improve neuronal survival under situations of glucose disruption by upregulating their respective monocarboxylate transporters (MTCs) which transfers lactate from astrocytes to neurons (127; 417). While astrocytes may increase their release of lactate after TBI, there is some controversy regarding the possible benefit of this release, as neurons appear less capable of taking up the lactate depending on their level of damage (216; 337). Increased release of lactate by astrocytes may contribute to lactic acidosis which can exacerbate ischemia induced oxidative stress (346). High lactate levels in the cerebrospinal fluid (CSF) of TBI patients have been linked worse clinical outcomes, which is blamed on neuronal mitochondrial dysfunctions, neuronal inability to uptake lactate, and subsequent necrosis in the brain (395). Increased lactate was also seen in patients after they had seizures caused by severe TBI, with astrocytes potentially releasing lactate as an energy source for these overactive neurons (12; 434). Under normal homeostasis and conditions of mild to moderate injury, astrocytes act to maintain neuronal survival by providing energy resources and maintaining the energy balance of the extracellular environment of the brain, but these actions can produce further damage if the CNS is already severely compromised.

E. Astrocyte mitigation of iron toxicity

Astrocytes are responsible for the transfer through the BBB of a variety of nutrients required for brain tissue homeostasis, including iron (39). Iron performs multiple functions within the brain, serving as an essential cofactor in several enzymatic reactions including those involved in the remyelination of neurons after injury (72; 366). Iron levels are tightly regulated in the brain via specific transport proteins and metabolic pathways, but dysregulation can occur under pathological conditions (125). Iron deficiency in the brain, due to causes such as dietary insufficiency or anemia, can produce cognitive impairments (35; 478). However, an excess of iron, due to TBI, hemorrhagic stroke or neurodegenerative diseases, causes neurotoxicity (125; 478; 485).

When present at high levels, ferrous iron (Fe^{2+}) interacts with hydrogen peroxide to generate toxic levels of hydroxyl radicals through the Fenton reaction (116). Neuronal susceptibility to iron-mediated necrotic, apoptotic, and autophagic cell death is likely due to their inability to effectively buffer the resulting ROS to combat redox stress (64). This is in marked contradiction to astrocytes which are highly effective at detoxification of ROS (209; 333) (79). Excess iron induces lipid peroxidation, protein and DNA oxidation, and cell death in neurons (125; 161). Disruptions in free iron handling within the CNS have been observed after acute injuries such as TBI as well as in chronic neurodegenerative disorders (446; 460). Iron and other transition metals within the brain bind to A β a peptide that accumulates in Alzheimer's Disease, causing greater neuronal death and toxicity

than A β alone (29; 132). Similarly, in a murine model, it was demonstrated that TBI results in an increase in iron deposition in the brain starting as early as four hours post-injury and extending for at least three weeks after initial damage (302). These findings support the proposal that acute deregulation of iron homeostasis may participate in long-lasting pathogenic effects that underlies neuronal damage and death (249; 460) with associated cognitive impairment.

Astrocytes utilize several distinctly different mechanisms to directly regulate free iron in the CNS. As discussed above, astrocytes utilize parallel mechanisms including increased expression of Nrf2, glutathione and catalase to combat redox stress that is likely one of the consequences of excessive free iron (79; 234). Astrocytes may also protect neurons from iron-induced cell damage under normal and pathological conditions by sequestering free iron through transient receptor potential canonical (TRPC) channels and divalent metal transporter (DMT1), respectively (77; 312). TRPC channels are best known for their proposed role in calcium influx after activation, though they transport multiple cation types across the cell membrane (68; 389). In a cell culture model, it was demonstrated that overexpression of TRPC6 can increase basal levels of intracellular iron as well as increasing iron presence after stimulation, suggesting that iron transfer through TRPC channels may occur under basal conditions (279). In contrast, DMT1 expression is controlled by pro-inflammatory cytokines. The pro-inflammatory cytokines tumor necrosis factor alpha (TNF- α), lipopolysaccharide, and interleukin-6 (IL-6) increase DMT1 expression in astrocytes while simultaneously decreasing ferroportin 1 (FPN-1) expression (329; 431). FPN-1 is an iron efflux transporter so the result of this activity then is to increase total iron uptake and storage in astrocytes after injury. Excess iron in the microenvironment of astrocytes upregulates the expression of ferritin, a rapidly inducible protein which binds and neutralizes ferrous iron, thus preventing its effects on oxidative stress (332). Ferritin functions by first converting ferrous iron to its less reactive state of ferric iron then nucleating this ferric iron (Fe³⁺) and storing it within ferritin's iron core (149). Together, the upregulation of iron transporters plus the upregulation of ferritin allows astrocytes to act as iron stores, resulting in reduced free ferrous iron in the microenvironment where it may contribute to neuronal toxicity.

F. Modulation and regulation of immune responses in the CNS

Immunological activity in the CNS is relevant for the prevention of pathogenic infection as well as responses to injury such as stroke and TBI when the BBB is compromised (193). Astrocytes play a complex role in responding to such CNS insults, and their inflammatory status as well as regulation of immune cells is controversial. Astrogliosis, is the defensive reaction of astrocytes to trauma, ischemic damage, inflammation, or pathological neurodegeneration (311). During astrogliosis, astrocytes increase at the site of the lesion, exhibit altered morphology with increased thicknesses of cellular processes, and display changes in gene expression related to altered function (311; 456). The increase in astrocytes at the site of injury is believed to be due to proliferation of astrocytes adjacent to the

lesion, and not due to astrocyte migration from neighboring areas of the brain (311). Astrocytes can be activated to a pro-inflammatory or anti-inflammatory phenotype with an associated alteration in their secretome (80; 108; 202; 473). The overall “defensive response” of astrocytes following injury is highly complex and has been shown in some studies to exacerbate inflammation while generally, it is found to mitigate it (311).

The pro-inflammatory activation of astrocytes and their expression of pro-inflammatory cytokines are dependent upon the nature of the stimulation they receive and their location in the brain (385). The activation of pro-inflammatory astrocytes can occur through interactions with microglia, the response to cytokines such as IL-1, IL-6, oncostatin M, leukemia inhibitory factor (LIF), and transforming growth factor α (TGF- α), in response to overt physical damage following brain injury, from interaction with A β plaques, or as a result of calcium-dependent phosphatase calcineurin activation (287; 311; 481). Under normal circumstances astrocytes aid in the morphological and physiological development of neurons and synaptogenesis (388). However, cell culture studies suggest that inappropriate activation or over-activation of astrocytes can induce the production of TNF- α and other cytotoxic factors that inhibit neurite growth and synapse formation (282). Additionally, exposure of astrocytes in cell culture to cytokines, such as interferon- γ (IFN- γ), can induce their production of nitric oxide which drives the formation of its toxic metabolite, peroxynitrite (399). In cell culture, this does not harm astrocytes but can lead to mitochondrial dysfunction and eventual cell death in co-cultured neurons (400).

Astrocytes can also modulate the immune system to reduce inflammation. Normal human astrocytes were shown to suppress both monocyte and T cell activation in cell cultures (202). It was found that astrocytes reduced monocyte activation, not by secreting IL-10, but by blocking CD80 induction on the monocytes through an undefined mechanism (202). Astrocytes can also function in a manner to recruit and direct white blood cells, both leukocytes and monocytes, to an area of injury, while at the same time protecting healthy tissue from inflammatory consequences of white blood cell invasion (51; 126; 441). Importantly, ablation of activated astrocytes in a murine model of spinal cord injury resulted in greater inflammation, increased neuronal degeneration, and negatively impacted subsequent motor function, suggesting that the activated astrocytes control the extent and location of inflammation following injury (109). The mechanisms of suppression of inflammation by astrocytes require further investigation.

G. Tissue homeostasis under conditions of DNA damage

DNA repair and synthesis are necessary for normal tissue homeostasis. DNA repair in neurons has been demonstrated to occur in a non-uniform and, in some cases, inefficient manner. Due to a decreased antioxidant response, neurons display increased chromosomal and mitochondrial DNA lesions that can result in cell death (148). As compared to astrocytes, neurons are slower in rejoining DNA double

strand breaks following radiation exposure, and they display greater cell death after episodes of DNA damage (136). Interestingly, DNA damage in neurons can induce the production of cell cycle enzymes, cyclin B, cyclin E and proliferating cell nuclear antigen (PCNA) (367; 471). But this cell cycle progression precedes apoptotic cell death rather than survival and proliferation in neurons (211; 212). Administration of cell cycle inhibitors after traumatic brain injury was shown to decrease neuronal cell death (135). The reason for this behavior could be related to the hypothesis that slower cycling cells repair DNA more efficiently (420). In contrast, a murine model of stroke indicated that a pause of several days occurred in the cell cycle of astrocytes before they continue to proliferate after exposure to injury (191). Such differences in cell cycle control may mean the difference between life and death at the cellular level. An ability to repair effectively before allowing for cell proliferation may explain astrocyte survival post injury (420).

Neurons do display a limited ability to repair DNA in both wild type and 7,8-dihydro- 8-oxoguanine glycosylase (OGG1) deficient mice in response to ischemia (240). OGG1, a DNA glycosylase involved in base excision repair, protects neuronal mitochondrial DNA from oxidative damage under ischemic conditions (240). However, the effectiveness of this repair has been called into question and may depend on the location of DNA damage within the chromosome of the mitochondria. Studies of DNA damage from radiation in NTERA-2 derived neurons showed that DNA was efficiently repaired for transcribed genes but inefficiently repaired in non-transcribed areas, suggesting that chromosomal organization plays an important role in the effectiveness of DNA repair mechanisms in neurons (288).

In contrast to neurons, astrocytes display robust DNA repair capacities for both nuclear and mitochondrial DNA (377). In cell culture assays, astrocytes exposed to menadione, an agent which induces oxidative stress, displayed a lower mitochondrial DNA strand break frequency and more efficient DNA repair as compared to all other cell types of the brain (221). The mechanisms induced to repair DNA in astrocytes are multifaceted and include upregulation of both primary double strand break pathways: non-homologous end joining and homologous recombination (53; 364). Accordingly, astrocytes are better able to protect themselves after DNA damage as compared to neurons. To do this they utilize a hierarchy of mechanisms. Protection of astrocyte DNA enables prevention of mutations and subsequent loss of function or induction of cell transformation and carcinogenesis. This resilience allows astrocytes to respond to and aid in the protection of neurons and other cell types after brain damage.

IV. A Comparison of Common Forms of Brain Injury: Traumatic Brain Injury and Radiation

Arguably, the two most common types of injury to the brain are traumatic brain injury (TBI), usually physical injury which can be due to automobile or other accidents, and radiation injury, from clinical radiotherapy. Traumatic brain injury is

one of the leading causes of death and disability world wide; estimations of TBI injury per year range 1.7-3.8 million (218; 244). Radiation therapy for metastatic brain cancers is required in roughly 30% of patients presenting with solid tumors and impacts about 170,000 Americans each year on top of the 40,000 Americans who are diagnosed with primary brain tumors (143; 219; 267). The total dosage of radiation used to treat brain tumors varies widely, depending upon the location of the tumor, the number of tumors, and whether the tumor is primary or metastatic. However, the range can be typically between 12 – 56 Gy total ((184; 196) Previously reported dose rates for patients receiving radiation therapy to the brain range from 0.5 Gy/min to 1.7Gy/min (184). Note, this places the dose rate used in our experiments (1.15 Gy/min) squarely in the appropriate therapeutic range.

Treatments for both radiation and traumatic brain injuries have been separately proposed, including the use of free radical scavengers, administration of growth factors, and use of stem cell grafts (6; 54; 134; 145; 314; 316). However little attention has been paid to the possibility of crossover treatments in which a treatment for radiation damage is utilized for traumatic brain injury or vice versa. It would therefore be useful to find effective treatments for both. It is within reason that studying radiation injury and developing treatments for it may aid in development of treatments for TBI. Therefore, to study radiation is not only to treat the long term impact of radiation therapy on patients but potentially also to treat individuals who have suffered TBIs.

A. Injury patterns in TBI and Radiation

The initial pattern of response to these two types of injury differs in the early response phase. In TBI, as the level of severity increases, death of the tissue proximal to the site of physical injury occurs regardless of cell type within the first hours to days after traumatic brain injury (269; 324; 347). In contrast, radiation damage to cells within the field shows selectivity: cells which are least differentiated and which proliferate most quickly are the first to die, while death of terminally differentiated cells is typically observed only at high total exposures of radiation and high dose rates (158). Since the brain is composed of non-uniform regions fully differentiated and undifferentiated cells, damage by radiation may vary, depending upon the region and total area of exposure.

For both TBI and radiation injury, there are similarities in the secondary responses: 1) astrocytes play a fundamental role in homeostasis and are relevant to certain repair processes after damage; and 2) secondary damage pathways overlap. Secondary injury cascades from the initial damage include: decreased ATP production, increased reactive oxygen species, increased inflammation, breakdown of the blood brain barrier, and increased tau protein expression and phosphorylation (180; 187; 229; 248). In addition to specific signaling cascades, the by-stander effect in radiation is thought to play a significant role in the spread of radiation damage; the bystander effect, which will be discussed at length in chapter 4. The secondary events are thought to spread the area of the initial damage from

both TBI and radiation, and are associated with long term deficits in memory, attention and processing that are observed in humans in the weeks, months, and years following either radiation therapy or mild traumatic brain injuries (91; 430).

B. Models of TBI and Radiation

While multiple animal models of TBI exist and may provide new insights into this pathogenesis there are shortcomings to these models which must be considered. First, the terms mild, moderate and severe TBI are used there is no complete consensus on these terms (466). Secondly, there are multiple models of TBI in order to study varying aspects of this injury with variations in ability to control for impact strength, location of impact and reproducibility of impact across cohorts (466). Third, while mild TBI is the most common form suffered in humans, it has been difficult to produce a suitable animal model of this type of damage (91).

Three main models of TBI are currently in use: controlled cortical impact (CCI), weight drop, and fluid percussion. Animal models have also been developed to study the effects of clinical radiation on the brain. In CCI the animal receives a craniotomy and then receives a physical impact from a rigid impactor on its dura; this method is thought to be more accurate than fluid percussion or weight drop (342). However, due to the surgical craniotomy prior to impact on exposed dura this model has been criticized for failing to fully represent the clinical presentation of TBI patients (296). In weight drop percussion, a standardized, guided weight is dropped on the animal's head to produce a blunt force injury on an intact skull; control of weight and height of drop is possible to modulate degree of injury (115). In fluid percussion, the animal first receives a craniotomy. In one model this is followed by rapidly injected saline, while in another model a weighted pendulum hits a fluid filled cylinder which in turn strikes the animal (9; 321). The common use of anesthesia in these models to reduce animal pain/suffering during experimental TBI may produce confounding variables and unanticipated protective effects not observed in human patients with TBI (296).

Many aspects of radiation therapy – from total dose of exposure, to dose rate, to accuracy of targeting – are highly controlled (25). Initial work in radiation began with the use of dogs and monkeys in the 1930s with a slow transition to mice and rats becoming the dominant animals used in these experiments (469). This has been due in part to ethical and monetary concerns surrounding the use of primates in radiation research (469). In addition cell culture models for cancer therapy and normal tissue response to therapeutic radiation have also been widely utilized (272). Experiments studying the role of radiation induced brain injury have utilized multiple or single fractionations, variations in dose rates, variations in radiation type, primarily X-ray and gamma irradiation, and variations in total dose in an effort to model all the variations in therapeutic uses of radiation (351). Anesthesia is utilized in some animal models and is also sometimes used for human clinical radiation therapy; in this case, the use of anesthesia in the animal model allows the model to more closely mimic clinical radiotherapy (13).

C. Damage and Subsequent Response to Radiation Injury

Failure of cells to repair DNA after ionizing radiation injury is cited as the main reason for cell death (190). Unlike damage to other macromolecules, damaged DNA cannot be easily destroyed and replaced by the cell (343). DNA damage repair is possible but mistakes can be carcinogenic or deadly (16). The stakes are high for cell repair to occur accurately and efficiently. DNA double strand breaks are particularly common after radiation injury and are the hardest to repair with the highest potential for mistakes (387; 424). The two main pathways utilized to repair after radiation induced DNA double strand breaks are: non-homologous end-joining (NHEJ) and homologous recombination (HR) (131). The choice between HR and NHEJ is cell cycle dependent and cell type dependent (390). The pathway may even be injury dependent with NHEJ occurring more frequently when DNA double strand breaks produce blunt ends or the breaks are less complex (176; 443).

Ionizing radiation induced production of ROS through radiolysis of water in the cell is credited with being the main pathway of cellular damage (162). Antioxidants have been studied for years due to their ability to protect cells from radiation induced ROS (38). Upregulation of antioxidants has been linked to greater protection of DNA and subsequent survival after radiation injury (119). Upregulation of antioxidants can protect nearby cells from cytotoxicity and subsequent apoptotic cell death (361). This may be due in part to their ability to upregulate DNA double strand break repair (175). Due to the well-known positive impact of DNA repair and antioxidant upregulation on survival after ionizing radiation these two pathways will be studied for their role in protection of astrocytes after ionizing radiation injury.

D. Response of the Brain to Radiation Injury

As previously stated, radiation tends to more severely impact undifferentiated, rapidly dividing cells. Fully differentiated cells also exhibit damage from radiation, but their responses vary. The responses of the different mature brain cell types to radiation have been studied to varying degrees.

The fate of adult stem cells in the brain has been of particular concern for the use of radiotherapy. A variety of stem cells are produced even in adulthood to replenish some cell type populations (255). Radiation injury disrupts and kills many of these immature cell types. Of particular concern in radiation therapy are the neural stem and progenitor cells located in the subventricular of the hippocampus and the subgranular zone of the dentate gyrus (169). These neural stem and progenitor cells may develop into neurons or astrocytes, but irradiation appears to drive them to apoptosis or adoption of an astrocyte differentiation pattern, effectively inhibiting neurogenesis (155; 169; 488). Adult precursors in the brain also exist for oligodendrocytes, responsible for the production of myelin sheaths around neuronal axon (95). Oligodendrocyte precursors fail to develop after

radiation; the loss of oligodendrocyte precursors was linked to white matter loss months after the irradiation (28).

Mature cells of the brain include neurons, glia and microglia, endothelial cells, pericytes, oligodendrocytes, and smooth muscle cells. Not all of these cell types have been specifically studied for their response to radiation. Endothelial cells, pericytes, microglia and a specific type of astrocytes make up the blood brain barrier (BBB) which controls entry of molecules from other parts of the body into the brain (81). Disruption of the BBB peaks around 24 hours after radiation and takes several weeks to repair (488). This disruption is partly attributed to death and/or damage to endothelial cells. Irradiated endothelial cells exhibit increased expression of adhesion molecules which encourages invasion of the brain by the leukocytes (480; 488). Recently pericyte and smooth muscle death were shown to undergo necrotic cell death in regions adjacent to treated tumors in the months following radiotherapy in patients treated for glioblastoma (225). Neurons may display death, changes in morphology including decrease in dendritic spine expression, or dysfunction after radiation injury including alterations in long term potentiation in the hippocampus (375; 406; 461). Findings from studies of microglia are more controversial, with some studies in murine models reporting decreased levels of microglia after radiation injury while others studies suggest that microglia may proliferate after radiation injury in the cerebellum (275; 449). This report of microglial proliferation may not be due to survival of microglia native to the brain but could be the result of peripheral macrophages and monocytes gaining entry to the brain after radiation injury, and subsequently developing features similar to microglial residents of the brain (274; 275).

As previously noted astrocytes show a robust survival response to other forms of oxidative stress which suggests a capability in responding to damage. However, research into the response of astrocytes to radiation has provided conflicting findings, with some studies reporting proliferation after radiation while others studies have reported astrocyte cell death (63; 70; 444). This difference in findings may be attributed to differences experimental conditions, such as the dose rate. Studies utilizing dose rates of 10.23 Gy/min have reported astrocyte cell death (63), and proliferation of astrocytes was observed in experiments that utilized more clinically relevant doses of 2.38 Gy/min (70). Our study utilized a dose rate of 1.157Gy/min which more closely matches the survival observed in the study utilizing a lower dose rate. A higher dose rate is linked to more clustered DNA damage and lower cell survival (237; 394; 397). In addition, dose rate has been shown to impact the subsequent cellular attempt at DNA repair (436). Therefore, we posit the results we display below are different from results of some other researchers due to differences in dose rate.

VII. Summary and Conclusions

We now recognize astrocytes play a vital role in modulating neuronal

synaptic activity, modulating CNS immune response, and in general homeostasis of the CNS environment (34). The rise in understanding of the importance of astrocyte function ought then to be met with increased investigation of their survival mechanisms. It is necessary to understand astrocyte response not only to understand how they survive but to understand what implications this may have for the CNS environment. Prior research indicates astrocytes under oxidative stress may act to protect themselves and other nearby cells by upregulation and release of antioxidants. However, astrocytes may also act selfishly to protect only themselves by upregulation of DNA double strand break repair. The methods by which astrocytes defend themselves against damage has implications for not only their survival but also survival of nearby cells. The purpose of my dissertation is to focus specifically on astrocytes and the mechanisms they utilize to respond to a specific type of stressor: ionizing radiation. We hypothesized that normal human astrocytes would demonstrate qualities of a radiation resistant cell type through the use of DNA repair and antioxidant response.

Radiotherapy induces cognitive impairments in over 50% of adult patients who receive fractionated dose therapy (178). These cognitive impairments may occur in the absence of obvious physiological injury (22). There is currently no treatment to ease the symptoms observed after radiation therapy. While research has previously focused on the impact of radiation injury on neuronal survival little research has been done to focus directly on survival and response to injury of astrocytes. Yet as described in this Chapter, astrocyte survival and response to injury is closely tied to neuronal survival and CNS repair. We sought to understand why prior evidence implicates astrocytes in protection of neurons and yet the astrocyte response fails to alleviate these cognitive symptoms of radiation injury. We anticipated secondary cascades of damage would be similar between radiation injury and other forms of oxidative stress. However, no prior studies have focused on response pathways after different forms of oxidative stress in normal human astrocytes. We accepted that it was possible that astrocytes would not upregulate typical pathways associated with response of astrocytes to oxidative stress and would fail to display radiation resistance. We therefore investigated the response of astrocytes to radiation injury, as the response of this cell type may be critical for the survival of other cell types, especially neurons. The purpose of this dissertation is to elucidate the impact of radiation injury on astrocyte survival and response to injury. The brain is not a homogenous organ, and each cell type displays variations in their ability to effectively respond to radiation injury.

Chapter 2

Abstract

Radiotherapy is a common modality for treatment of brain cancers, but it can induce long-term physiological and cognitive deficits. The responses of normal human

brain cells to radiation is not well understood. Astrocytes have been shown to have a variety of protective mechanisms against oxidative stress and have been shown to protect neurons. We investigated the response of cultured normal human astrocytes (NHA) to X-ray irradiation. Following exposure to 10 Gy X-irradiation, NHA exhibited DNA damage as indicated by the formation of γ -H2AX foci. Western blotting showed that NHA displayed a robust increase in expression of non-homologous end joining DNA repair enzymes within 15 min post-irradiation and increased expression of homologous recombination DNA repair enzymes ~2 h post-irradiation. The cell cycle checkpoint protein p21/waf1 was up-regulated from 6-24 h, and then returned to baseline. Levels of DNA repair enzymes returned to basal ~48 h post-irradiation. NHA reentered the cell cycle and proliferation was observed at 6 days. In contrast, normal human mesenchymal stem cells (MSC) failed to up-regulate DNA repair enzymes and instead displayed sustained up-regulation of p21/waf1, a cell cycle checkpoint marker for senescence. Ectopic overexpression of Ku70 was sufficient to protect MSC from sustained up-regulation of p21/waf1 induced by 10 Gy X-ray. These findings suggest that increased expression of Ku70 may be a key mechanism for the radioresistance of NHAs, preventing their accelerated senescence from high dose radiation. These results may have implications for development of novel targets for the radiation countermeasure development.

Introduction

Radiation therapy is frequently used in combination with surgery and chemotherapy for the treatment of brain cancers. Each of these modalities have serious limitations for treatment of brain cancers. Poor penetrance of the blood brain barrier can limit the efficacy of chemotherapeutic drugs, and the position of tumors within the brain can limit the utility of surgery (104). Radiation damage to neurons is also a critical concern, and neuronal damage is a limiting factor for the use of radiation for cancer treatment (114). Adult neural stem cells in the subventricular zone near the lateral ventricles and in the dentate subgranular zone in the hippocampus, sites believed to be involved in neurogenesis in the adult, can undergo apoptosis after radiation injury (111). The long-term adverse effects of radiation injury on the human central nervous system (CNS) are manifestations of neuronal loss and include memory impairment, attentional deficits, poor coordination, blindness, seizures, and paralysis (7; 76; 101), and some patients receiving chronic radiation treatment develop dementia over time (140; 362). These cognitive deficits were correlated with structural abnormalities in the white matter of the corpus callosum, and deficits in the basal ganglia and thalamus following radiation treatment (379). Cell death, unrepaired damage to surviving brain cells, and neuroinflammation are recognized as three hallmarks of radiation-induced CNS pathology (3; 31; 112; 226).

Different tissues display variations in tolerance to radiation-induced damage, but the mechanisms underlying these variations are not completely understood, and this remains a fundamental question in radiation biology (401). The survivability of

brain neurons from any stress insult is dependent, in part, upon the support they receive from astrocytes (52; 153; 298; 345; 447). Astrocytes are the most abundant cells in the human brain; they play integral roles in neuronal development, signaling, and plasticity (398; 429). Astrocytes protect neurons from oxidative stress, including that induced by ethanol, hydrogen peroxide, and glutamate-induced excitotoxicity, suggesting that astrocytes may serve a critical function in the brain's response to oxidation and toxicity (46; 89; 150; 452).

Radiation can directly damage macromolecules within the cell or it can induce indirect damage through radiolysis of water molecules, generating reactive oxygen species (ROS) (19). In some cases, the vulnerability of tissues to radiation injury is predicted by the Law of Bergonie and Trebondeau, which states that radiation is generally more damaging in rapidly dividing and undifferentiated cells. This law is related to a widely accepted dogma in the field of radiation biology: that DNA is the most important molecular target of radiation because of its critical role in cell replication and proliferation. Radiation damage to DNA, especially DNA double strand breaks (DSBs) that are not repaired or are repaired incorrectly is believed to be a primary cause of radiation-induced cell senescence and death (85). There are two major pathways repair of DSBs: non-homologous end joining (NHEJ), which is most active during the G₀/G₁ phases of the cell cycle; and homologous recombination (HR), which is believed to be most active during the S/G₂ phases of the cell cycle (110; 254). The proteins Ku70 and Ku80 act as DSB sensors which bind to damaged DNA and initiate NHEJ by recruiting additional proteins necessary for DNA repair (254; 319; 338). Ligation of repaired DNA strands occurs when DNA ligase IV interacts with X-ray repair Cross Complementing 4 (XRCC4) (71). In contrast, HR repair begins with DNA damage sensing proteins inducing a signaling cascade which activates the trimer replication protein A (RPA) via phosphorylation at multiple sites including ser4/8 (174; 292; 396; 482). RPA binds to single stranded DNA (ssDNA) to stabilize it and prevent formation of secondary structures (289). A protein complex including RAD51 then retrieves the ssDNA from RPA (230). RAD51 is the integral recombinase involved in search homology and strand invasion of the sister chromatid which allows DNA repair and subsequent synthesis of the damaged strand (50; 154).

Because of the importance of astrocytes for the protection of neurons and other cells during oxidative stress (328; 377; 453), and studies demonstrating that astrocytes have endogenous mechanisms of protection against redox stress (52), we investigated the response of normal human astrocytes (NHA) to X-ray radiation and compared them to those observed in human mesenchymal stem cells (MSC), a well-established model system for the investigation of radiation cellular effects. We found that NHA, unlike MSC, are resilient to up to 10 Gy X-ray irradiation, with the capacity to proliferate following a dramatic up-regulation of DNA repair enzymes, with a transient pause of the cell cycle. Our research, therefore, has revealed the ability of astrocytes to robustly respond to radiation injury, with a central role for the DNA repair enzymes.

Materials and Methods

Reagents

Unless otherwise noted, reagents were obtained from Sigma-Aldrich (St Louis, MO).

Cell Culture and X-irradiation

Normal human astrocytes (NHA) (cat. #CC2565, lot #0000402839, 0000412568), astrocyte specific media (cat. #CC3186), trypsin (cat. #CC-5012), human mesenchymal stem cells (MSC) (cat. #PT2501, lot # 0000423370, 0000463158, 0000471980), and MSC media (cat. #PT3001) were obtained from Lonza (Walkersville, MD). At least two independent isolates of MSC and NHA were used. Note that other lots of NHA did not all display Ku70 upregulation. Cells (passages 3-6) were plated and cultured (37°C and 5% CO₂) according to the vendor's protocols. Jurkat cells, A549 cells, and HEK293T cells were obtained from American Type Culture Collection (ATCC, Manassas VA). Dulbeccos Modified Eagles Media (DMEM) high glucose formulation was obtained from Thermo Fisher Scientific (Waltham, MA). A549 and HEK293T cells were grown in DMEM media supplemented with 10% fetal bovine serum (Gemini Bioproducts, Woodland, CA), penicillin (100 units/ml), and streptomycin (100 µg/ml). Jurkat cells were grown in RPMI1640 with glutamine (Thermo Fisher Scientific), 10% fetal bovine serum, with penicillin (100 units/ml) and streptomycin (100 µg/ml). Cells were grown in a humidified 5% CO₂ incubator at 37 °C. X-ray irradiation was conducted using a RS2000 Biological Irradiator (Rad Source Technologies, Alpharetta, GA). The RS2000 provided a cone-shaped radiation field with irradiation at 12.39 to 40.50 cm from the X-ray source. For dose measurements, irradiation was performed with the following settings: 160 kVp, 25 mA, 90 sec irradiation time, and 0.3 mm Cu beam filtration. The approximate half value layer (HVL) provided by the manufacturer was 0.62 mm Cu. Cells were irradiated at a dose rate of 0.989 Gy/min (Approx. 14 inches from source at level 2). Prior to irradiation cells received fresh, pre-warmed media before being transported to the irradiator room at room temperature. Cells were irradiated at room temperature then returned to the incubator at 37°C, or used for experimentation.

Cell proliferation assay

Equal numbers of cells were plated to yield approximately 50% confluence prior to X-ray irradiation injury. NHA and MSC were collected 4, 5, and 6 days post radiation exposure. Media was changed before radiation injury and 4 days post-irradiation. Cells were trypsinized, collected by centrifugation (5 min, room temp, 1700-2000×g), resuspended in pre-warmed media and then counted by hemocytometer; at least 100 cells per plate per condition were counted.

Assay of phosphorylated H2AX to assess DNA damage

Cells were irradiated then cultured for 24, 48 or 72 h. Cells were washed (3 times with phosphate buffered saline [PBS]), then treated for 20 min with 4% paraformaldehyde (room temperature) and washed again (3 times with PBS). Cells were permeabilized with 70% ethanol (5 min, room temperature), blocked with 5%

normal donkey serum in PBS for 1 h at 37°C, and incubated for 1 h at 37°C with anti- γ -H2AX antibody (#9718S, Cell Signaling, Danvers, MA) diluted 1:400 in 5% normal donkey serum in PBS. Cells were washed 3 times with PBS and then incubated for 1 h in Donkey anti-rabbit IgG (Thermo Fischer, A21206) in PBS. Cells were washed (3 times, PBS), incubated with DAPI (4',6-diamidino-2-phenylindole) (5 min, room temperature, in dark) diluted 1:1000 in PBS, then mounted and visualized with an Olympus BX61 fluorescence microscope (Olympus, Center Valley, PA) using 10 $\times$ magnification at 488nm. Immunoreactive γ -H2AX foci were counted in 100 DAPI-positive cells per plate.

Annexin V stain

The Annexin V-FITC detection kit (Cell signaling, cat#6592) staining was slightly modified from manufacturer's protocol (Cell Signaling). Briefly, MSC and NHA were grown on glass coverslips for ease of use. Jurkat cells were grown in suspension. All cell types were irradiated at 10 Gy then incubated at 37°C for 24 h. Jurkat cells were centrifuged 220 $\times$ g for 5 min 4°C twice and washed with 1 $\times$ PBS. MSC and NHA were washed twice with 1 $\times$ PBS. Cells were then incubated 15 min on ice in the dark in 1 $\times$ binding buffer containing Annexin V-FITC conjugate at a concentration of approximately 1:200. MSC and NHA were then directly imaged. Jurkat cells were pipetted unto coverslips and then imaged. Imaging was performed on Olympus BX61 fluorescence microscope (Olympus, Center Valley, PA) using 10 $\times$ magnification at 488nm or using phase contrast.

β -Galactosidase Senescence Assay

β -Galactosidase assays were performed as described previously by Panganiban *et al.*, (2013) (304). Briefly, cells were irradiated at 50-70% confluence, to avoid false positives which can occur in confluent cell cultures (470), and assayed at 24, 72 and 120 h post- irradiation. Cultures were washed twice with PBS and then fixed with 3.7% formaldehyde in PBS for 5 min at room temperature. Plates were then washed twice with PBS, exposed to X-gal solution (1 mg/ml 5-bromo-4-chloro-3-indoyl β -galactopyranoside, 150 mM NaCl, 2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, citric acid/sodium phosphate buffer [pH 6]) and maintained at 37°C for either 16 h (MHCs) or 18 h (NHAs) without CO₂. Cells were washed with PBS, treated for 5 min with methanol, and air dried before observing by microscopy. At least 100 cells were scored in random fields for expression of β -galactosidase; all cells in each field were scored.

Transfection

Flag-tagged pCMV_Ku70 and a flag-tagged control vector were generous gift of Dr. Shigemi Matsuyama, Case Western Reserve University, Cleveland, OH (360). Lipofectamine 2000 (Cat#11668027, Thermofisher) was used at a 3:1 ratio of Lipofectamine to vectors, diluted in Opti-mem media (cat#31985070 Thermofisher). The pCMV_Ku70 flag tagged vector or control plasmid was co-transfected into MSCs with an EGFP-CMV expression plasmid (courtesy of the Dr. Shigemi Matsuyama, Division of Hematology, Case Western University, Cleveland,

OH) at a ratio of 1:2. Cells were incubated for 18 h before Lipofectamine was discarded and normal growth media was reapplied. Radiation occurred 24 h after transfection.

Western Blotting

Cells were irradiated at 70-90% confluence and lysates were prepared at specific time points post-radiation injury. Cells were washed 3 times with PBS and then extracted with RIPA buffer (50mM Tris (pH 8), 150mM NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate and 1× Halt Protease and phosphatase inhibitor (cat# 78443, Thermofisher, Rockville MD). Primary antibodies were obtained for full length caspase 3 (cat. #9662S, Cell Signaling, Danvers, MA) (1:1000), cleaved caspase 3 (cat. #9661S, Cell Signaling) (1:1000), and p21 (cat. #9sc-397, Santa Cruz Biotechnology, Inc, Dallas TX) (1:500), p-RPA32 (cat. #A300-245A, Bethyl, Montgomery, TX) (1:500), RPA32 (cat#A300-244A, Bethyl) (1:500), Rad51 (cat. #98875S, Cell Signaling) (1:500), Ku70 (cat. #9sc-9033, Santa Cruz Biotechnology) (1:1000), and XRCC4 (cat. #sc-8285, Santa Cruz Biotechnology) (1:300). Proteins were detected with species-matched horseradish peroxidase-linked secondary antibodies (1:500-1:2000, R&D Systems) and Novex ECL Chemiluminescent Substrate (Cat # WP20005, Thermofisher). WCIF ImageJ software was used for densitometry analysis (NIH, Bethesda MD <https://imagej.nih.gov/ij/download.html>).

Statistical Analysis

Means $\pm$ standard deviations (SD) were calculated, and statistically significant differences between two groups were determined by the Student's *t* test. For three or more groups, statistical analysis was performed using one-way ANOVA, followed by the Tukey's post-analysis, as appropriate; $p < 0.05$ was considered statistically significant. Statistical software for all analysis was SPSS Statistics (IBM, Bethesda, MD).

Results

Normal human astrocytes are resistant to radiation-induced growth arrest and apoptosis as well as senescence after 10 Gy exposure

Astrocytes have been demonstrated to be resistant to oxidative stress, with improved cell survival and reduced cell death following oxidative stress (89; 263; 454). Additionally, astrocytes have been demonstrated to provide protection to neurons and other cell types against a variety of stresses, including redox stress, in vivo and in vitro, through a variety of mechanisms (52; 328). Here we sought to better understand the mechanisms involved by comparing radiation responses of NHA with those of MSC, a model cell type used in the investigation of radiation biology (65). MSC were previously demonstrated to be relatively resistant radiation-induced damage, requiring 2 Gy to reduce the surviving fraction of cells to 37% (65). Here we investigated a higher dose of radiation, 10 Gy, which was previously demonstrated in MSC to reduce the surviving population to <1% (65). NHA and MSC

were exposed 10 Gy X-ray (0.989 Gy/min) during exponential growth phase, and cells were counted at 4, 5, and 6 days post-exposure (Fig. 2). Total cell counts revealed increased numbers of NHA between day 4 and day 6 ($p < 0.05$) in both the sham and irradiated conditions. In contrast, only sham irradiated MSC had increased cell numbers between days 4 and 6, and irradiated MSC failed to proliferate over this time period. Imaging of NHAs after radiation was done to further corroborate proliferation assay counts (Suppl Fig 1). Clonogenic assays are often used to demonstrate sensitivity of cells to radiation. However, repeated attempts at performing clonogenic assays on NHA were not successful due to their slow growth and failure to form colonies.

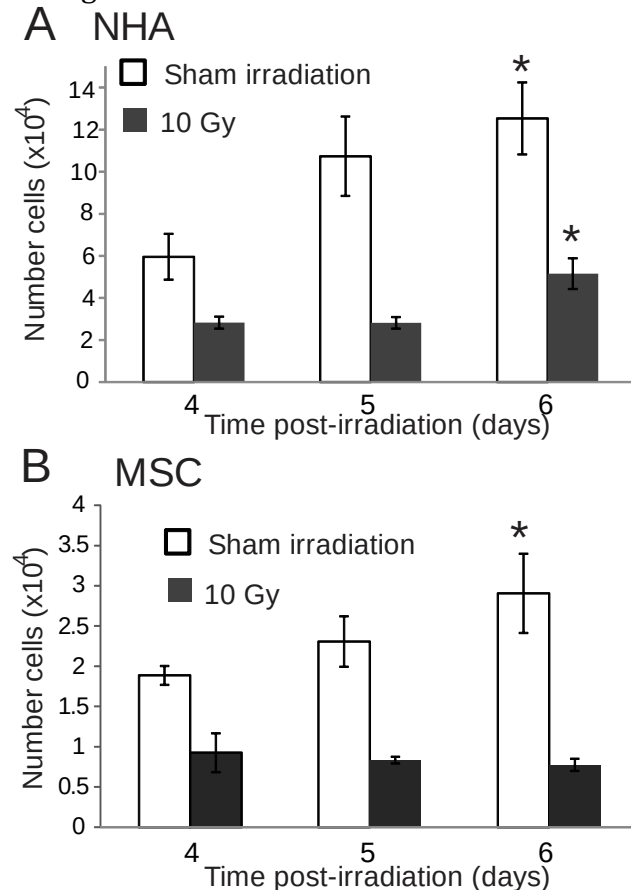


Figure 2 Effect of 10 Gy X-ray radiation injury on normal human astrocytes and mesenchymal stem cell proliferation.

*Normal human astrocytes (NHA, A) or normal human adult mesenchymal stem cells (MSC, B) were grown to 50% confluence and sham irradiated or exposed to 10 Gy X-ray. Cells were counted by hemocytometer at the indicated time points. Graphs show means of three independent experiments performed in triplicate $\pm$ SEM; * indicates $p < 0.05$ from control.*

Following exposure to oxidative stress, cells attempt to activate pathways to promote repair and survival, but if the biomolecular damage is too extensive, normal cells will activate either death or senescence pathways (120). Apoptotic cell death is one of the most common forms of cell death after radiation injury (90; 306). Accordingly, a series of experiments were conducted to investigate apoptotic

responses in NHA and MSC irradiated with 10 Gy. In addition to NHAs and MSCs, radiation sensitive Jurkat cells were used as a positive control. Annexin V immunohistochemistry was used to detect apoptotic cells. While NHAs and MSCs displayed no positive staining for Annexin V as expected Jurkat cells displayed positive Annexin V expression (Fig.3). This is consistent with reports in the literature indicating that Jurkats undergo apoptosis after radiation injury (67). Our findings in NHA and MSC were verified by western blotting for activated caspase-3, the common downstream caspase in both intrinsic and extrinsic apoptosis (320). No activated caspase-3 was observed in western blots of extracts of irradiated MSCs or NHAs (data not shown). Additionally, we determined that NHAs did not exhibit radiation-induced necrosis as judged by the release of lactate dehydrogenase (at 72 h), a marker for necrosis (data not shown).

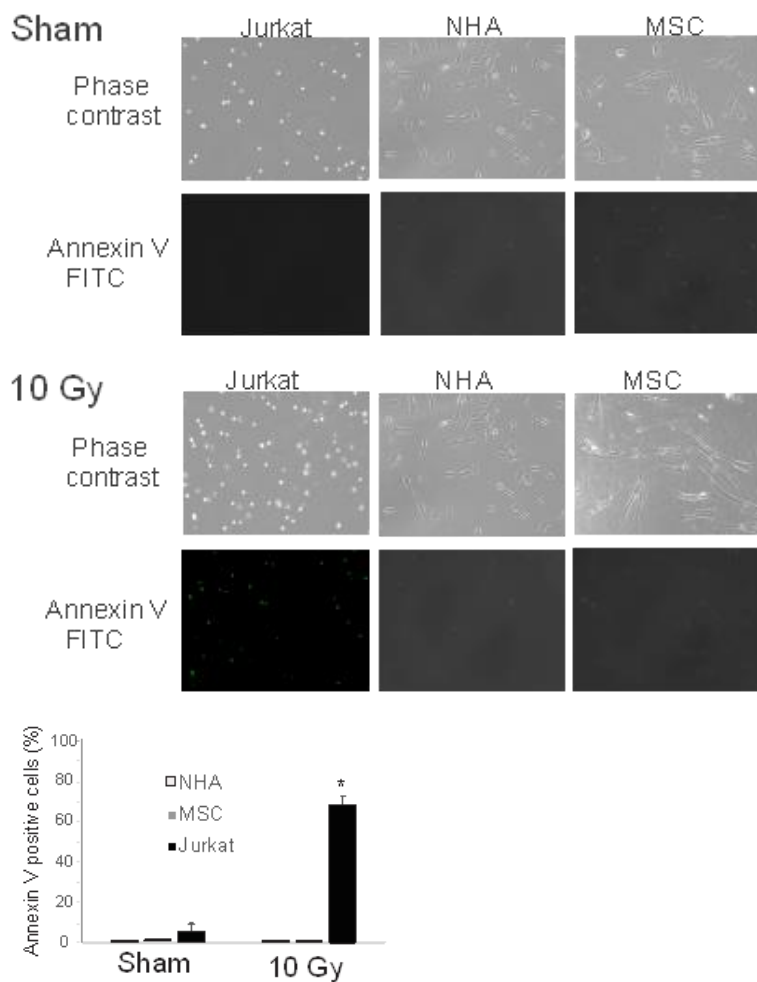


Figure 3 10 Gy X-ray irradiation does not induce significant apoptosis in human astrocytes or mesenchymal stem cells.

Normal human astrocytes (NHA), normal human adult mesenchymal stem cells (MSC) and Jurkats, an immortalized human T cell line were grown to 50% confluence and sham irradiated or exposed to 10 Gy X-ray. Annexin V staining was performed 24 h post-irradiation. Panels show control and irradiated cells for Annexin V FITC and phase contrast imaging. All cells were counted in random fields to reach a minimum of 100 cells total to

determine percentage expressing Annexin V staining. Graph indicates means of three independent assays $\pm$ SEM; * indicates $p < 0.05$ from control

Previous findings from our laboratory demonstrated that in primary non-transformed endothelial cells and keratinocytes the primary response to ionizing radiation exposure was cellular senescence, rather than cell death (304-306). Extensive oxidative stress and DNA damage trigger premature, permanent cell cycle arrest marked by an increase of β -galactosidase activity, termed senescence-associated β -galactosidase (SA- β -gal), and up-regulation of several cell cycle arrest proteins including p21/waf1 (18; 199; 214; 341). We investigated β -galactosidase activity and p21/waf1 expression following 10 Gy X-ray exposure in NHAs and MSCs. MSCs, but not NHAs, showed a statistically significant increase in β -galactosidase activity at 24 and 72 h after radiation exposure (Fig. 4A). Western blot analysis of p21/waf1 in NHAs revealed a transient increase in p21/waf expression at 6 h and 24 h post-irradiation, followed by a return to baseline levels by 48 h (Fig. 4B) in NHAs. In contrast, MSCs showed a significant increase in p21/waf1 protein expression by 6 h post-exposure ($p < 0.05$) which was sustained through 72 h, consistent with premature senescence as a result of 10 Gy irradiation.

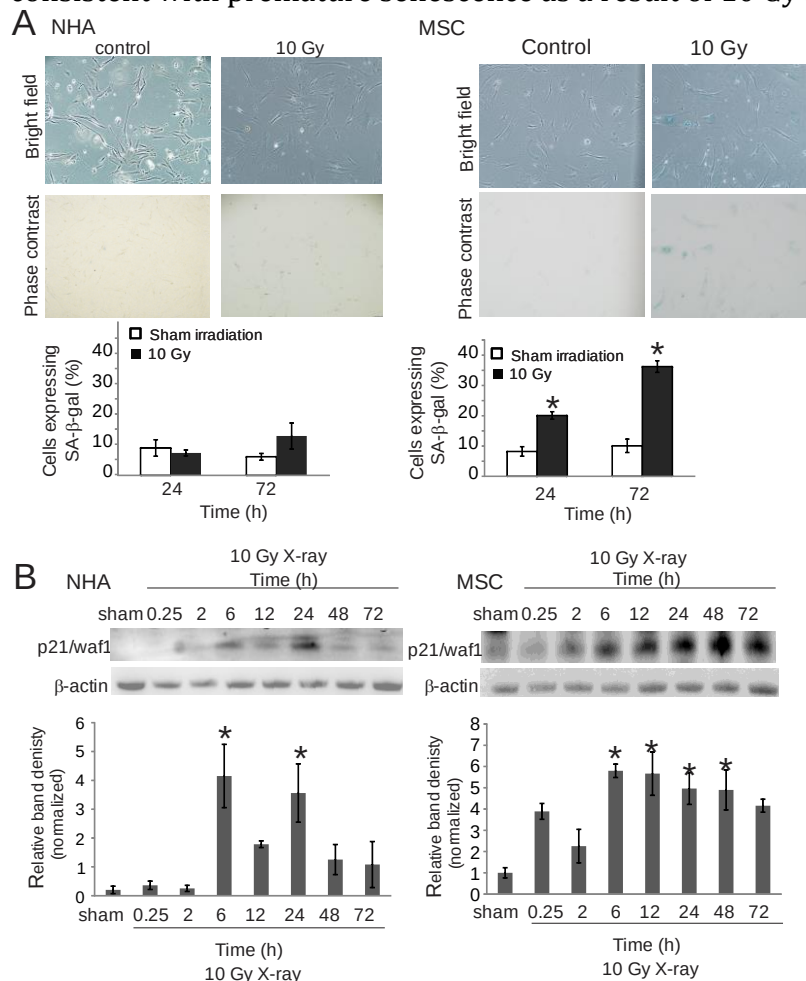


Figure 4 X-ray irradiation induces accelerated senescence in mesenchymal stem cells, but not in normal human astrocytes.

*Normal human astrocytes (NHA) or normal human adult mesenchymal stem cells (MSC) were grown to 50% confluence and sham irradiated or exposed to 10 Gy X-ray. A. β -galactosidase assays were performed at 24 h and 72 h post-irradiation. All cells were counted in random fields to reach a minimum of 50 cells total to determine percentage of cells with positive for β -galactosidase expression. Bar graphs indicate means of three independent assays $\pm$ SEM; * indicates $p < 0.05$. B. NHA and MSCs were grown to 70% confluence and sham irradiated or exposed to 10 Gy X-ray irradiation. Cell lysates were prepared at the indicated time points, and western blots were performed for p21/waf1 or for β -actin as a loading control. Representative data are shown from n=3 independent experiments. Graph indicates means of three independent assays $\pm$ SEM; * indicates $p < 0.05$ from control.*

Both non-homologous end joining and homologous recombination DNA repair mechanisms are activated by radiation exposure in normal human astrocytes but not in mesenchymal stem cells

Since radiation-induced unrepaired DNA damage is associated with senescence, mutations, and cell death (356), we investigated the expression of DNA DSBs in NHAs and MSCs. The H2AX histone is phosphorylated on serine 139 to form γ -H2AX, which is present in foci surrounding DSBs that are undergoing repair. Therefore, γ -H2AX is used as an indicator of the DNA repair process (199; 214; 376). In both cell types, 10 Gy irradiation induced a significant increase in γ -H2AX levels at all time points post-irradiation as compared to controls. In contrast to MSCs, NHAs displayed a statistically significant decrease in γ -H2AX foci between 24 h and 48 h (Fig. 5A), suggesting that DSB repair had occurred and that a significant percentage of γ -H2AX foci were resolved. MSCs did not display a significant decrease in γ -H2AX until 72 h post-irradiation (Fig. 5B). These findings suggest that DSB repair occurs more rapidly in NHAs than in MSCs.

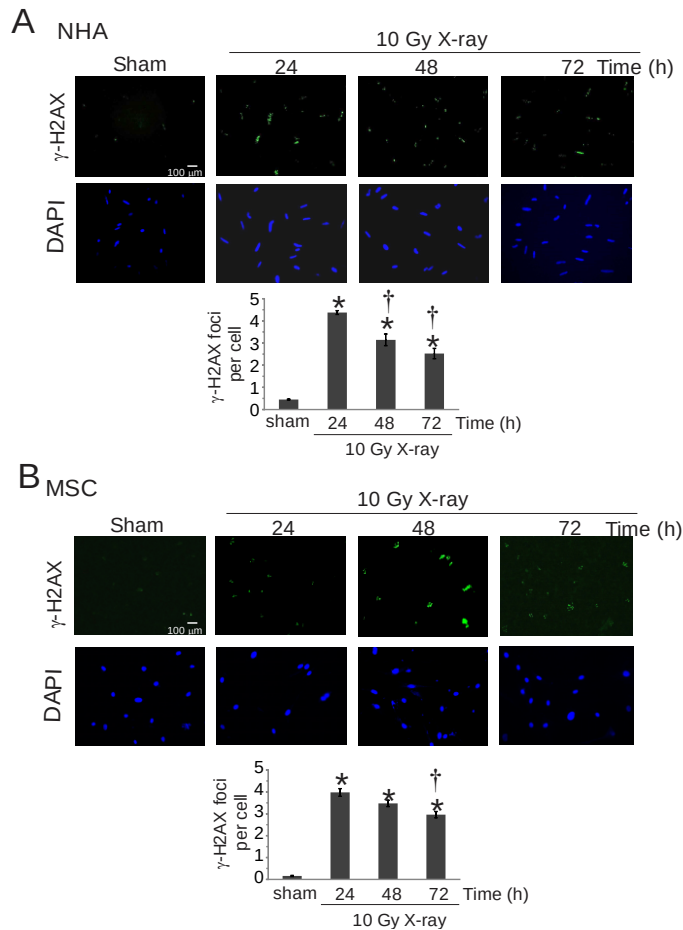


Figure 5 Both normal human astrocytes and mesenchymal stem cells demonstrate removal of γ -H2AX foci following radiation exposure

. Double Strand Break (DSB) repair was detected using γ -H2AX immunohistochemistry in normal human astrocytes (NHA, A) and mesenchymal stem cells (MSC, B). NHA or MSC were grown to 70% confluence and sham irradiated or exposed to 10 Gy X-ray irradiation. Immunohistochemistry was performed at the indicated time points. Panels show representative images of γ -H2AX or DAPI staining. The numbers of foci in all cells were counted in random fields to reach a minimum of 50 cells total to determine average numbers of foci per cell for $n = 3$ independent experiments. Bar graphs indicate means of numbers of foci per cell $\pm$ SEM. * indicates $p < 0.05$ from control; † indicates $p < 0.05$ from 24 h time point.

We next examined the impact of radiation exposure on levels of DNA repair enzymes in NHAs and MSCs. The two main pathways for DNA repair are non-homologous end-joining (NHEJ) and homologous recombination (HR) (378). We first examined the effects of radiation exposure on the expression of markers for NHEJ, Ku70 and XRCC4 and Ku70. Ku70 binds directly to DNA DSB and acts as both a scaffold and signaling molecule for other proteins to induce end joining (45). Ku70 was demonstrated to be upregulated in some cell types in response to DNA damage (47; 110; 254). In some systems it is believed that Ku70 is highly expressed, and may not be subject to regulation by DNA damage (188; 322). Because of this controversy, we first compared the levels of basal Ku70 expression in NHA and MSC with two cancer cell lines that express high levels of Ku70 (A549 and HEK293)(Fig. 6A). We observed that the relative abundance of Ku70 (compared to β -actin) in NHA

was <50% that of A549 and HEK293 cells. The relative abundance of Ku70 in MSC was less than 10% that of A549 and HEK293 cells. This suggests that in both NHA and MSC, the levels of Ku70 are significantly lower than in cancer cells, where Ku70 is considered to be highly expressed and no longer regulated by DNA damage. We examined Ku70 protein levels in response to 10 Gy X-ray exposure. We found that Ku70 increased ~10-fold in NHAs from 15 min through 6 h post-irradiation ($p < 0.05$), but no increase in Ku70 was observed in MSCs after 10 Gy irradiation (Fig. 6 B,C, respectively).

XRCC4, a protein which binds both DNA and DNA ligase IV, aids in ligation of newly repaired DNA and was shown to be increased in response to DNA damage (71; 317). XRCC4 increased >2-fold in NHAs at 2 h post radiation injury ($p < 0.05$) (Fig. 6D), but XRCC4 expression was below detection in MSCs at all time points (data not shown). Collectively, the findings with Ku70 and XRCC4 indicate that NHAs are more robust in activating the NHEJ repair mechanisms as compared to MSCs, following radiation exposure.

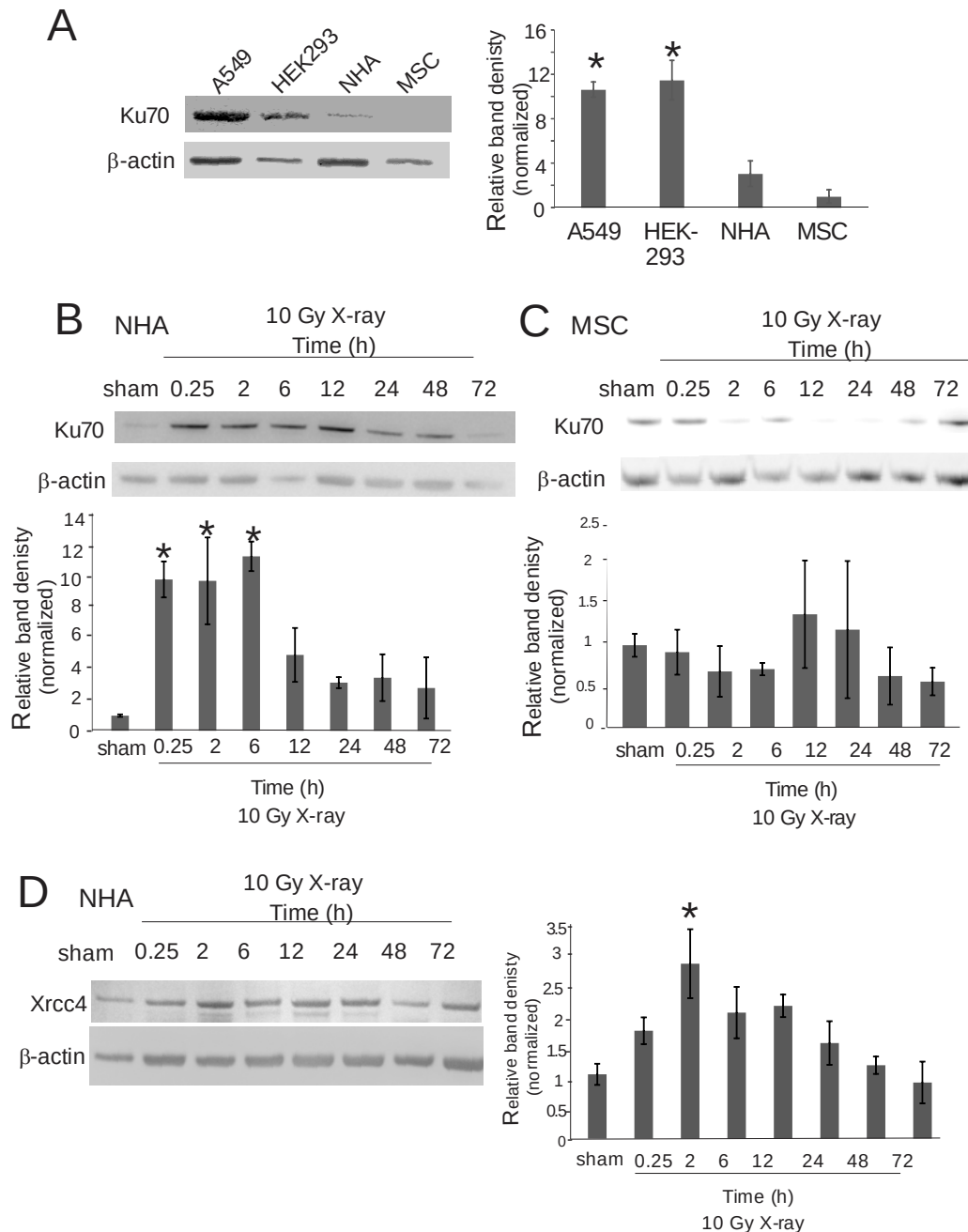


Figure 6 Normal human astrocytes, but not mesenchymal stem cells, display increased levels of proteins involved in non-homologous end joining

*A. Comparison of Ku70 levels in A549 lung cancer cells, HEK293T cells, NHA or MSC. Equal amounts of protein were used for western blots for Ku70; blots were stripped and reprobed for β -actin to normalize. Graphs show means of levels of Ku70 as a ratio of β -actin for each cell type. B-D. NHA and MSC were grown to 70% confluence and sham irradiated or exposed to 10 Gy X-ray. Cell lysates were prepared at the indicated time points, and western blots were performed for Ku70 (B,C) or for Xrcc4 (D) or for β -actin (A-C) as a loading control. Representative data are shown from $n=3$ independent experiments. Graphs show means $\pm$ SE of three independent experiments; * indicates $p \leq 0.05$.*

Homologous recombination (HR), the second major repair pathway for DSBs, relies on homology search and strand invasion of a non-damaged sister chromatid

(230). RAD51, another protein in the HR pathway, and its mediators then obtain the ssDNA from RPA and initiate strand invasion of the undamaged sister chromatid which allows for repair of the double strand break (208). During HR, replication protein A (RPA) is phosphorylated on its 32 kDa subunit (RPA32) which activates it, allowing ssDNA binding (413). NHAs displayed a significant increase in RAD51 expression after radiation injury, and a trend towards significance in MSCs. (Fig. 7A and B). Only NHAs displayed an increase in both total RPA and serine 4/8 phosphorylation of the 32 kDa subunit of RPA at 2 h post radiation injury (Fig. 7C) whereas, phosphorylation of RPA and total RPA were below detection in MSCs at all time points (data not shown). Together these data indicate that both NHEJ and HR are robustly up-regulated in NHAs, but not in MSCs.

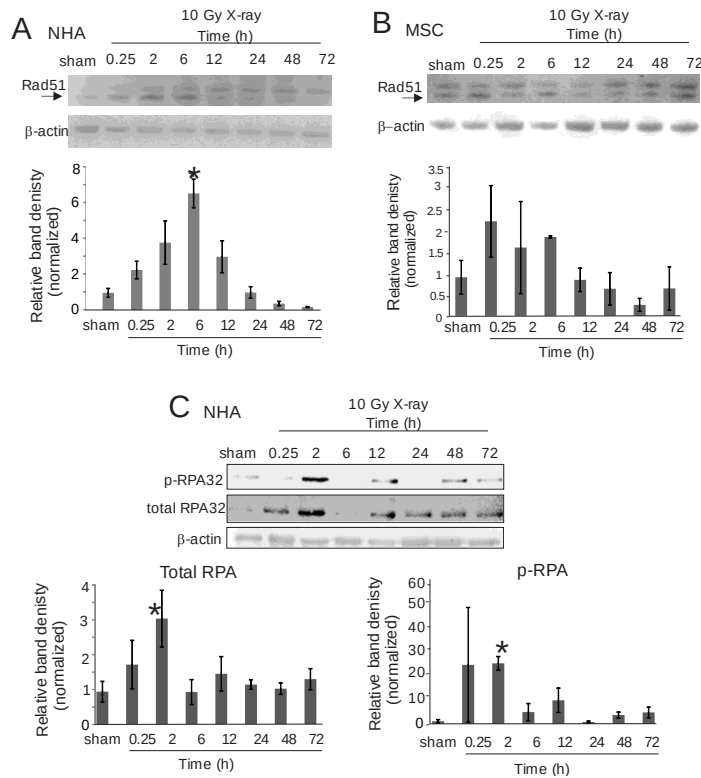


Figure 7 Normal Human Astrocytes display upregulation of Rad51 and total and p-RPA32.

Normal human astrocytes (NHA) or normal human adult mesenchymal stem cells (MSC) were grown to 70% confluence and sham irradiated or exposed to 10 Gy X-ray. Cell lysates were prepared at the indicated time points, and western blots were performed for RAD51 (A,B) or for total and p-RPA32 (serine 4/8) (C) or for β -actin (A-C) as a loading control. Representative data are shown from $n=3$ independent experiments. Graphs show normalized means $\pm$ SE of three independent experiments; * indicates $p \leq 0.05$.

Ku70 is sufficient to protect against radiation-induced accelerated senescence in MSC.

Our experiments indicated that in NHAs, Ku70 expression was increased within 15 min of radiation exposure, and had the highest level of increased expression, suggesting that this repair enzyme may play a critical role in NHA

radiation resistance. Previous literature in tumor cells has also shown a correlation between Ku70 expression and radio-sensitivity (201; 458). Accordingly, we next sought to determine if increased Ku70 expression could rescue MSCs from undergoing radiation-induced senescence. Ku70 was ectopically expressed in MSCs for 24 h prior to 10 Gy X-ray irradiated or sham irradiation. Co-expression of eGFP was used to identify transfected cells; overall transfection efficiency was ~15%, comparable to published findings for this cell type (253). Expression of p21/waf was detected by immunohistochemistry at 24 h post-irradiation as a marker of senescence after radiation exposure (Fig. 8). In cells transfected with a control vector, 10 Gy radiation significantly increased the number of cells with up-regulated p21/waf1. In contrast, in cells transfected with Ku70, radiation exposure lead to a statistically lower percentage of cells that exhibited an increase in p21/waf1 expression.

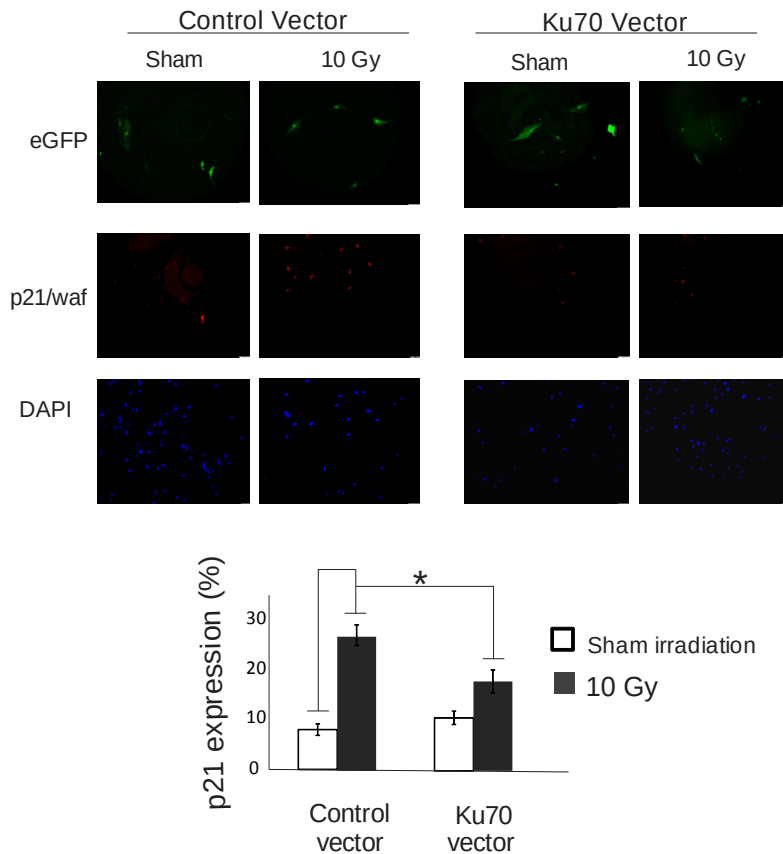


Figure 8 Overexpression of Ku70 decreases expression of p21 in MSCs after radiation injury.

Human mesenchymal stem cells were grown to 70% confluence before transfection with Ku70 expression vector + eGFP expression vector or control vector + eGFP then incubated overnight. Cells were then sham irradiated or exposed to 10 Gy x-ray. Immunohistochemistry for p21/waf1 was done at 24 h post-irradiation. DAPI staining was performed to label all nuclei; eGFP expression, which served to identify transfected cells. Bottom panel shows overlay of three other panels; blue arrows indicate eGFP positive cells (transfected) that are positive for p21/waf1 and white arrows indicate eGFP negative cells (untransfected) that are positive for p21/waf1. Panels show 20× magnification. Transfected cells (eGFP positive cells) were counted and scored for p21/waf1 upregulation to reach 50 cells per plate (approximately 20 viewing frames, counting all transfected cells in each frame), for n = 3 independent experiments. Graph shows means ± SE of three experiments; * indicates $p \leq 0.05$.

Discussion

Here we have shown that NHAs are highly resistant to radiation damage, displaying the ability to proliferate after 10 Gy X-ray exposure with minimal levels of senescence and no evidences of apoptosis. Our findings indicate that NHAs robustly up-regulated DNA repair enzymes of the NHEJ pathway as well as the HR pathway following X-irradiation. In contrast to NHAs, we observed that MSCs displayed a radiation sensitive phenotype following 10 Gy X-ray exposure, as reflected by the activation of SA- β -gal, a sustained increase in p21/waf1 levels, and an inability to proliferate. The present findings that markers of NHEJ DNA repair are robustly upregulated in NHAs versus MSCs, and the demonstration that ectopic expression of the NHEJ repair enzyme Ku70 is sufficient to rescue MSCs from undergoing radiation-induced senescence, supports the proposal that enhanced NHEJ underlies the radio resistance of NHAs

Our findings indicate that radiation initially induced similar levels of DNA damage, as indicated by similar numbers of γ -H2AX foci in both NHA and MSC. However, NHA displayed a more rapid reduction of γ -H2AX foci. These findings suggest that the persistence of DNA damage in MSC may be associated with the development senescence in these cells, with increased SA- β -gal activity and sustained up-regulation of p21/waf1. Senescent cells display a host of aberrant biological characteristics including an inability to proliferate and migrate, increased pro-inflammatory protein secretion, and genomic instability (85; 278). These characteristics of senescent cells may be related to tissue dysfunctions observed following radiation injury, including repair failure, persistent inflammation, fibrotic remodeling, and predisposition to the development of cancer (85; 278). The resistance of astrocytes to senescence may be a part of the protective phenotype that these cells display in neuronal tissues.

In our investigation of DNA repair pathway regulation after radiation injury, we found significant differences in the capacity of NHA and MSC to regulate the enzymes of both NHEJ and HR. We found that XRCC4 and Ku70, two proteins integral to NHEJ, were up-regulated in NHAs but not MSCs. Although Ku70 is often considered for its role in DNA repair, recent studies indicate that in the cytoplasm, Ku70 may also regulate apoptosis and p53 expression (217; 442). It is not known whether these functions also play a role in the protection of astrocytes following radiation exposure. The protection of MSC by over-expression of Ku70 also may involve both nuclear and cytoplasmic roles of Ku70. Taken together our data suggest that Ku70 is sufficient to protect cells from radiation induced senescence. Previous research demonstrated the resilience of astrocytes and their ability to proliferate after exposure to redox stress or ionizing radiation *in vivo* in rodents (22; 479). Cell culture investigations of astrocytes have also provided evidence for resistance to redox stress (52; 427; 474). Our finding of the resistance of astrocytes to radiation damage is consistent with the pro-survival effect of astrocytes on other cells following radiation exposure or redox stress (52; 130; 377). Our findings expand the current knowledge of astrocyte resistance to X-ray-induced cell death or

senescence. Further research is necessary to understand the specific mechanisms by which enzymes of NHEJ and HR are robustly upregulated in astrocytes following radiation exposure.

Chapter 3

Abstract

Production of reactive oxygen species (ROS), ionization of biological macromolecules, and subsequent mitochondrial dysfunction are believed to play a major role in cell death after injury. Normal human astrocytes have previously been shown to upregulate antioxidant enzymes and detoxifying proteins in response to other forms of oxidative stress, such as hydrogen peroxide, traumatic brain injury, and ALS mouse models. We hypothesized astrocytes would similarly demonstrate a strong antioxidant response after radiation exposure. To test this we first used MitoSOX and dichlorofluorescein to observe ROS expression at multiple time points after 10 Gy x-ray radiation. We next tested ATP presence in both NHAs and MSCs at time points 24, 48 and 72h post radiation as a method of determining mitochondrial health. Western blotting was done to determine protein expression levels of MnSOD and catalase. Immunofluorescence was used as an indirect method to determine activity of the proteins thioredoxin and Nrf2 by translocation to the nucleus. Surprisingly, we did not see the expected upregulation of protein or activity levels after radiation injury in the antioxidants examined: MnSOD, glutathione, catalase, thioredoxin and Nrf2. Additionally, mitochondrial oxidative stress and ATP production were not significantly perturbed at later time points after radiation injury. This suggests that antioxidant response pathways are not strongly activated in normal human astrocytes in response to radiation exposure.

Introduction

The destruction of normal tissue is the major concern that limits the use of radiation therapy for the treatment of malignant tumors (24). Thus, while radiation therapy is a first line treatment for brain and spinal cord tumors and metastases there is concern over the long term neuronal damage, including cognitive impairments, typically associated with its use (141; 261). The duration of cellular responses to radiation can vary greatly from restoration of normal expression occurring within 24h to continued dysfunction and dysregulation continuing out to 2 months (83; 428). Thus, the brain and other neurological tissues may experience profound and long lasting effects of radiation exposure, and the effectiveness of protective cellular responses are critical for mitigating potential injury and promoting tissue repair. This variation is likely due to differing cell type, types of radiation, dose rates of radiation, and total dose.

Initial tissue damage from radiation occurs extremely rapidly, but can continue through the cascading events. The initial cellular damage in response to

radiation exposure occurs in less than 1 picosecond; additionally, subsequent generation of reactive oxygen species including superoxide and hydroxide radicals are mostly quenched within 5 minutes after radiation exposure (76; 220; 335). However, even after the dispersion or quenching of initial ROS species, ionizing radiation induces longer lasting increases in oxidative stress. This is due to decreases in cellular antioxidants and detoxification molecules, and damage induced mitochondrial dysfunction which causes secondary production of ROS (374; 476). Homeostasis of the nervous system relies heavily on a careful redox balance, with reactive oxygen species (ROS) having impacts on cell fate, neurogenesis, cell survival, and apoptosis and senescence (168). The antioxidant response element (ARE) is responsible for the transcriptional activation of antioxidant genes under basal and oxidative stress conditions (283). Nuclear factor E2-related factor 2 (Nrf2), a potent transcription factor activated under conditions of redox stress, is credited with binding and activation of ARE to produce one of the cell's most potent and immediate antioxidant defenses in response to ROS exposure (265). Activation of ARE produces an increase in phase II antioxidant proteins including glutathione S-transferase (GST), manganese superoxide dismutase (MnSOD), catalase, and thioredoxin (THX) (21; 393). Under basal normoxic conditions Nrf2 is post transcriptionally regulated by Kelch-like erythroid cell-derived protein (Keap1) which binds Nrf2, preventing its entry into the nucleus and aids in ubiquitylation and degradation of Nrf2 (438). Oxidative stress causes Nrf2 release from Keap1, allowing it to translocate to the nucleus and induce expression of the phase II antioxidant genes to improve cell survival in both normal and tumorigenic cells (172; 198; 326; 380). Knockdown of Nrf2 induces increased cellular sensitivity to oxidative stressors in murine models and in vitro tumor and normal tissue (163; 175; 380).

Survival after radiation injury may be attributed to at least two mechanisms: quenching of ROS and effective DNA repair (368). Research into cancer cells has indicated that higher expression and activity of antioxidant enzymes and their products is key to survival after oxidative stress. Radiation resistant glioma subtypes demonstrate increased activity of MnSOD, glutathione peroxidase (GPx), and catalase compared to their more radiation sensitive parent strain both before and after radiation injury (222). Similarly exogenous overexpression of GPx, MnSOD or THX reduces apoptosis and improves survival after oxidative stressors from ionizing radiation or staurosporine in murine models as well as tumor cells and normal cells (20; 92; 105; 165; 409; 412; 464).

Nrf-2 controls key antioxidant response pathways that are necessary for survival of cancer cells in vitro and in murine models following radiation exposure. Specific antioxidant enzymes downstream of Nrf2 activation include MnSOD, GPx, THX, and catalase (107; 423). Upregulation of Nrf2 to activate early responses to control redox stress promote cell survival. Additionally, knockdown of Keap1 (a negative regulator of Nrf-2), disruption of Keap1-Nrf2 binding (to allow Nrf-2 activation), or pharmacological amplification of Nrf2 produces cytoprotective

responses compared to controls when subjected to oxidative stress both in murine models and in vitro in NCM460 colonic cells, primary neonatal rat astrocytes (82; 250; 295).

Depletion or knockdown of antioxidants together or separately can produce sensitization to oxidative stressors in both murine models and tissue culture. Complete knockdown of MnSOD/SOD2 produces a neonatal lethal mutation in mice in which offspring are produced but die weeks after birth (486). Downregulation of catalase expression is associated with the autosomal disorder Granular Corneal Dystrophy Type II which promotes oxidative stress and apoptosis in corneal fibroblasts even in the absence of injury (74). Prolonged depletion of glutathione or thioredoxin significantly decreases survival in murine models as well as tumors and normal cells in culture (146; 147; 281; 310; 340). Failure to control ROS can produce damage to DNA and other macromolecules as well as damage to organelles including the mitochondria which controls energy production (353). This damage to the mitochondria and its respiratory chain complexes leads to failure to produce energy which results in apoptotic or necrotic cell death; this pathway choice may be dependent on tissue type (257; 450).

Based on this evidence, we hypothesized that NHAs would upregulate multiple antioxidant pathways to effectively handle the production of ROS which occurs after radiation injury. This ability to handle radiation injury was set to be measured in the cytoplasm by DCF and in the mitochondria by MitoSOX. Further, long term damage from ROS would be measured by survival of the cells and ability to produce ATP, as this is a mechanism for measuring mitochondrial function.

Methods

Reactive Oxygen Species Assay

MSCs (Lonza) and NHAs (Lonza) were irradiated or sham irradiated at 70% confluence in 96 well assay black bottom plates (Corning, Corning NY, cat#3603) then treated with 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Thermofisher, Waltham MA, cat#D399) or MitoSOX (Thermofisher, cat#m36008). Briefly, cells were washed twice in pre-warmed PBS then incubated with 5 μ M MitoSOX for 20 minutes at 37°C or with 10 μ M H₂DCFDA for 20min at 37°C. Cells were then read on a microplate reader at excitation and emission wavelengths as recommended by the company. The negative control was done for each time point and each plate. It consisted of an empty well containing no cells which was filled with H₂DCFDA or MitoSOX. Numbers obtained from irradiated cells were subtracted from the negative control of their own plate at the same time point. Numbers from sham irradiated cells were subtracted from the negative control of their own plate. The standardized irradiated cell numbers were divided by the standardized sham irradiated cell number. This was performed in triplicate and the average is shown. A ratio in which the irradiated cell number was the same as the

sham irradiated cell number would yield a ratio of 1:1. Samples were done in triplicate and N=4 was performed for each condition and time point.

ATP measurements

ATP measurement were performed in collaboration with Drs. Snehalata Pawar and Sudeep Banerjee (University of Arkansas for Medical Sciences). Cells were washed with warm PBS then trypsinized and counted once they reached approximately 1.5×10^6 or 4.5×10^6 . They were then centrifuged at 1000xg for 5min at 4°C. Cells were then washed twice with cold PBS and centrifuged at 1,000xg for 5min. After the second wash the cells were resuspended in 1mL PBS and centrifuged 10,000xg for 30 seconds. Supernatant was discarded and pellets were treated with 0.1% triton x-100 for 1 h on a shaker in the cold room. Cells were then centrifuged at 14,000xg for 10 minutes at 4°C. The supernatant was moved to a new tube then flash frozen in liquid nitrogen. Once all samples were collected they were shipped on dry ice to Drs. Pawar and Banerjee.

Nrf2 and thioredoxin expression and activation after radiation injury

Cells were irradiated then cultured for 30 min, 2h and 6h or were sham irradiated and cultured for 6h. Cells were washed (3 times with phosphate buffered saline [PBS]), then treated for 10 min with 4% paraformaldehyde (room temperature) and washed again (3 times with PBS). Cells were permeabilized with 70% ethanol (10 min, room temperature), blocked with 5% normal donkey serum in PBS, 1 h, 37°C, and incubated for overnight at 4°C with Nrf2 antibody (Novus Biologicals, MAB3925) diluted 1:400 in 5% normal donkey serum in PBS. Anti-thioredoxin antibody was obtained from Novus Biologicals (cat H00007295-M01). Primary antibody was used 1:400, Donkey anti-mouse IgG secondary (Thermofisher) was used 1:1000. Cells were washed 3 times with PBS and then incubated for 1 h in Donkey anti-mouse IgG (Thermo Fischer, A21206) in PBS at room temperature. Cells were washed (3 times, PBS), incubated with DAPI (4',6-diamidino-2-phenylindole) (5 min, room temperature, in dark) diluted 1:1000 in PBS, then mounted and visualized with an Olympus BX61 fluorescence microscope (Olympus, Center Valley, PA) using 10× magnification at 488nm. Nrf2 positive nuclei were counted. Approximately 100 DAPI-positive cells per plate were counted. Nrf2 positive nuclei were measured as a percentage with cells containing positive nuclei divided by total cells in a random field then multiplied by 100. One way Anova was performed to obtain significance.

Western Blotting

Cells were grown to 70% confluence then irradiated and collected 15 minutes, 2h, 6h, 12h, 24h, 48h, 72h post radiation injury. These cells were then treated with RIPA buffer and Holt protease and phosphatase cocktail. Cells were sonicated for 10 seconds in a cold room before being centrifuged at 15000xg for 15 minutes at 5°C. B-actin (Santa Cruz, sc-47778, 1:1000), MnSOD (Abcam, cat#ab86087), catalase (Santa Cruz, cat# sc-50508, 1:500) . N=3 was performed for all time points after radiation injury and the sham irradiated condition and t-test

was used to compare sham expression of catalase or MnSOD between NHAs and MSCs. When comparing sham values to irradiated values the sham value was multiplied by a number to make the sham value equal to 1. The irradiated values were multiplied by the same number in order to get a modified value to compare to the sham value.

Superoxide Dismutase Activity assay

The activity levels of SOD was measured via an SOD determination kit (Fluka/Honeywell, Morris Plains NJ, cat19160). Cells were grown to 70% then irradiated and extracted at indicated time points using a modified buffer (0.5% sodium deoxycholate, 1% triton X, Holt Protease and Phosphatase inhibitor) in 1xPBS. Enzyme working solution and WST working solutions were added and blanks were used according to manufacturer's protocol. Samples were incubated in a 96 well plate for 20min at 37°C then read at 450nm to calculate SOD activity. SOD activity was obtained via proprietary equation (Fluka) Activity rate = inhibition potential (%) = $[(\text{blank 1} - \text{blank 3}) - (\text{sample} - \text{blank 2})] / (\text{blank 1} - \text{blank 3}) \times 100$. Blank 1 contained water, WST and enzyme working solution with no dilution buffer. Blank 2 contained sample and WST but no enzyme solution and dilution buffer while blank 3 contained working solution, water and dilution buffer. WST is a tetrazolium salt which demonstrates color change in the presence of a superoxide anion produced with the aid of the enzyme solution. This process is inhibited by the activity of SOD within the samples. Higher SOD activity of samples causes inhibition of WST color change. Activity for NHAs and MSCs at 24h, 48h and 72h or sham irradiated were initially collected and measured separately. 30min and 2h or sham irradiated were collected and measured simultaneously to observe potential differences in activity levels between sham irradiated MSCs and NHAs when no obvious change in activity was observed at later time points. One way or two way ANOVA was utilized. N=3 was performed for all time points and sham irradiated condition.

GSH assay

Cells were irradiated or sham irradiated and collected at 24, 48, or 72 h post injury. This assay was performed as previously described (86). Briefly, cells were trypsinized, collected, then 500ul was taken for BCA assay. The remaining solution was spun at 200xg, 5 min, 4°C, then washed 2x with 1x PBS on ice. 0.1% perchloric acid was used to resuspend the cells before they were sonicated 10 sec in a cold room. Cell lysates were centrifuged 14,000 x g for 10 min at 4°C. Samples were stored in -20°C until used. Frozen cell lysates were thawed on ice then brought to pH 6.5-7.0 by potassium hydroxide-3-(N-morpholino)propanesulfonic acid (MOPS). Then samples were sonicated and centrifuged 14,000 x g at 4°C for 10 min. Oxidized glutathione of varying concentrations between 1-10nmol were used as reference. The Assay buffer solution (0.1M sodium phosphate (mono and dibasic were mixed evenly) with 5 mM EDTA, pH 7.4) was used to dilute standards and samples. 5,5-dithio-bis-(2-nitrobenzoic acid (DTNB), NADPH, and glutathione reductase were utilized as described to produce reaction mixtures which were

added to samples and standards (426). The reaction was measured at 410nm absorbance for 3 min at 1 min intervals on Synergy H1 BioTek spectrophotometer (Winooski, Vermont). Oxidized glutathione, glutathione reductase, DTNB, and NADPH were all purchased from Sigma Aldrich. N=3 was performed for all time points and sham irradiated condition.

Statistical Analysis

Comparisons between sham samples of the two cell types for catalase and MnSOD concentrations utilized t-test. Comparisons of irradiated samples and sham control utilized one way ANOVA. For the MitoSOX and ROS assay, one way ANOVA was used to compare different time points for one cell type. To compare time points between two cell types, two way ANOVA was utilized on SPSS software where $p > 0.05$ was taken to indicate statistical significance.

Results

ROS expression was increased at early time points post radiation injury

Radiation injury can produce long lasting alteration in ROS expression in cells in either the cytoplasm or the mitochondria (350). Increased and uncontrolled ROS are linked to cell dysfunction and death. To measure this potential change in the cell DCF and MitoSOX were used to measure ROS in cytoplasm and mitochondria, respectively.

Levels of ROS after radiation injury measured by DCF or MitoSOX varied depending on the experiment, though general trends could be observed. Findings varied for the peak of ROS generation as well as cell type generation of ROS. Positive controls for the experiments provided consistent results, indicating that the variation in findings was due to response to radiation induced ROS, and not experimental error. The DCF assay indicated increased levels of ROS generation occurred in the cytoplasm between 24-72 h, but the peak increases in ROS were observed at varying times in each experiment (performed in triplicate). In addition, two experiments demonstrate statistically significant increases in DCF expression in NHAs, and only one experiment demonstrated statistically significant DCF expression in MSCs but not in NHAs. An example of peak at 72 h post-irradiation in NHAs shown in Fig. 9. These data suggest that radiation induces increased ROS with a significant delay post-irradiation, but that the timing of the ROS generation varies. Potential sources of variation could include the passage number of the cells or the cell cycle at the time of radiation exposure. Both cell types were used within passage 3-6 for this experiment. For other assays this difference in passage number did not result in significant alteration of the experimental findings. We were unable to synchronize the cells, since serum depletion induced senescence or apoptosis.

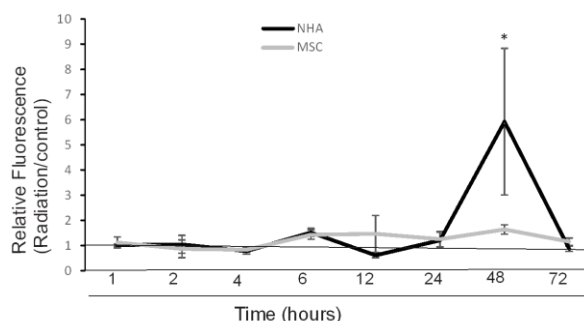


Figure 9. Effect of 10 Gy X-ray radiation exposure of normal human astrocyte and mesenchymal stem cell on the production of reactive oxygen species in the cytoplasm.

Normal human astrocytes (NHA) and normal human adult mesenchymal stem cells (MSC) were plated at ~70% confluence in 96 well plates in triplicate. Cells were sham irradiated or exposed to 10 Gy X-ray. DCF was applied as previously described and read at the indicated time points post-irradiation. Data from a single representative experiment is shown. Graphs show means $\pm$ SEM. Negative controls were subtracted from irradiated and control samples, then irradiated samples were divided by controls. A value of 1 indicates readings for irradiated cells were equivalent to readings for control cells. * indicates statistical significance between NHAs and MSCs was observed at 48h.

In MSCs and NHAs the MitoSOX assay indicated increased ROS (superoxide) production at 2-6h post-irradiation (Fig. 10). The MitoSOX assay also provided varying results with each experiment for the peak of ROS generation. As stated for the DCF assay, changes in time points of peaks for mitochondrial ROS production was potentially affected by passage number of cells and an inability to serum starve and control cell cycle. The induction of mitochondrial ROS corresponded roughly with the time for the induction of some changes in DNA repair enzymes (See Chapter 2).

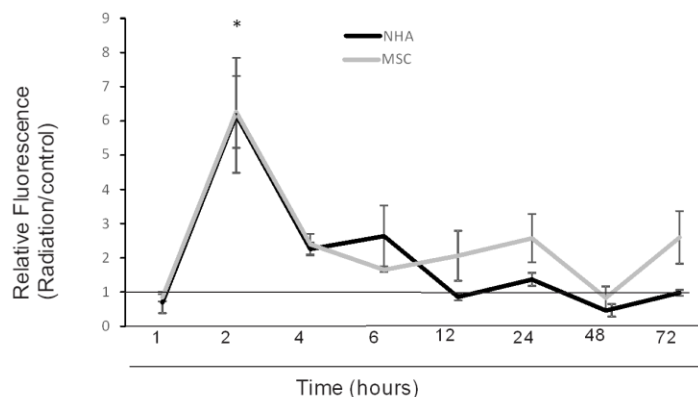


Figure 10. Effect of 10 Gy X-ray radiation exposure of NHA and MSC on the production of reactive oxygen species in the mitochondria by MitoSOX assay.

NHAs and MSCs were plated at approximately 70% confluence in 96 well plates and sham irradiated or exposed to 10Gy X-ray. Samples were run in triplicate but n=1 shown. MitoSOX was applied as previously described and read via spectrophotometer at the indicated time points. Graphs show cells plated in triplicate $\pm$ SEM. Negative controls were subtracted from irradiated and control samples, then irradiated samples were divided by controls. A value of 1 indicates readings for irradiated cells were equivalent to readings for control cells. To compare time points within

cell type one way ANOVA was used * indicates $p < 0.05$. To compare time points between cell types, two way ANOVA was used.

ATP production did not statistically drop after radiation damage

To investigate if increases in ROS production were relevant for cell death and mitochondrial dysfunction we examined ATP production. ATP levels are expected to decrease in cells with significant mitochondrial damage. ATP production was not significantly decreased in either cell type (Fig. 11). These findings are consistent with our MitoSOX assays that indicated that mitochondrial ROS generation occurred at a peak of ~2-6 h post-irradiation, and then ROS generation returned to normal levels. Together these findings suggest that radiation did not induce catastrophic mitochondrial damage in either cell type.

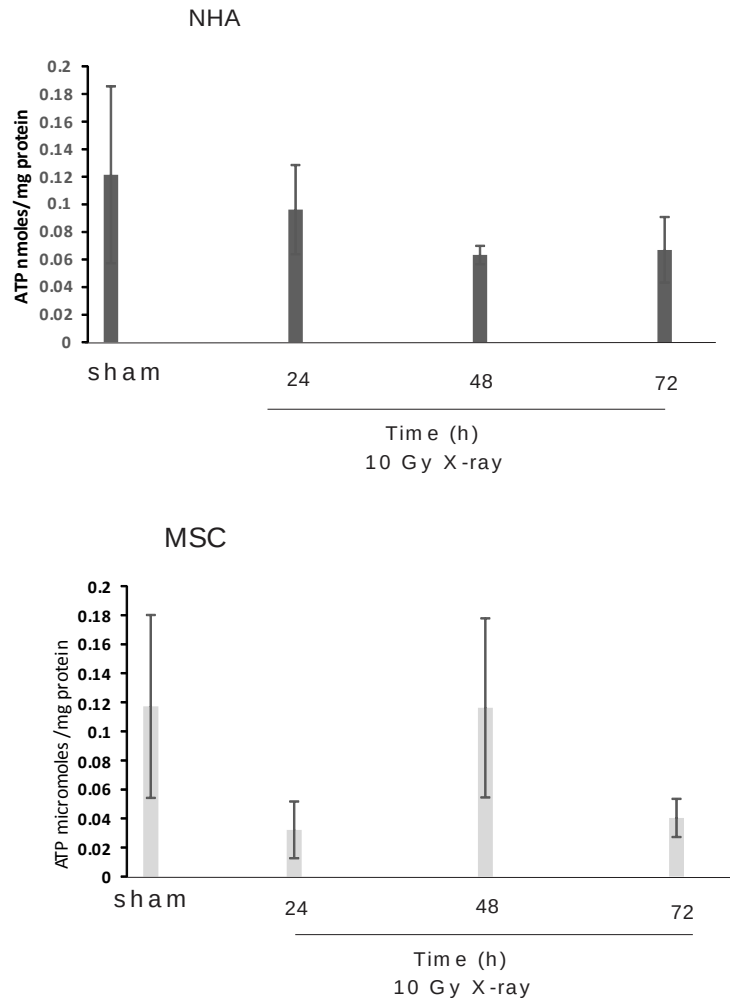


Figure 11. Radiation does not significantly decrease ATP levels in MSCs or NHAs.

ATP concentrations were determined per total protein concentration of NHAs and MSCs at indicated time points. Representative data are shown from $n=3$ independent experiments. Graphs show means $\pm$ SEM of three independent experiments. No significance observed using one way ANOVA

Nrf2 localization in the nucleus was observed in NHAs but not MSCs following X-ray irradiation.

Prior studies in A549 lung cancer cells, immortalized T cell Jurkats, and immortalized human corneal endothelial cells have shown that ionizing radiation is capable of upregulating Nrf2 expression within 6h post-radiation (238; 310; 487). Therefore, we anticipated that increased Nrf2 expression and nuclear localization would occur for NHAs and MSCs in response to radiation. However, Nrf2 expression was below detection in MSCs at all time points (Fig. 13). In contrast, NHAs displayed a basal level of Nrf2 expression and with ~35% of cells displaying localization to the nucleus (Fig. 12). Unexpectedly, there was no significant increase of Nrf2 entry into the nucleus at any time point post radiation injury in NHAs. These findings suggest that significant Nrf2 activation does not occur in NHA over the time course examined.

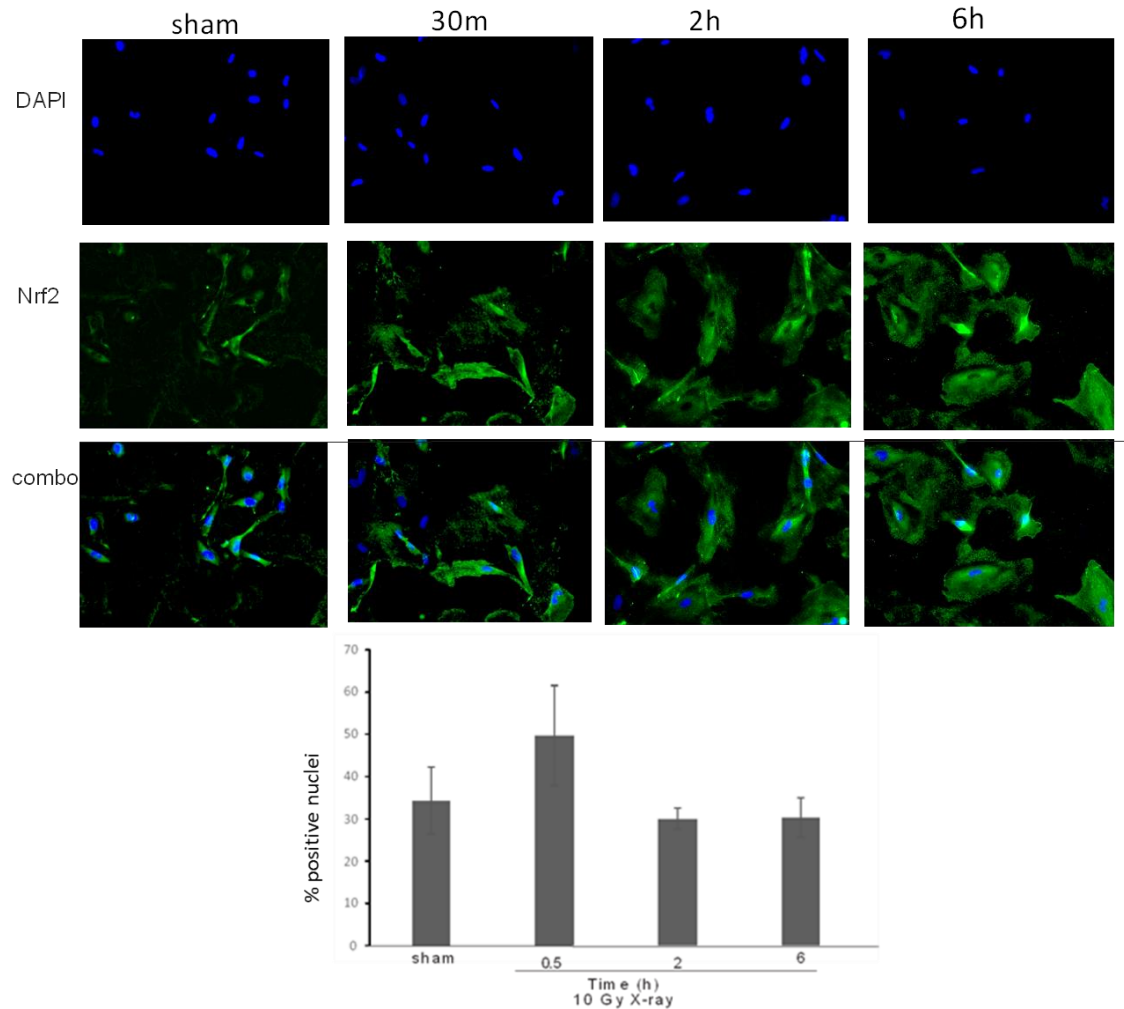


Figure 12. Nrf2 expression is present in NHAs but increase in nuclei after radiation does not occur

NHA were sham irradiated or exposed to 10 Gy X-ray irradiation. Upper panel: immunohistochemistry was performed at the indicated time points. Panels show representative images of Nrf2 or DAPI staining. 10x magnification is shown. Lower panel: Nrf2 expression within nuclei, as measured by overlap between DAPI and Nrf2 were counted in random fields to reach a minimum of 100 cells total to determine average numbers of Nrf2 presence in the nuclei. Three independent experiments were performed. Bar graphs indicate percentage of Nrf2 in nuclei of all cells counted $\pm$ SEM. No significance was observed using One Way ANOVA.

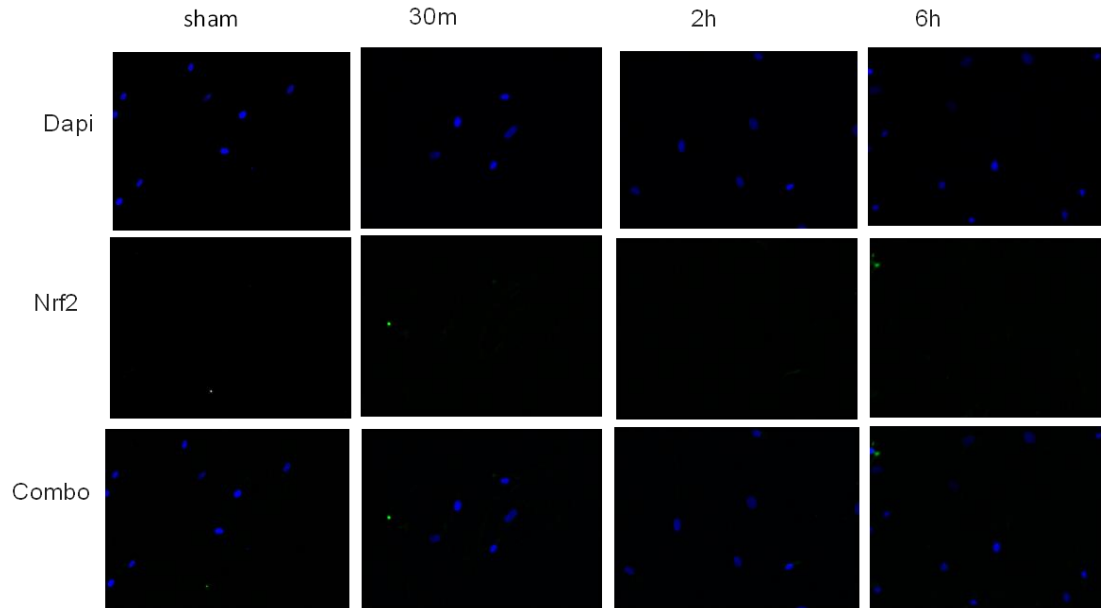


Figure 13 MSCs display no basal level of Nrf2 nor do they display upregulation after radiation injury.

MSC were grown to 70% confluence and sham irradiated or exposed to 10 Gy X-ray irradiation. Immunohistochemistry was performed at the indicated time points. Panels show representative images of Nrf2 or DAPI staining. Nrf2 expression was below background. 10x magnification is shown.

Effect of ionizing radiation on manganese superoxide and catalase in NHA and MSC

Manganese superoxide dismutase (MnSOD), an inducible enzyme located in the mitochondrial matrix, catalyzes the conversion of superoxide to H_2O_2 and oxygen (55). H_2O_2 can then be hydrolyzed to water and oxygen by either catalase or the glutathione system. MnSOD knockout results in embryonic lethality in mice, indicating its expression and activity are essential for development (457). Several studies have shown that MnSOD is increased in cells in response to oxidative stress (55; 69; 200). We therefore hypothesized that MnSOD expression would increase after radiation injury, to respond to oxidative stress. Our studies revealed that there was no increase in MnSOD protein levels in NHAs (Fig. 14A). However, a statistically significant increase was observed in MSCs at 12 h post-irradiation (Fig. 14B). Since activity of an enzyme is not always correlated with expression levels we then sought to measure the activity of MnSOD at both short (15 min and 2 h) and longer time points (24, 48, 72 h) following 10 Gy X-ray irradiation. We did not observe any statistical difference in activity between control and irradiated samples for either NHA or MSC at any time point (Fig. 15).

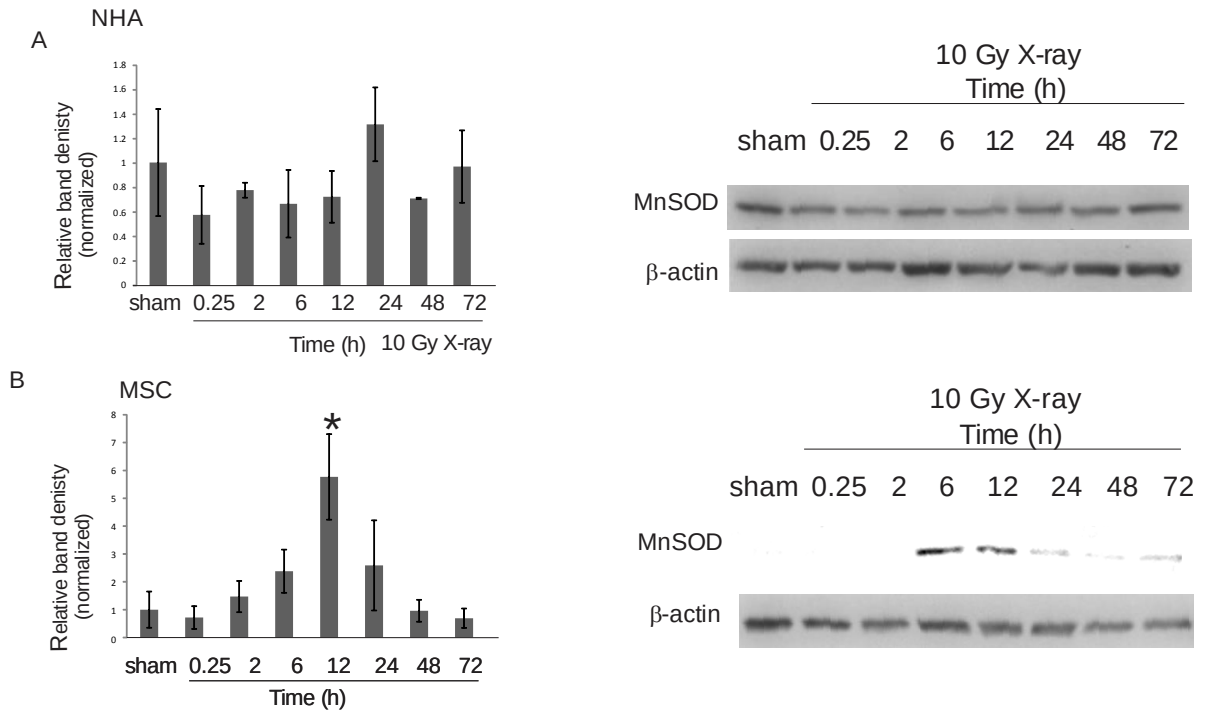


Figure 14. MSCs but not NHAs display a statistically significant increase in MnSOD at 12 h after radiation injury MnSOD.

Normal human astrocytes (NHA) or normal human adult mesenchymal stem cells (MSC) were grown to 70% confluence and sham irradiated or exposed to 10 Gy X-ray. Cell lysates were prepared at the indicated time points, and western blots were performed for MnSOD and for β -actin (A) as a loading control. Representative data are shown from three independent experiments. Graphs show means $\pm$ SEM, $n=3$; * indicates $p \leq 0.05$ via One way ANOVA

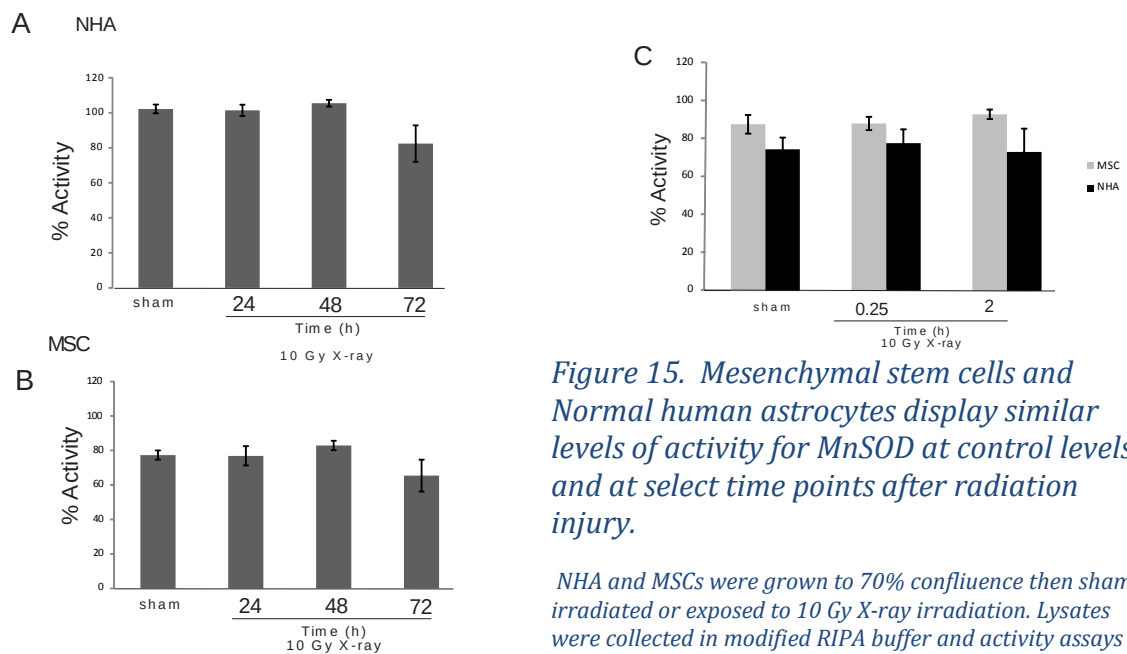


Figure 15. Mesenchymal stem cells and Normal human astrocytes display similar levels of activity for MnSOD at control levels and at select time points after radiation injury.

NHA and MSCs were grown to 70% confluence then sham irradiated or exposed to 10 Gy X-ray irradiation. Lysates were collected in modified RIPA buffer and activity assays were performed according to manufacturer's protocol at

the indicated time points, $n=3$. Data show means $\pm$ SD for all graphs.

Catalase expression has previously been shown to protect cells from radiation damage. Knockdown of catalase in a glioma cells produced sensitization to radiation and hydrogen peroxide induced oxidative stress (384). It was anticipated that catalase expression would increase as a mechanism to handle radiation induced oxidative stress. However, no significant change in expression was observed after radiation injury in either cell type, at any time point (Fig. 16). Additionally, no difference in basal expression levels were observed for MnSOD or catalase in MSCs and NHAs (Fig. 17). Together our data indicate that neither MnSOD nor catalase are regulated by X-ray irradiation over the time course examined. This is in agreement with our data showing that Nrf2 is not regulated in these cell types by X-ray irradiation, as Nrf2 is the transcriptional regulator believed to be activated for induced expression of MnSOD and catalase after ROS exposure.

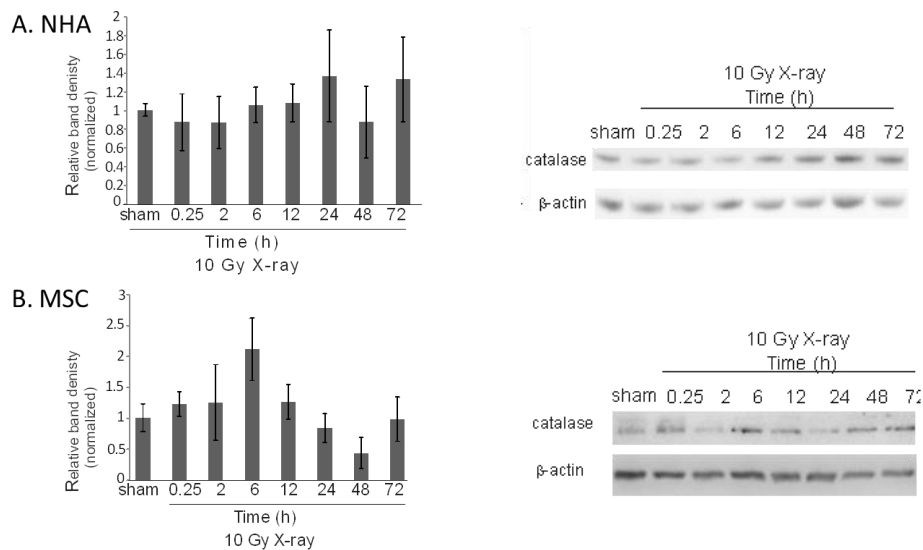


Figure 16. Neither MSCs nor NHAs display increased catalase expression after radiation injury.

NHAs and MSCs were grown to 70% confluence and irradiated at 10 Gy or sham irradiated and harvested at indicated time points. Westerns were performed for catalase; blots were stripped and reprobed for β -actin as a loading control. $N = 3$ for each time point. No statistical significance observed via One way ANOVA.

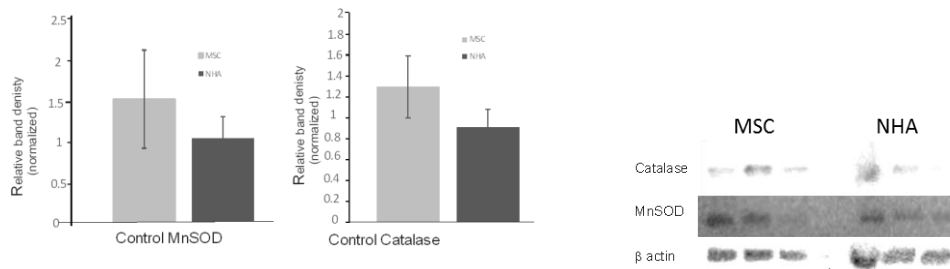


Figure 17. NHAs and MSCs show equivalent expression of MnSOD and catalase in control cells.

NHAs and MSCs were grown to 70% confluence and irradiated at 10 Gy or sham irradiated and harvested at indicated time points. Westerns were performed for catalase, MnSOD and β -actin. N=3. Expression levels of MnSOD and catalase were normalized to actin. No statistical significance via t-test.

Thioredoxin nuclear localization in NHA and MSC following radiation exposure

Thioredoxins act as electron donors and ROS scavengers through their disulfide active sites in enzymatic reactions that protect cells from oxidative stress (41). Thioredoxin requires thioredoxin reductase (TrxR) for the reduction of its active site; TrxR expression directly impacts the reduced or oxidized state of thioredoxins within the cell (224). Thioredoxin was demonstrated to translocate from the cytoplasm to the nucleus within an hour after 400 J/m² UVB or 10 Gy X-ray irradiation stimulation in HeLa, MCF-7 and immortalized human keratinocytes (HSC-1)s (160; 186). Once inside the nucleus, thioredoxin aids in modulation of redox factor 1 (Rel-1) and activator protein 1 (AP-1) factors, potentially for the regulation of oxidative stress responses. Thioredoxin entry into the nucleus improves cell proliferation and DNA repair through this cascade involved in DNA repair and cell proliferation (159). To measure thioredoxin expression and localization, immunofluorescence was performed on NHAs and MSCs (Fig. 18, 19 respectively). Thioredoxin was expressed in the nucleus in control conditions and did not rise to significance after radiation injury in either cell type, at any time.

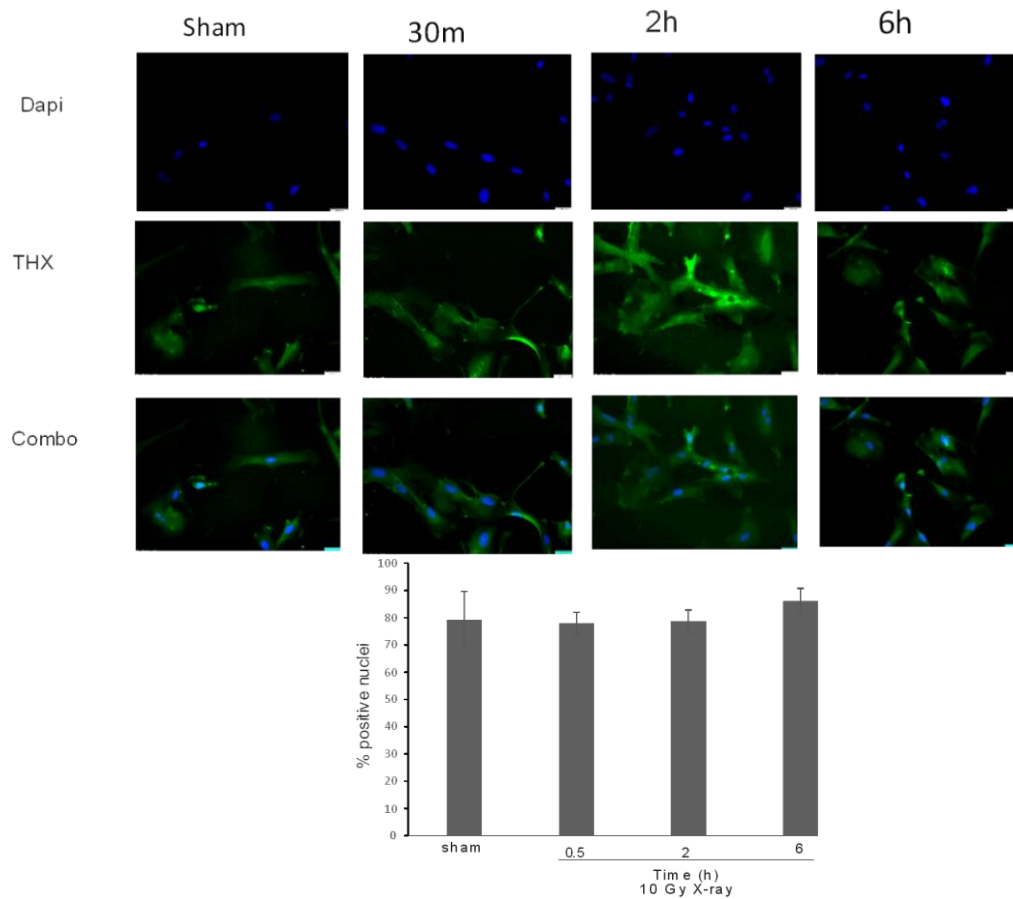


Figure 18. Thioredoxin is present in nuclei of NHAs at basal levels, but is not significantly altered after radiation exposure.

NHAs were sham irradiated or irradiated with 10 Gy X-ray at indicated time points. Upper panel: representative images of THX or DAPI staining 10x magnification is shown. Lower panel: THX expression within the nucleus, as measured by overlap between DAPI and THX were counted in random fields to reach approximately 100 cells. Three independent experiments were performed. Bar graphs indicate percentage of THX in nuclei of all cells counted $\pm$ SEM. $N=3$ for each time point. Sham cells treated at same time as 6h time point. No significant changes were determined by One way ANOVA

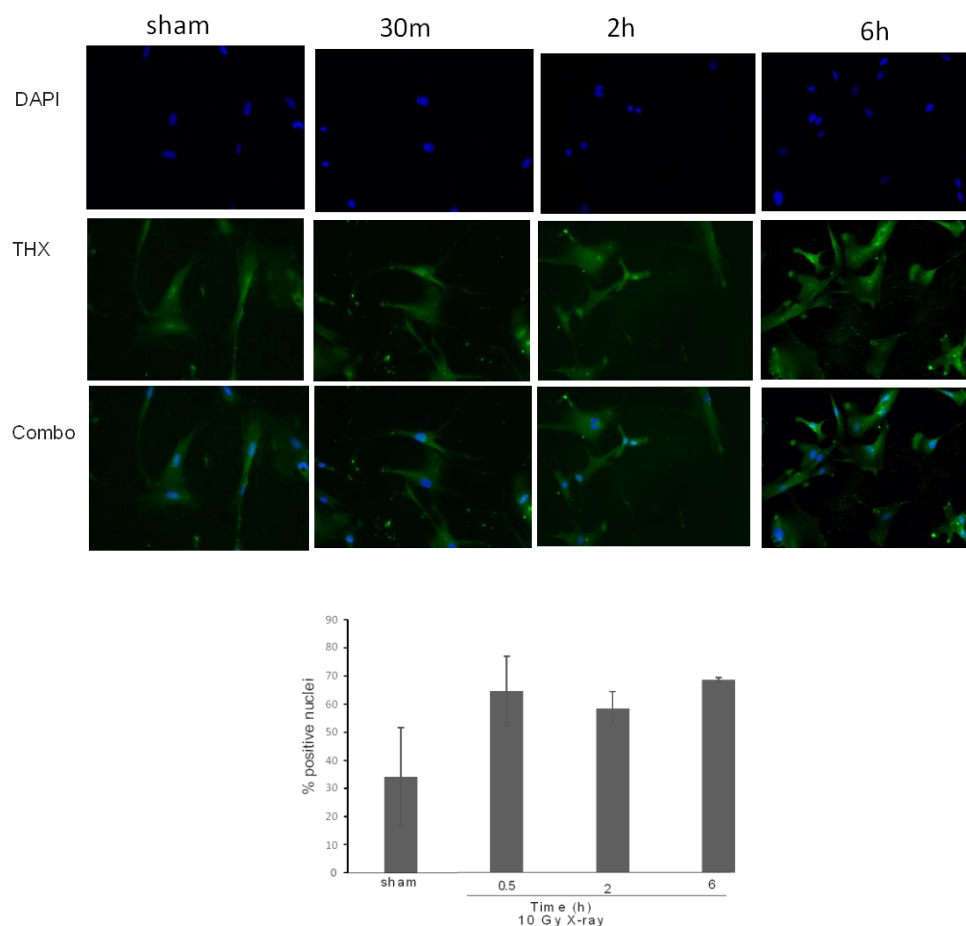


Figure 19. Thioredoxin is present in nuclei of MSCs at basal levels, and this does not rise to a statistically significant degree after radiation exposure.

MSCs were sham irradiated or irradiated with 10 Gy x ray at indicated time points. Upper panel: representative images of THX or DAPI staining. 10x magnification is shown. Lower panel: THX expression within the nucleus, as measured by overlap between DAPI and THX were counted in random fields to reach approximately 100 cells. Three independent experiments were performed. Bar graphs indicate percentage of THX in nuclei of all cells counted $\pm$ SEM. N=3. Sham processed at the same time as the 6h time point. No significance was indicated by One way ANOVA.

Glutathione levels showed a statistically significant increase after radiation injury in NHAs

Previously it was shown in U373-MG and HCT116 cells that increased glutathione biosynthesis via activation of the (PKR)-like endoplasmic reticulum kinase (PERK) pathway was associated with increased radiation resistance and enhanced survival (349). Similar increases in B16M melanoma tumor cell and HeLa cell survival, metastatic capacity, and radiation resistance were linked to high GSH content (294; 463). Conversely, depletion of GSH in B16M and modified radiation resistant HeLa cells (HeLaR) caused increased sensitivity to ROS and radiation (57; 463). We hypothesized that higher levels of glutathione would be associated with radiation resistance in the NHA. Interestingly, glutathione levels increased significantly at 24h post radiation injury in the NHAs, but then declined to near baseline levels at 48 and 72h post-irradiation (Fig. 20). Interestingly, MSCs had a higher basal concentration of glutathione compared to NHAs. MSC glutathione levels

did not change significantly at any time point following radiation exposure, although the levels remained higher than glutathione levels in NHAs at 48 and 72h post-irradiation. These findings suggest that glutathione levels are not primary indicators of radiation resistance for NHA. Additionally, glutathione levels are not an indicator of radiation resistance in MSC compared with NHA.

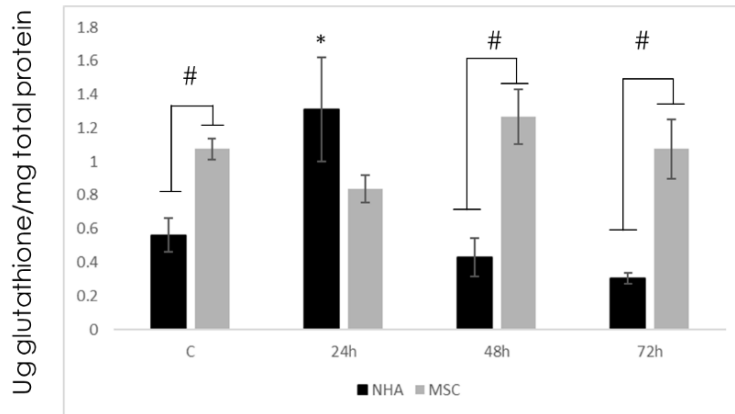


Figure 20. MSCs maintain higher glutathione levels up to 72h after radiation compared to NHAs.

*NHAs and MSCs were grown to 70% confluence then prepared for glutathione assay or BCA assay to establish protein concentrations. Cell lysates were prepared at the indicated time points then glutathione concentrations were determined. Representative data are shown from 3 independent experiments, each with n=3. Graphs show means $\pm$ SE of three independent experiments. N=3. * indicates significance between time points, one way ANOVA $p < 0.05$. # indicates statistical significance between cell types, two way ANOVA $p < 0.05$.*

Discussion

Our previous research indicated NHAs have the capacity to proliferate after exposure to high doses of X-ray irradiation (10 Gy) while MSCs undergo senescence following this level of radiation exposure. We hypothesized that NHAs would display a higher level of antioxidant capacity and/or upregulate antioxidant enzymes compared with MSC. We further hypothesized that NHA would have lower levels of intracellular ROS following radiation exposure compared with MSC. We investigated a numbers of antioxidant response pathways and enzymes that have previously been demonstrated to be elevated in radiation-resistant cells and/or to be activated in response to oxidative stress: Nrf2, thioredoxin, MnSOD, catalase or glutathione. Surprisingly we did not observe higher levels of antioxidant capacities in NHA compared with MSC, and we also did not observe upregulation of antioxidant proteins in response to radiation exposure in the NHA. Interestingly, we also did not observe a consistent upregulation of intracellular ROS following exposure to ionizing radiation in NHA. The control of ROS production was not clearly linked to upregulation of the antioxidant response markers we studied. We do not see catastrophic ATP loss or mitochondrial damage. This suggests that the antioxidant response of astrocytes is a mechanism for survival after radiation injury. However, we acknowledge that this response does not rely on the proteins we examined.

Previous research in normal and cancerous cell cultures have indicated that ROS may persist after radiation injury (19). Hematopoietic stem cells, a radiation sensitive cell type, display a statistically significant increase in intracellular ROS between 24-72h after 4 Gy X-ray exposure (468). This consistent and prolonged increase in ROS after radiation is observed in vivo as well. Bone marrow taken from mice that received 4Gy y ray showed increases in intracellular and mitochondrial ROS 12 days post exposure (467). Intestine from mice receiving 2 Gy y ray demonstrate intracellular and mitochondrial ROS up to 1 year post radiation (83). Uncontrolled ROS may lead to senescence, mutagenesis or cell death. We observed statistically significant spikes in ROS expression in MSCs and NHAs utilizing MitoSOX and DCF. However, this increase in ROS was not sustained nor was it consistent between experiments. Interestingly, prior research into ROS production due to glucose deprivation has similarly shown no significant upregulation of mitochondrial ROS at 72h in mouse primary astrocytes (69). Suggesting this control of mitochondrial ROS after radiation in NHAs is consistent with other literature focused on control of ROS. We sought an additional method of indirectly determining mitochondrial damage due to ROS: production of ATP. An inability to produce ATP due to damage to the mitochondria would lead to cell dysfunction, senescence or cell death. Interestingly, we did not see statistically significant drops in ATP production for either cell type. Together these data are consistent, and suggest that these two cell types recover from initial radiation exposure and suppress delayed ROS generation.

The importance of a strong antioxidant response is a key consideration in radiation resistant cancer cell types. Glioblastomas are reportedly radiation resistant and tend to contain high concentrations of these antioxidants including glutathione, peroxidoredoxin, catalase and Nrf2 (78; 383; 384). Decreases in these antioxidants leads to radiation sensitization in cancer cells (78; 383). Similar studies in normal human astrocytes have also indicated that these antioxidant proteins, particularly glutathione, are relevant for survival and protection of neurons after oxidative stress via hydrogen peroxide, nitric oxide and glucose deprivation (66; 97; 307; 373). Shockingly, NHAs do not display significant regulation of Nrf2 in response to radiation. Nrf2 is widely believed to be a primary regulator of MnSOD, glutathione, and catalase following oxidative stress. Therefore, our data is consistent in the lack of activation of Nrf2 and upregulation of MnSOD, glutathione, and catalase.

In sum, our findings suggest that while ROS production is suppressed in NHA post-irradiation, commonly activated antioxidant response pathways are not activated in these cells. Prior research indicates that astrocytes may utilize a host of different responses to protect and respond to injury including activation of the Hippo pathway and YAP, DNA repair and upregulation of growth factors such as fibroblast growth factor (FGF) (138; 197; 376). This study suggests that the majority of the antioxidants studied here may not be relevant to survival in the hours to days after radiation injury. Further research is necessary to determine whether other

antioxidant response pathways are activated in NHA to contribute to their survival following radiation exposure.

Chapter 4: Summary and Conclusions

I. Major Findings of this Investigation

Radiotherapy has been used for the clinical treatment of cancer since 1896 (133). The development of ion beams allowed for the treatment of deep tissue cancers, and radiation treatment for brain cancer was initiated in the 1980's-1990's (404). Despite this, only a handful of investigations have focused on astrocyte response to radiation injury. Several of studies hypothesized the potential response of astrocytes to radiation based on astrocyte response to other forms of oxidative stress, such as hydrogen peroxide. Certain scientific reviews report that astrocytes protect neurons, oligodendrocytes and endothelial cells from oxidative stress induced by radiation injury when in fact the referenced papers do not utilize radiation as its source of oxidative stress (113; 271). Other studies examined radiation effects on transformed glial cells (gliomas), showing an upregulation of antioxidant proteins, and extrapolating these data to predict that normal astrocytes would function the same way (354). These predictions were in conflict with findings from other studies which indicated that astrocytes may become activated in response to radiation, producing pro-inflammatory cytokines which may be linked to late radiation necrosis (245; 273). Other studies that inferred the responses of astrocytes to radiation also relied heavily on whole brain murine models and were not focused on the specific astrocyte response (70; 170; 418).

The research presented here is unique in focusing on the endogenous mechanisms of radiation resistance and repair in cultured normal human astrocytes (NHA). The main findings of this study is that astrocytes survive and proliferate after relatively high acute exposure to radiation (10 Gy) and that this survival is associated with robust upregulation of DNA double strand break repair pathways. NHAs significantly upregulated proteins in both the non-homologous end joining (NHEJ) and homologous recombination (HR) pathways as rapidly as 30 min following radiation exposure. Surprisingly, our studies failed to demonstrate significant upregulation of antioxidant responses in NHAs following 10 Gy radiation exposure. This contradicts prior literature postulating that astrocytes protect neurons and themselves against radiation injury through an antioxidant response.

II. Sufficiency of DNA Double Strand Break Repair for Radiation Resistance

Radiation injury induces DNA damage, which if left unrepaired can cause genomic instability leading to cancer or cell death (262; 358). Deficiencies in DNA repair have been noted in cell types shown to be more radiation sensitive (131; 235). In contrast, cells which upregulate DNA double strand break repair pathways demonstrate improved cell survival after radiation injury (37). Our data

demonstrates that astrocytes upregulate DNA repair for both NHEJ and HR pathways responses following radiation exposure. The earliest increase in DNA repair enzymes was observed for Ku70, a key enzyme in NHEJ. To determine whether increased expression of Ku70 was sufficient to protect cells from radiation-induced accelerated senescence, we investigated the effect of Ku70 overexpression in a more sensitive primary cell type, human bone marrow mesenchymal stem cells (MSC). When MSCs were transfected with Ku70 these cells also demonstrated significant protection against radiation-induced senescence, as determined by the expression of p21/waf, a marker for senescence. This data provides further support to the relative importance of activating double strand break repair mechanisms, and specifically for the upregulation of NHEJ, to prevent senescence in normal, untransformed, non-immortalized cells.

Only one other study focused on double strand DNA repair mechanisms of astrocytes. Schneider et al. (2012) examined the response of astrocytes to ionizing radiation, but found deficient DNA repair response (364). However, major experimental differences exist between the Schneider study and our findings. The 2012 investigation used modified astrocytes, which were produced from murine embryonic stem (ES) cell derived neural stem cells (NSC), and not cultured directly from adult brain tissue (364). The difference in DNA damage repair from astrocytes differentiated from ES-cell-derived NSC and cultured adult astrocytes has not been investigated. Additionally, the study by Schneider et al. compared DNA repair in the derived astrocytes to the ES-cell derived NSC themselves. In contrast our studies utilized normal human astrocytes which displayed robust upregulation of DNA double strand break markers compared to MSCs.

These findings indicated that both the NHEJ and HR repair pathways are upregulated in normal human astrocytes following radiation exposure. It is possible that upregulation of the HR pathway alone could sufficiently protect the astrocytes, and that after knockdown of both pathways would we see senescence or death after radiation. However, as a limitation of our study, we were unable to evaluate the relative contributions of the two DNA repair pathways for astrocyte survival following radiation. Experiments using RNAi to knockdown Ku70 in human astrocytes resulted in reduced survival of the astrocytes within 36 h, and experiments examining the effects of radiation exposure could not be conducted. Methods have been published for effective RNAi transfections in murine astrocytes and human glioma cell lines (U87 MG) (36; 177). Cancer cells are not an optimal replacement for normal tissue so the substitution of glioma cells for NHA was discarded. We considered the use of murine astrocytes as a fast solution to this problem. But attempts to grow mixed cortical mouse astrocytes (Lonza) and rat astrocytes (Lonza) have shown these cells to be even slower growing and more temperamental than NHAs (Supp Fig. 2). Future experiments to solve this problem could utilize lentiviral vectors expressing short hairpin shRNA to knockdown Ku70 mRNA in NHAs. Lentiviruses have previously been used successfully in normal human astrocytes (246; 330).

Our studies utilized a surrogate marker of DNA repair, γ -H2AX, to infer DNA repair rates in response to radiation. Both NHAs and MSCs displayed decreases in γ -H2AX over time, but MSCs became senescent while NHAs continued to proliferate. Decreased γ -H2AX levels in NHA positively correlated with our observations of increased DNA repair enzyme expression in NHAs. A second potential limitation of our study was the reliance on the expression of γ -H2AX as a marker of DNA DSB repair. γ -H2AX is an accepted and well-studied method for measurement of DNA damage and subsequent repair after radiation injury (331; 363), but it does not directly measure DNA double-stranded break (DSB) repair. A number of other methods are available to directly measure efficacy of DNA DSB repair. Long qPCR experiments directed at both genomic and mitochondrial DNA, a method utilized by Furda et al. (2014) (122), relies on the assertion that long PCR products can only be obtained from DNA strands that have been repaired correctly; using this method, DNA repair failure resulted in no product or short products (121). Experiments utilizing this method to measure DSB repair in our laboratory were inconclusive. Alternative methods to directly measure DNA repair include the dicentric assay and the micronuclei assay. However, both techniques are reportedly labor intensive and require expertise for effectively scoring results (4; 87; 363). Other options are to directly measure oxidation of DNA bases, sugar moieties or DNA crosslinking by mass spectrometry (94). Future investigation may use a more direct measurement of DSB repair to elucidate the rate and accuracy of DNA repair mechanisms in NHA compared with other cell types.

III. Antioxidant Response

Astrocytes have been shown to protect neurons from ROS via the glutathione system (98; 100). Similarly, the antioxidant response system has repeatedly been cited as a mechanism for astrocytic survival after various stressors (26; 433). It seemed reasonable to hypothesize that astrocytes would utilize their antioxidant response to protect against ionizing radiation. However, our investigation failed to demonstrate an increase in a number of antioxidant enzymes - including MnSOD, catalase, and thioredoxin - after radiation injury. Although we did see an almost significant increase in glutathione concentration in NHAs 24 h after radiation injury this concentration was roughly similar to the level of glutathione concentration in MSCs. NHAs did not have a higher concentration of glutathione than MSCs. Under basal conditions and 48 and 72 h after radiation injury MSCs displayed a statistically higher concentration of glutathione compared to NHAs. Additionally, the basal levels of catalase and MnSOD in MSCs seemed to be almost statistically higher than in NHAs. The only experiment where this trend was noticeably different was Nrf2 expression and location. NHAs displayed a higher basal level of Nrf2 while MSCs did not display any noticeable Nrf2. This did not translate to increased localization of Nrf2 in the nucleus after radiation injury. Only one study has linked Nrf2 activation with the HR DNA repair pathway (175). Further investigation into the role of Nrf2 in modulation of HR could provide a more insight into radiation repair and proliferation after injury.

IV. Astrocytes and the Bystander Effect

The Bystander Effect in radiation refers to the negative impact of radiation on cells or tissues outside of the radiation field. It may also refer to the damage and subsequent development of genomic instability, changes in gene expression and function of normal tissue despite initial avoidance of direct injury from radiation damage (382). Exposure of one side of an animal to irradiation while shielding the other side has shown to produce DNA damage and upregulation of DNA damage responses in the protected side (204; 206). The mechanism of the Bystander Effect is poorly understood and is postulated to be anything from scattered low levels of radiation to distress signals from damaged cells or epigenetic changes (203; 205). This is a particularly serious issue in the CNS where neurons, oligodendrocytes and endothelial cells display low levels of antioxidants (339). These low levels of antioxidants are blamed for some of the radiation sensitivity seen in these cells (233; 440). Additionally, there may be a role for gap junctions or other cell-cell contacts that may allow transmission of damaged proteins or signal transduction molecules (42).

Findings from the literature indicate that astrocytes can release a number of factors to modulate survival of other cell types following oxidative stress (see Chapter 1). Our investigation suggests that astrocytes do not upregulate antioxidant enzymes following radiation exposure. However, it must be understood astrocytes may release other types of molecules in order to modulate cell survival in other cell types. Glioblastoma stem cells show decreased radiation sensitivity which is credited to their increase activation of the JAK/STAT pathway after radiation injury via co-culture with normal human astrocytes through an unknown mechanism (328). Melanoma cells treated with chemotherapy were protected from death by astrocytes who took up excess calcium released because of the treatment (236). Upregulation of miR-29 in astrocytes has aided in protection of neurons after brain ischemia (298). Astrocytes have also released other factors including heat shock protein 70 (HSP70) and interleukin-6 (299). Other studies suggest that some factors released by astrocytes after radiation injury may be initially beneficial but could be linked to long term death of other cells such as upregulation of vascular endothelial growth factor (VEGF). VEGF upregulation in astrocytes and gliomas is associated with cell survival and proliferation for these cells (308), but is associated with BBB death due to endothelial dysfunction and radiation necrosis in white matter tracts (88; 459). Additional research is necessary to determine whether astrocytes can protect against the bystander effect following radiation exposure.

V. Astrocytes: Iconic or Atypical Radiation Resistant Mammalian Cell Type and Future Directions

The purpose of this investigation was to determine the radiation sensitivity/resistance of normal human astrocytes to ionizing radiation, and to determine the mechanism(s) utilized by NHAs to protect against radiation injury. Our experiments provide evidence that normal human astrocytes are one of the

most radiation resistant mammalian cell types studied to day. Our data indicate that robust DNA repair is critical to astrocyte survival following radiation exposure. Our data also indicate that regulation of common antioxidant enzymes do not play a role in astrocyte radioresistance. This has obvious implications for protection of other cells present in the CNS. The antioxidants we studied did not become upregulated after radiation injury, suggesting paracellular protection does not occur through these antioxidant pathways at the time points studied. Astrocytes may still protect neurons and other cells of the CNS, but it must occur through other mechanisms. It is also unknown whether the pattern of astrocyte responses to radiation occurs in other cell types.

Based on the findings presented here, it is reasonable to suggest further attention should be focused on the efficacy of astrocyte DNA repair after radiation injury. As observed in our data, upregulation of the NHEJ protein Ku70 in MSCs protected them from radiation induced senescence. Understanding the ability of normal, primary cells to effectively repair DNA may be highly relevant to protecting radiation sensitive cells from cytotoxicity and subsequent death or senescence. However other mechanisms besides antioxidant regulation and DNA repair may also play a role in astrocyte radioresistance. Other mechanisms to further study include the release of growth factors, control of gap junctions to decrease cytotoxicity, and the regulation of microRNAs. An improved understanding of mechanisms for radiation resistance by normal mammalian cells may provide insights for the development of radiation countermeasures for the protection of normal tissues in cancer radiotherapy and for protection against accidental radiation injuries.

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