Radiation resistance of normal human astrocytes: the role of non-homologous end joining DNA repair activity

Michelle A. Bylicky¹, Gregory P. Mueller¹ and Regina M. Day²,*

¹Department of Anatomy, Physiology, and Genetics, The Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, USA
²Department of Pharmacology and Molecular Therapeutics, The Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, USA
*Corresponding author. Department of Pharmacology and Molecular Therapeutics, Building C Room 2023, 4301 Jones Bridge Road, Bethesda, MD 20814–4799, USA. Tel: +1-301-295-3236; Fax: +1-301-295-3220; Email: Regina.day@usuhs.edu

(Received 14 June 2018; revised 3 August 2018; editorial decision 6 September 2018)

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 (NHAs) to X-ray irradiation. Following exposure to 10 Gy X-irradiation, NHAs exhibited DNA damage as indicated by the formation of γ-H2AX foci. Western blotting showed that NHAs 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 upregulated from 6–24 h, and then returned to baseline. Levels of DNA repair enzymes returned to basal ~48 h post-irradiation. NHAs re-entered the cell cycle and proliferation was observed at 6 days. In contrast, normal human mesenchymal stem cells (MSCs) failed to upregulate DNA repair enzymes and instead displayed sustained upregulation of p21/waf1, a cell cycle checkpoint marker for senescence. Ectopic overexpression of Ku70 was sufficient to protect MSCs from sustained upregulation of p21/waf1 induced by 10 Gy X-rays. 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 the development of novel targets for radiation countermeasure development.

Keywords: X-ray irradiation; astrocytes; mesenchymal stem cells; non-homologous end joining; homologous end joining; Ku70

INTRODUCTION

Radiation therapy is frequently used in combination with surgery and chemotherapy for the treatment of brain cancers, but each of these modalities has serious limitations. Poor penetration 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 [1]. Radiation damage to neurons is also a critical concern, and neuronal damage is a limiting factor for the use of radiation for cancer treatment in the central nervous system (CNS) [2]. 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 [3]. The long-term adverse effects of radiation injury on the CNS are manifestations of neuronal loss and include memory impairment, attentional deficits, poor coordination, blindness, seizures, and paralysis [4–6]. Some patients who receive multiple fractionations of doses of between 1.5 and 3 Gy with total exposures of 20–60 Gy develop dementia months to years after radiation therapy [7–9]. These cognitive deficits are correlated with structural abnormalities in the white matter of the corpus callosum, and deficits in the basal ganglia and thalamus following radiation treatment [9, 10]. Cell death,
unrepaired damage to surviving brain cells, and neuroinflammation are recognized as three hallmarks of radiation-induced CNS pathology [11–14]. Different tissues display variations in tolerance to radiation-induced damage, but the mechanisms underlying these variations are not completely understood, and this variation remains a fundamental question in radiation biology [15]. The survivability of brain neurons from any stress insult is dependent, in part, upon the support they receive from astrocytes [16–20]. Astrocytes are the most abundant cells in the human brain; they play integral roles in neuronal development, signaling, and plasticity [21, 22]. 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 [23–26].

Radiation can directly damage macromolecules within the cell or it can induce indirect damage through radiolysis of water molecules, generating reactive oxygen species (ROS) [27]. In some cases, the vulnerability of tissues to radiation injury is predicted by the Law of Bergonié 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 [28]. There are two major pathways for 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 [29, 30]. 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 [30–32]. DNA-dependent protein kinase catalytic subunit (DNA-PKcs) phosphorylates and activates multiple enzymes in this process, often under conditions of complex DSBs, although DNA-PKcs activation is not detected under all DNA damage conditions [33, 34]. Upon phosphorylation, Artemis gains endonuclease activity to cut 5’ and 3’ overhangs when complexed with and activated by DNA-PKcs [35]. DNA-PKcs activity is also relevant in regulating end processing and ligation of repaired DNA strands [36]. Ligation of repaired DNA strands occurs when DNA ligase IV interacts with X-ray Repair Cross Complementing 4 (XRCC4) [37]. In contrast, HR repair begins with 5’ DNA end resection, which produces a 3’ overhang [38]. This resection is performed by multiple proteins, including the Mre11-Rad50-Nbs1 (MRN) complex made of Mre-11, Nbs-1, and Rad50 as well as CtIP [39]. In addition, the MRN complex blocks NHEJ by inhibiting DNA ligase end joining [40]. Subsequently, ATM, ATR and DNA-PK (a key player in NHEJ) activate replication protein A (RPA) and phosphorylate its 32 kDa subunit at multiple sites, including ser4/8 [41–45]. RPA binds to single-stranded DNA (ssDNA) to stabilize it and prevent formation of secondary structures [46, 47]. A protein complex including RAD51 then retrieves the ssDNA from RPA [48]. RAD51 is the integral recombinase involved in search homology and strand invasion of the sister chromatid that allows DNA repair and subsequent synthesis of the damaged strand [49, 50]. Because of the importance of astrocytes for the protection of neurons and other cells during oxidative stress [24, 51, 52], and studies demonstrating that astrocytes have endogenous mechanisms of protection against redox stress [20], we investigated the response of normal human astrocytes (NHAs) to X-ray radiation and compared them with those observed in human mesenchymal stem cells (MSC), a well-established model system for the investigation of radiation cellular effects. We found that NHAs, unlike MSCs, are resilient to up to 10 Gy X-ray irradiation, with the capacity to proliferate following a dramatic upregulation 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 (NHAs) (cat. #CC2565, lot #0000402839, 0000412568), astrocyte-specific media (cat. #CC3186), trypsin (cat. #CC-5012), human mesenchymal stem cells (MSCs) (cat. #PT2501, lot #0000423370, 0000463158, 0000471980), and MSC media (cat. #PT3001) were obtained from Lonza (Walkersville, MD). At least two independent isolates of MSCs and NHAs were used. Note that other lots of NHAs 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 irradiation 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 s 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 (35.56 cm from the 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
Culture dishes (60 mm for NHAs and 35 mm for MSCs) containing ~50% confluent cells were plated prior to X-ray irradiation injury. NHAs and MSCs 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 temperature, 170–200g), 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 plated on 35 mm culture dishes, and then irradiated and cultured for 24, 48 or 72 h. Culture dishes containing cells were washed [three times with phosphate-buffered saline (PBS)], then treated for 20 min with 4% paraformaldehyde (room temperature) and washed again (three 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 three times with PBS and then incubated for 1 h in Donkey anti-rabbit IgG (Thermo Fischer, A21206) in PBS. Cells were washed (three times with 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 488 nm. 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 the manufacturer’s protocol (Cell Signaling). Briefly, MSCs and NHAs 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 at 220g for 5 min at 4°C twice and washed with ×1 PBS. MSCs and NHAs were washed twice with ×1 PBS. Cells were then incubated for 15 minutes on ice in the dark in ×1 binding buffer containing Annexin V-FITC conjugate at a concentration of ~1:200. MSCs and NHAs were then directly imaged. Imaging was performed on an Olympus BX61 fluorescence microscope (Olympus, Center Valley, PA) using ×10 magnification at 488 nm or using phase contrast.

β-galactosidase senescence assay
Cells were irradiated at 50–70% confluence, to avoid false positives, which can occur in confluent cell cultures [53, 54], 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-indolyl 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 the generous gift of Dr Shigemi Matsumura, Case Western Reserve University, Cleveland, OH [55]. Lipofectamine 2000 (Cat#11668027, Thermofisher) was used at a 1:3 ratio of Lipofectamine to vectors, diluted in Opti-mem media (cat#31985070, Thermofisher). The pCMV_Ku70 flag-tagged vector or control plasmid was con-transfected into MSCs with an EGF-CMV expression plasmid 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 three times with PBS and then extracted with RIPA buffer (50 mM Tris (pH 8), 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate and α1 Halt Protease and phosphatase inhibitor (cat# 78444, 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. #98297, Santa Cruz Biotechnology, Dallas TX) (1:500), p-RPA32 (cat. #A300–245A, Bethyl, Montgomery, TX) (1:500), RPA32 (cat#A300–244A, Bethyl) (1:500), Rad51 (cat. #98095S, Cell Signaling) (1:500), Ku70 (cat. #9261–9033, 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 ± standard deviations (SDs) 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. The statistical software used for all analysis was SPSS Statistics (IBM, Bethesda, MD).

RESULTS
Normal human astrocytes were 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 [25, 56, 57]. 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 [20, 51]. Here we sought to better understand the mechanisms involved by comparing radiation responses of NHAs with those of MSCs, a model cell type used in the investigation of radiation biology [58]. MSCs were previously demonstrated to be relatively resistant to radiation-induced damage, requiring 2 Gy to reduce the surviving fraction of cells to 37% [58]. Here we investigated a higher dose of radiation, 10 Gy, which was previously demonstrated in MSCs to reduce the surviving population to <1% [58]. NHAs and MSCs were exposed 10 Gy X-rays (0.989 Gy/min) during exponential growth phase, and cells were counted at 4, 5 and 6 days post-exposure (Fig. 1). Total cell counts revealed increased numbers of NHAs between Day 4 and Day 6 (P < 0.05) in both the sham and irradiated conditions. The increase in cell number could also be observed by light microscopy (Fig. S1). In contrast, only sham-irradiated MSCs had increased cell numbers between Days 4 and 6, and irradiated MSCs failed to proliferate over this time period. Clonogenic assays are often used to demonstrate the sensitivity of cells to radiation. However, repeated attempts at performing clonogenic assays on NHAs were not successful due to their slow growth and failure to form colonies.

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 [59]. Apoptotic cell death is one of the most common forms of cell death after radiation injury [60, 61]. Accordingly, a series of experiments were conducted to investigate apoptotic responses in NHAs and MSCs 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. 2). This is consistent with reports in the literature indicating that Jurkats undergo apoptosis after radiation injury [62]. Our findings in NHAs and MSCs were verified by western blotting for activated caspase-3, the common downstream caspase in both intrinsic and extrinsic apoptosis [63]. 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).

Findings in the literature demonstrated that, in primary non-transformed endothelial cells and keratinocytes, the primary response to ionizing radiation exposure was cellular senescence, rather than cell death [53, 61, 64]. Extensive oxidative stress and DNA damage trigger premature, permanent cell cycle arrest marked by an increase in β-galactosidase activity, termed senescence-associate β-galactosidase (SA-β-gal), and upregulation of several cell cycle arrest proteins, including p21/waf1 [65–67]. 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. 3A). Western blot analysis of p21/waf1 in NHAs revealed a transient increase in p21/waf1 expression at 6 h and 24 h post-irradiation, followed by a return to baseline levels by 48 h (Fig. 3B) 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.

Both non-homologous end joining and homologous recombination DNA repair mechanisms were 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 [68], we investigated the levels of DNA DSBs in NHAs and MSCs. DNA damage initiates signaling cascades that phosphorylate the H2AX histone on serine 139 to γ-H2AX, which is present in foci surrounding DSBs [69]. γ-H2AX foci are surrogate markers of the DNA repair process [52, 66, 70]. In both cell types, 10 Gy irradiation induced a significant increase in γ-H2AX levels at all time points post-irradiation as compared with controls (Fig. 4). NHAs displayed a statistically significant decrease in γ-H2AX foci between 24 h and 48 h (Fig. 4A), suggesting that DSB repair had occurred and that a significant percentage of γ-H2AX foci were resolved. MSCs displayed a significant decrease in γ-H2AX at 72 h post-irradiation (Fig. 4B).

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 NHEJ and HR [71]. We first examined the effects of radiation exposure on the expression of markers for NHEJ—Ku70, XRCC4. Ku70 binds directly to DNA DSBs and acts as both a scaffold and signaling molecule for other proteins to induce end joining [72]. Ku70 has been demonstrated to be upregulated in some cell types in response to DNA damage [29, 30, 73]. In some systems, it is believed that Ku70 is highly expressed, and may not be subject to regulation by DNA damage [74, 75]. Because of this controversy, we first compared the levels of basal Ku70 expression in NHAs and MSCs with that in two cancer cell lines that express high levels of Ku70 (A549 and HEK293) (Fig. 5A). We observed that the relative abundance of Ku70 (compared with β-actin) in NHAs was <50% that in A549 and HEK293 cells. The relative abundance of Ku70 in MSCs was <10% that in A549 and HEK293 cells. This suggests that in both NHAs and MSCs, 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 till 6 h post-irradiation (P < 0.05), but no increase in Ku70 was observed in MSCs after 10 Gy irradiation (Fig. 5B and C, respectively).

XRCC4, a protein that binds both DNA and DNA ligase IV, aids in the ligation of newly repaired DNA and has been shown to be increased in response to DNA damage [37, 76]. XRCC4 increased >2-fold in NHAs at 2 h post radiation injury (P < 0.05) (Fig. 5D), 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 with MSCs, following radiation exposure.

HR, the second major repair pathway for DSBs, relies on homology search and strand invasion of a non-damaged sister chromatid [48]. 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 [77]. During HR, RPA is phosphorylated on its 32 kDa subunit (RPA32), which activates it, allowing ssDNA binding [78]. NHAs displayed a significant increase in RAD51 expression after radiation injury; a trend towards significance for upregulation of RAD51 was also observed in MSCs (Fig. 6A 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. 6C), 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 upregulated in NHAs, but not in MSCs.

Ku70 was sufficient to protect against radiation-induced accelerated senescence in MSCs

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 concerning tumor cells also showed a correlation between Ku70 expression and radiosensitivity [79, 80]. Accordingly, we next sought to determine whether 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 irradiation or sham irradiation. Co-expression of eGFP was used to identify transfected cells; the overall transfection efficiency was ~15%, comparable with published findings for this cell type [81]. Expression of p21/waf1 was detected by immunohistochemistry at 24 h post-irradiation as a marker of senescence after radiation exposure (Fig. 7; higher magnification images shown in Fig. S2). In cells transfected with a control vector, 10 Gy radiation significantly increased the number of cells with upregulated 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.

DISCUSSION

Here we have shown that NHAs were highly resistant to radiation damage, displaying the ability to proliferate after 10 Gy X-ray exposure with minimal levels of senescence and no evidence of apoptosis. Our findings indicate that NHAs robustly upregulated DNA repair enzymes in 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 were robustly upregulated in NHAs versus MSCs, and the demonstration that ectopic expression of the NHEJ repair enzyme Ku70 was sufficient to rescue MSCs from undergoing radiation-induced senescence, supports the proposal that enhanced NHEJ underlies the radioresistance 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 NHAs and MSCs. However, NHAs displayed a more rapid reduction in γ-H2AX foci. However, caution must be used in interpreting these results, because controversy exists regarding the ability of γ-H2AX to measure the efficiency and accuracy of DSB repair [70, 82, 83], since foci may linger after DSB repair has occurred [84]. While upregulation of DNA repair enzymes in conjunction with γ-H2AX results suggested a role for DNA repair in NHA survival and proliferation, further investigation into repair accuracy is necessary. Nonetheless, these findings suggest that the persistence of DNA damage in MSCs may be associated with the development of senescence in these cells, as indicated by increased
SA-β-gal activity and sustained upregulation 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 [28, 85]. These characteristics of senescent cells may be related to the tissue dysfunctions observed following radiation injury, including repair failure, persistent inflammation, fibrotic remodeling, and predisposition to the development of cancer [28, 85]. 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

Fig. 2. Irradiation with 10 Gy X-rays did not induce significant apoptosis in human astrocytes or mesenchymal stem cells. Normal human astrocytes (NHAs), normal human adult mesenchymal stem cells (MSCs) and Jurkats (an immortalized human T cell line) were grown to 50% confluence and sham irradiated or exposed to 10 Gy X-rays. Annexin V staining was performed 24 h post-irradiation. Panels show control and irradiated cells for AnnexinV 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 ±SEM; asterisk indicates p < 0.05 from control.
Fig. 3. X-ray irradiation induced accelerated senescence in mesenchymal stem cells, but not in normal human astrocytes. Normal human astrocytes (NHAs) or normal human adult mesenchymal stem cells (MSCs) were grown to 50% confluence and sham irradiated or exposed to 10 Gy X-rays. (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 positive for β-galactosidase expression. Bar graphs indicate means of three independent assays ±SEM; asterisk indicates \( P < 0.05 \). (B) NHAs 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 ±SEM; asterisk indicates \( P < 0.05 \) from control.

NHAs and MSCs to regulate the enzymes of both NHEJ and HR. The relative roles of HR and NHEJ in DNA protection has been discussed in the literature, with the consensus that HR is less error prone but only occurs during G2 and S phase [77]. Our experimental attempts to control the cell cycle in NHAs and MSCs prior to radiation injury through serum starvation resulted in a high...
Fig. 4. Both normal human astrocytes and mesenchymal stem cells demonstrated removal of γ-H2AX foci following radiation exposure. Double-strand break (DSB) repair was detected using γ-H2AX immunohistochemistry in normal human astrocytes (NHAs, A) and mesenchymal stem cells (MSCs, B). NHAs or MSCs 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 ±SEM. Asterisk indicates \( P < 0.05 \) from control; †indicates \( P < 0.05 \) from 24 h time point.
proportion of senescent cells, even without radiation exposure. However, prior research has indicated that RPA plays a role in preventing premature recombination through Rad51, and decreases the likelihood of mitotic catastrophe from inappropriate repair [86]. Therefore, Rad51 upregulation alone, as we observed in MSCs, may not be sufficient for DNA repair without additional regulation of RPA32.

We found that XRCC4 and Ku70, two proteins integral to NHEJ, were upregulated in NHAs but not in MSCs. Although Ku70 is often acknowledged for its role in DNA repair, recent studies indicate that in the cytoplasm, Ku70 may also regulate apoptosis and p53 expression [87, 88]. It is not known whether these functions also play a role in the protection of astrocytes following radiation exposure. The protection of MSCs by overexpression of Ku70

Fig. 5. Normal human astrocytes, but not mesenchymal stem cells, displayed increased levels of proteins involved in non-homologous end joining. (A) Comparison of Ku70 levels in A549 lung cancer cells, HEK293T cells, NHAs or MSCs. 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) NHAs and MSCs were grown to 70% confluence and sham irradiated or exposed to 10 Gy X-rays. 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 ±SE of three independent experiments; asterisk indicates P ≤ 0.05.
may involve both nuclear and cytoplasmic roles for Ku70. Taken together, our data suggest that Ku70 was sufficient to protect cells from radiation-induced senescence. However, limitations to the study must be acknowledged. Although we were able to observe a suppression of p21/waf1 by Ku70 transfection, we were unable to determine whether ectopic Ku70 expression rescued the proliferative abilities of the irradiated MSCs. Further research is necessary to determine the extent of the protection by Ku70.

Previous research demonstrated the resilience of astrocytes and their ability to proliferate after exposure to redox stress or ionizing radiation in vivo in rodents [89, 90]. Cell culture investigations of astrocytes have also provided evidence for resistance to redox stress [20, 91, 92]. Our finding of resistance of astrocytes to radiation damage is consistent with the pro-survival effect of astrocytes on other cells following radiation exposure or redox stress [20, 52, 93]. Our findings expand the current knowledge of astrocyte resistance.

Fig. 6. Normal human astrocytes displayed upregulation of Rad51, total and p-RPA32, whereas mesenchymal stem cells only showed upregulation of Rad51. Normal human astrocytes (NHAs) or normal human adult mesenchymal stem cells (MSCs) were grown to 70% confluency and sham irradiated or exposed to 10 Gy X-rays. 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 ±SE of three independent experiments; asterisk indicates P ≤ 0.05.
Further research is necessary to understand the specific mechanisms by which enzymes of NHEJ and HR are robustly upregulated in astrocytes following radiation exposure, as well as to directly measure the rates of DSB repair in astrocytes.

**ACKNOWLEDGEMENTS**

We would like to thank Dr Ognoon Mungunsukh for technical advice and training, and Mr Michael Woolbert for assistance with the use of the RS2000 X-ray irradiator. Some of the authors are employees of the US Government, and this manuscript was...
prepared as part of their official duties. Title 17 U.S.C. §105 states that ‘Copyright protection under this title is not available for any work of the United States Government.’ Title 17 U.S.C. §101 defines a US Government work as a work prepared by a military service member or employees of the US Government as part of that person’s official duties. The views in this article are those of the authors and do not necessarily reflect the views, official policy, or position of the Uniformed Services University of the Health Sciences, Department of the Navy, Department of Defense, or the US Federal Government.

SUPPLEMENTARY DATA
Supplementary data are available at Journal of Radiation Research online.

CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

FUNDING
This work was supported by a predoctoral fellowship from NASA’s Langley Research Center in Hampton Virginia, Administered through Universities Space Research Association (USRA) to M.B.

REFERENCES
1. Eom H, Park H, Jo S et al. Ionizing radiation induces altered neuronal differentiation by mGluR1 through PI3K–STAT3 signaling in C17.2 mouse neural stem-like cells. PLoS One 2016; 11:e0147538.
2. Fishel M, Vasko M, Kelley M. DNA repair in neurons: so if they don’t divide what’s to repair? Mutat Res 2007;614:24–36.
3. Fike J, Kola R, Limoli C. Radiation response of neural precursor cells. Neurosurg Clin N Am 2007;18:115–27.
4. Al-Mefty O, Kersh J, Routh A et al. The long-term side effects of radiation therapy for benign brain tumors in adults J Neurosurg 1990;73:502–12.
5. Citrin D, Cotrim A, Hyodo F et al. Radioprotectors and mitigators of radiation-induced normal tissue injury. Oncologist 2010; 15:360–71.
6. Duffner P. Long-term effects of radiation therapy on cognitive and endocrine function in children with leukemia and brain tumors. Neurologist 2004;10:293–310.
7. Schatz J, Kramer J, Ablin A et al. Processing speed, working memory, and IQ: a developmental model of cognitive deficits following cranial radiation therapy. Neuropsychology 2000;14: 189–200.
8. Greene-Schloesser D, Moore E, Robbins M. Molecular pathways: radiation-induced cognitive impairment. Clin Cancer Res 2013;19:2294–300.
9. Vigliani MC, Duyckaerts C, Hauw JJ et al. Dementia following treatment of brain tumors with radiotherapy administered alone or in combination with nitrosourea-based chemotherapy: a clinical and pathological study. J Neurooncol 1999;41:137–49.
10. Simo M, Vaquero L, Ripolles P et al. Brain damage following prophylactic cranial irradiation in lung cancer survivors. Brain Imaging Behav 2016;10:283–95.
11. Acharya M, Lan M, Kan V et al. Consequences of ionizing radiation–induced damage in human neural stem cells. Free Radic Biol Med 2010;49:1846–55.
12. Fike J, Rosi S, Limoli C. Neural precursor cells and central nervous system radiation sensitivity. Semin Radiat Oncol 2008;19: 122–32.
13. Lee Y, Cho H, Lee W et al. Whole brain radiation-induced cognitive impairment: pathophysiological mechanisms and therapeutic targets. Biomol Ther 2012;20:357–70.
14. Belka C, Budach W, Kortmann R et al. Radiation induced CNS toxicity—molecular and cellular mechanisms. Br J Cancer 2001; 85:1233–9.
15. Stone H, Coleman C, Anscher M et al. Effects of radiation on normal tissue: consequences and mechanisms. Lancet Oncol 2003;4:529–36.
16. Hayakawa K, Esposito E, Wang X et al. Transfer of mitochondria from astrocytes to neurons after stroke. Nature 2016;535: 551–5.
17. Rosenberg PA, Aizenman E. Hundred-fold increase in neuronal vulnerability to glutamate toxicity in astrocyte-poor cultures of rat cerebral cortex. Neurosci Lett 1989;103:162–8.
18. Ouyang YB, Xu L, Lu Y et al. Astrocyte-enriched miR-29a targets PUMA and reduces neuronal vulnerability to forebrain ischemia. Glia 2013;61:1784–94.
19. Wang XF, Cynader MS. Pyruvate released by astrocytes protects neurons from copper-catalyzed cysteine neurotoxicity. J Neurosci 2001;21:3322–31.
20. Bylicky M, Mueller G, Day R. Mechanisms of endogenous neuroprotective effects of astrocytes in brain injury. Oxid Med Cell Longev 2018;2018:16.
21. Stevens B. Neuron–astrocyte signaling in the development and plasticity of neural circuits. Neurosignals 2008;16:278–88.
22. Tsai H, Li H, Fuentealba L et al. Regional astrocyte allocation regulates CNS synaptogenesis and repair. Science 2012;337: 358–62.
23. Haskew-Layton R, Payappilly J, Smirnova N et al. Controlled enzymatic production of astrocytic hydrogen peroxide protects neurons from oxidative stress via an Nrf2-independent pathway. Proc Natl Acad Sci U S A 2010;107:17385–90.
24. Watts L, Rathinam M, Schenker S et al. Astrocytes protect neurons from ethanol-induced oxidative stress and apoptotic death. J Neurosci Res 2005;80:655–66.
25. Desagher S, Glowinski J, Premont J. Astrocytes protect neurons from hydrogen peroxide toxicity. J Neurosci 1996;16:2553–62.
26. Brown D. Neurons depend on astrocytes in a coculture system for protection from glutamate toxicity. Mol Cell Neurosci 1999; 13:379–89.
27. Azzam E, Jay-Gerin J, Pain D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Lett 2014;327:48–60.
28. Day R, Snow A, Panganiban R. Radiation-induced accelerated senescence: a fate worse than death? Cell Cycle 2014;13:2011–2.
44. Olson E, Nievera C, Liu E et al. The Mre11 complex mediates DNA repair and DNA damage tolerance. *Cell Res* 2008;18:99–113.
45. Budke B, Chan Y, Bishop D et al. Real-time solution measurement of RAD51- and RecA-mediated strand assimilation without background annealing. *Nucleic Acids Res* 2013;41:e130.
46. Helleday T, Lo J, Van Gent D et al. DNA double-strand break repair: from mechanistic understanding to cancer treatment. *DNA Repair* 2007;6:923–35.
47. Rath BH, Wahba A, Camphausen K et al. Coculture with astrocytes reduces the radiosensitivity of glioblastoma stem-like cells and identifies additional targets for radiosensitization. *Cancer Med* 2015;4:1705–16.
48. Li X, Heyer W. Homologous recombination in DNA repair and DNA damage tolerance. *Cell Res* 2008;18:99–113.
49. Porter A, Janicke R. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ* 1999;6:99–104.
50. Panganiban R, Snow A, Templeton D et al. The role of c-Jun N-terminal Kinase (JNK) in apoptosis induced by ultraviolet C and γ radiation. *J Biol Chem* 1996;271:31929–36.
51. Neben K, Staudt C et al. γ-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic Acids Res* 2008;36:5678–94.
67. Aubrey BJ, Kelly GL, Janic A et al. How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression? Cell Death Differ 2018;25:104–13.
68. Sanchez-Flores M, Pasaro E, Bonassi S et al. yH2AX assay as DNA damage biomarker for human population studies: defining experimental conditions. Toxicol Sci 2015;144:406–13.
69. Chandra A, Lin T, Zhu J et al. Prediction of tumor radiosensitivity in rectal carcinoma based on p53 and Ku70 expression. J Exp Clin Cancer Res 2003;22:223–8.
70. Kuo LJ, Yang LX. Gamma-H2AX foci paradigm: molecular mechanisms, in vivo significance, and redox considerations. Antioxid Redox Signal 2009;11:59–98.
71. Yuan H, Gaber M, Boyd K et al. Effects of fractionated radiation exposure on the brain vasculature in a murine model: blood-brain barrier permeability, astrocyte proliferation, and ultrastructural changes. Int J Radiat Oncol Biol Phys 2006;66:860–6.
72. Balentova S, Adamkov M. Molecular, cellular and functional effects of radiation-induced brain injury: a review. Int J Mol Sci 2015;16:27796–815.
73. Yong R, Yang C, Lu J et al. Cell transcriptional state alters genomic patterns of DNA double-strand break repair in human astrocytes. Nat Commun 2014;5:5799.
74. Titler A, Posimo J, Leak R. Astrocyte plasticity revealed by adaptations to severe proteotoxic stress. Cell Tissue Res 2013;352:427–43.
75. Genis L, Dávila D, Fernandez S et al. Astrocytes require insulin-like growth factor I to protect neurons against oxidative injury. F1000Res 2014;3:28.