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Angiotensin-(1-7) prevents radiation-induced inflammation in rat primary astrocytes through regulation of MAP kinase signaling

Elizabeth Moore^{1,2,3}, Mitra Kooshki^{2,3}, Linda Metheny-Barlow^{1,2,3}, Patricia E. Gallagher⁴, and Mike E. Robbins^{1,2,3}

¹Department of Cancer Biology, Wake Forest School of Medicine, Winston-Salem, NC 27157

²Department of Radiation Oncology, Wake Forest School of Medicine, Winston-Salem, NC 27157

³Department of Brain Tumor Center of Excellence, Wake Forest School of Medicine, Winston-Salem, NC 27157

⁴Department of Hypertension and Vascular Research Center, Wake Forest School of Medicine, Winston-Salem, NC 27157

Abstract

About 500,000 new cancer patients will develop brain metastases in 2013. The primary treatment modality for these patients is partial or whole brain irradiation which leads to a progressive, irreversible cognitive impairment. Although the exact mechanisms behind this radiation-induced brain injury are unknown, neuroinflammation in glial populations is hypothesized to play a role. Blockers of the renin-angiotensin system (RAS) prevent radiation-induced cognitive impairment and modulate radiation-induced neuroinflammation. Recent studies suggest that RAS blockers may reduce inflammation by increasing endogenous concentrations of the anti-inflammatory heptapeptide angiotensin-(1-7) [Ang-(1-7)]. Ang-(1-7) binds to the AT₍₁₋₇₎ receptor and inhibits MAP kinase activity to prevent inflammation. This study describes the inflammatory response to radiation in astrocytes characterized by radiation-induced increases in i) IL-1 β and IL-6 gene expression; ii) COX-2 and GFAP immunoreactivity; iii) activation of AP-1 and NF- κ B transcription factors; and iv) PKC α , MEK and ERK (MAP kinase) activation. Treatment with U-0126, a MEK inhibitor demonstrates that this radiation-induced inflammation in astrocytes is mediated through the MAP kinase pathway. Ang-(1-7) inhibits radiation-induced inflammation, increases in PKC α and MAP kinase pathway activation (phosphorylation of MEK and ERK). Additionally Ang-(1-7) treatment leads to an increase in dual specificity phosphatase 1 (DUSP1). Furthermore, treatment with sodium vanadate (Na₃VO₄), a phosphatase inhibitor, blocks Ang-(1-7) inhibition of radiation-induced inflammation and MAP kinase activation, suggesting Ang-(1-7) alters phosphatase activity to inhibit radiation-induced inflammation. These data suggest that RAS blockers inhibit radiation-induced inflammation and prevent radiation-induced cognitive impairment not only by reducing Ang II by also by increasing Ang-(1-7) levels.

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Corresponding Author: Elizabeth Moore, Department of Radiation Oncology, Room 412 C, Nutrition Research Center, Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157., Phone: 336-713-7629, Fax: 336-713-7639, elmoore2@wakehealth.edu.

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Keywords

Angiotensin-(1-7); Radiation-induced Inflammation; Rat Primary Astrocytes; MAP Kinase signaling; Renin-angiotensin system

Introduction

Over 1.6 million new cancer cases will be diagnosed in 2013[1] and approximately 500,000 of these individuals will develop brain metastases[2, 3]. The number of patients diagnosed with brain metastases continues to increase with enhanced neuroimaging, improved systemic treatment of primary tumors, and an aging population[3, 4]. The majority of metastatic brain tumor patients will receive some form of radiation therapy as part of their treatment[3-5], putting them at risk for developing late radiation-induced brain injury, including a progressive, irreversible decline in cognitive function which is associated with increased mortality and morbidity[3, 6, 7]. Currently, there are no proven successful long-term treatments or effective preventive strategies for radiation-induced cognitive impairment[3, 7].

Although the specific pathological mechanism(s) involved in the onset and progression of radiation-induced brain injury remains ill-defined, experimental evidence suggests that chronic inflammation/oxidative stress plays a major role[8-11]. Radiation activates co-cultures of microglia and astrocytes *in vitro*[12], increasing expression of pro-inflammatory mediators, including cyclooxygenase-2 (COX-2)[13], interleukin (IL)-1 β [14], IL-6[15, 16], and tumor necrosis factor- α (TNF- α)[17]. Additionally, radiation-induced increases in COX-2 have been reported in monocultures of astrocytes [13]. These effects may be partially mediated through radiation-induced activation/phosphorylation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MEK) [18-21]. To date there is a paucity of studies characterizing the radiation response of astrocytes. The results of this study demonstrate a important radiation-induced inflammatory signaling pathway in astrocytes.

Preclinical studies suggest that blockade of angiotensin (Ang) II, a pro-inflammatory peptide of the renin-angiotensin system (RAS), may serve as a potential therapeutic intervention for late radiation-induced organ injury[22]. Attenuation of Ang II signaling via RAS inhibition prevents radiation-induced late effects in the kidney, lung and brain[23]. Indeed, blockade of the RAS with an angiotensin converting enzyme inhibitor (ACEI) or angiotensin receptor blocker (ARB) reduces expression of inflammatory proteins[22], prevents AP-1 and NF- κ B activation[24], and reduces oxidative stress[23, 25] in the rodent brain. Previous studies in our lab demonstrate that administration of the ARB, L-158,809[26], or the ACEI, ramipril, before, during and after fractionated whole brain irradiation (fWBI) prevents cognitive impairment in the young adult male rat assessed 6 months post-irradiation[21].

RAS blockers not only attenuate Ang II signaling, but also increase the generation of the anti-proliferative, anti-fibrotic, anti-inflammatory peptide, Ang-(1-7)[26, 27]. Ang-(1-7), whose sequence is Asp-Arg-Val-Tyr-Ile-His-Pro, binds to the G protein-coupled AT₍₁₋₇₎ receptor and modulates MAP kinase inflammatory pathways which are activated by radiation[27, 28]. Ang-(1-7) decreases lipopolysaccharide (LPS)-stimulated inflammation in macrophages within the brain[29] and directly inhibits Ang II stimulated inflammation in endothelial cells[30] and leukocytes[31]. Ang-(1-7) attenuates mitogen-stimulated increases in COX-2[32] and prostaglandin E synthase (PGES)[33] and increases the dual specificity phosphatase 1 (DUSP1) [34], to reduce MAP kinase activation[35-37]. Thus, Ang-(1-7) treatment may prevent radiation-induced MAP kinase activation and inflammation to

ameliorate radiation-induced cognitive impairment. In this study, primary cultures of rat astrocytes were incubated with Ang-(1-7) to determine whether the heptapeptide hormone could effectively block the inflammatory response induced by radiation.

Materials and Methods

Materials

Ang-(1-7) (100 nM) and [D-Ala⁷]-Ang-(1-7) (1 μM) were dissolved in PBS(Bachem). The phosphatase inhibitor sodium vanadate (Na₃VO₄) (1 μM) (Sigma-Aldrich) was dissolved in water and the MEK inhibitor U-0126 (1 μM) (EMD Millipore), was dissolved in dimethyl sulfate Me₂SO₄. The following antibodies were used for this study: Goat anti-COX-2 (1:1000) (Santa Cruz Biotechnologies), rabbit anti-p-MEK1/2(1:1000)(Santa Cruz Biotechnologies), mouse-anti-p-ERK1/2 (1:1000)(Santa Cruz Biotechnologies), rabbit anti-PKCα (1:20,000) (Cell Signaling) rabbit anti-DUSP-1 (1:1000) (EMD Millipore), Total MEK (1:1000) (Santa Cruz Biotechnologies), Total ERK (1:1000) (Santa Cruz Biotechnologies) and mouse anti-β-actin (1:10,000) (Sigma-Aldrich).

Astrocyte Isolation, Irradiation and Treatments

Astrocyte isolation and cell culture—Astrocytes were isolated from mixed glial cultures as described previously[38]. In brief, brains taken from 1- to 3-day-old rat pups were minced, filtered, and plated in DMEM/F12 media(Invitrogen). At 100% confluence (1 week post plating), the mixed glial cultures were shaken at 200 rpm overnight at 37°C on a rotary shaker to separate the microglia and oligodendrocytes from the adherent astrocytes. The media, microglia and oligodendrocytes were removed; the astrocytes were trypsinized and subcultured in DMEM/F12 supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 IU/mL penicillin, 100 mg/mL streptomycin (Sigma-Aldrich), 100 mg/mL L-glutamine (Sigma-Aldrich) and 15mM HEPES (Sigma-Aldrich). Astrocytes were maintained at 37°C in 5% CO₂ and 95% air. Cultures used for experiments were determined to contain 99% astrocytes as assessed by, using double immunostaining for glial fibrillary acidic protein (GFAP) to identify astrocytes, and Iba1 (Wako) for microglia.

Irradiation—The culture medium was replaced with low serum media (0.5%) 24 h prior to irradiation to synchronize the cells. Cells were irradiated with a single dose of 10 Gy using a ¹³⁷Cs irradiator at a dose rate of 3.57 Gy/min. Irradiations were conducted at room temperature and control cells received sham-irradiation (same time in the irradiator without any radiation dose); culture dishes were returned to the incubator and maintained at 37°C in 5% CO₂ and 95% air following irradiation. Times after radiation that resulted in changes in inflammatory mediators and cytokines and MAP kinase activation were based on historical laboratory data[39, 40].

Treatment of Cultured Cells

Cultured astrocytes were incubated with 100 nM of Ang-(1-7), 1 μM of the AT₍₁₋₇₎ receptor antagonist D-Ala-Ang-(1-7), 1 μM of the MEK inhibitor U-0126 or 1 μM of the phosphatase inhibitor Na₃VO₄. For treatments with a single agent, astrocytes received Ang-(1-7), U-0126 or Na₃VO₄ for one hour prior to irradiation. When astrocytes were treated with a combination of the Ang-(1-7) and D-Ala-Ang-(1-7) or Na₃VO₄, cells were first treated with the inhibitor (ie, D-Ala-Ang-(1-7) or Na₃VO₄) for 15 min and then Ang-(1-7) was added 1 h prior to radiation.

Immunoblot Hybridization

Total cellular protein was harvested using M-PER lysis buffer (Pierce Biotechnology) supplemented with 1 mg/mL aprotinin (Sigma-Aldrich), 1 mM leupeptin (Sigma-Aldrich), 10 mg/mL phenylmethylsulfonyl fluoride (PMSF), 1 mM Na₃VO₄ (Sigma-Aldrich), and 150 mM NaCl. Lysates were centrifuged at 12,500 rpm for 30 min, and the supernatant was collected. Protein concentrations were measured using the Bradford assay method (Bio-Rad) at absorbance 595 nm. Five to 50 µg of protein were separated by SDS-PAGE. Protein was transferred to a polyvinylidenedifluoride membrane at 35 V overnight, blocked in 5% milk in TBST (0.02 M Tris, 0.015 M NaCl, 0.05% Tween 20, pH 7.5), and incubated overnight with primary antibody. Membranes were washed, incubated with the appropriate horseradish peroxidase-conjugated secondary antibody, developed using the ECL detection system (GE Healthcare), and processed using a Kodak processing system. Films were scanned and densitometry was conducted to quantify the signal intensity using Adobe Photoshop Elements 6.0 to express protein levels as fold changes, with β-actin used as a loading control. No changes were observed in total levels of MEK or ERK in response to radiation or Ang-(1-7) (Supplementary Figure 1). Thus, the changes in p-MEK and p-ERK were normalized to the loading control, β-actin.

RNA isolation and Quantitative Real Time Polymerase Chain Reaction with Taq-Man

RNA was harvested using Trizol reagent (Invitrogen) according to the manufacturer's protocol. DNA contamination was removed by chloroform extraction (Ambion). Real-time PCR amplifications were conducted in a 20-µl reaction volume containing 2 µl cDNA, 10 µl Taq-Man Master Mix (Applied Biosystems), 0.1 µM gene-specific probe, upstream and downstream primers (Applied Biosystems), and 7 µl nuclease-free water. Real-time PCR was carried out in an ABI Prism 7000 at 50 °C for 2 min, 95 °C for 2 min, and 45 cycles of 95 °C for 15 min, 55 °C for 30 s, and 72 °C for 30 s. The fold changes in IL-1β (Applied Biosystems), and IL-6 (Applied Biosystems) gene expression were calculated using the comparative Ct (cross threshold) method[41].

Electromobility-shift assay (EMSA)

Cells were lysed on ice with Buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT); lysates were homogenized using a Dounce homogenizer, which was followed by centrifugation at 12,000 rpm for 10 min. The nuclear pellets were lysed with Buffer C (5 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 25% v/v glycerol, 400 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 mg/mL aprotinin, 2 mg/mL leupeptin, and 1 mM Na₃VO₄) followed by centrifugation at 12,000 rpm for 10 min to extract nuclear proteins. Protein concentrations were measured using the Bradford assay (Bio-Rad) at absorbance 595 nm. The EMSA procedure was performed using the Promega Gel-Shift Core Assay following the manufacturer's protocol.

In brief, 10 µg of nuclear protein was incubated with 2 µl Binding Buffer (Promega) for 10 min. Consensus binding sequences of NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3' and 3'-TCAACTCCCCTGAAAGGGTCCG-5') and AP-1 (5'-CGCTTGATGAGTCAGCCGAA-3' and 3'-GCGAACTACTCAGTCGGCCTT-5') were labeled with 10 µCi of γ-32P (GE Healthcare) and T4 polynucleotide kinase (Promega). The consensus sequences were then incubated with the nuclear protein for 20 min and proteins were separated by electrophoresis on a 4% nondenaturing polyacrylamide gel. Band intensity on X-ray film was determined by densitometry and expressed as fold changes.

Statistical analysis

All data are expressed as mean and $\pm$ SEM. Each experiment was repeated a minimum of three (or more) independent times. All analyses were carried out using GraphPad software (GraphPad Prism 5.0). T-tests were used for analysis of experiments comparing only sham to irradiated samples ($*p < 0.05$). 2-Way ANOVAs were used to determine if there was a radiation ($*p < 0.05$) or drug ($\#$ or $\dagger p < 0.05$, Ang-(1-7), D-Ala-Ang-(1-7), U-0126 or Na_3VO_4) effect. Bonferroni post-tests were used for all pair-wise comparisons.

Results

Radiation induces inflammation and activation of MAP kinase signaling in primary rat astrocytes

A 10 Gy single dose of Cs^{137} radiation was administered to primary astrocytes to determine if radiation induced inflammation in monocultures of astrocytes. Radiation rapidly resulted in a 2-fold increase in inflammatory mediator IL-1 β mRNA at 1 h post-irradiation (Figure 1A) and a delayed 2-fold increase in cytokine IL-6 at 7 h post-irradiation as compared to sham controls (Figure 1A). Enhanced immunoreactivity of inflammatory proteins COX-2 and GFAP was also observed at 7 h post irradiation as compared to sham controls (Figure 1B). In addition to increasing inflammatory markers, radiation caused an immediate activation of MAP kinase signaling at 30 min post-irradiation, characterized by a 2-fold increase in total levels of PKC α , 1.5-fold increase in phosphorylation of MEK and a 2-fold increase in phosphorylation of ERK as compared to sham controls (Figure 1C). Furthermore, downstream MAP kinase pro-inflammatory transcription factors AP-1 and NF- κ B were activated 30 min post-irradiation as measured by EMSA and compared to sham controls (Figure 1D). Taken together, these data suggest that radiation causes an inflammatory response, potentially mediated through MAP kinase activation in rat primary astrocytes.

Radiation-induced inflammation in primary astrocytes occurs through activation of the MAP kinase pathway

The MEK inhibitor, U-0126 was used to block MAP kinase signaling to determine if MAP kinase activation is a key mediator of radiation-induced inflammation in rat primary astrocytes. Pretreatment with U-0126 1 h prior to irradiation prevented radiation-induced increases in phosphorylation of ERK and reduced basal levels of phosphorylated ERK as compared to sham controls (Figure 2A). Interestingly, MEK inhibition also prevented radiation-induced increases in upstream kinase PKC α , blocking any potential feedback loop in the MAP kinase cascade (Figure 2B). Radiation-induced inflammation was also abolished as a result of MEK inhibition. Radiation-induced increases in inflammatory mediators IL-1 β 1 h post irradiation as well as IL-6 and COX-2 at 7 h post irradiation were blocked with U-0126 treatment as compared to sham controls (Figures 2C, 2D, and 2E). Of note, U-0126 treatment in sham-irradiated astrocytes did not significantly alter inflammatory cytokine or mediator levels. These findings indicate that radiation-induced inflammation is likely mediated through MAP kinase activation in rat primary astrocytes.

Ang-(1-7) inhibits radiation-induced inflammation and MAP kinase activation through the AT₍₁₋₇₎ receptor

Rat primary astrocytes were pretreated with 100 nM Ang-(1-7) for 1 h followed by a 10 Gy single dose of radiation. Inflammatory markers and MAP kinase activation were evaluated to determine whether Ang-(1-7) attenuates radiation-induced inflammation. As shown in Figure 3A, a greater than 2-fold increase in IL-1 β mRNA compared to the control cell concentration was observed 1 h post-irradiation. A 1 h pre-incubation of the astrocytes with

Ang-(1-7) prevented the radiation-induced increase in IL-1 β gene expression. Radiation of rat primary astrocytes also up-regulated IL-6 mRNA at 7 h post irradiation and Ang-(1-7) treatment for 1 h before radiation prevented the increase (Figure 3B). The attenuation of radiation-induced increases in both IL-1 β and IL-6 gene expression by Ang-(1-7) in cultured rat astrocytes was effectively blocked by the AT₍₁₋₇₎ receptor antagonist D-Ala-Ang-(1-7) (Figures 3A and 3B), indicating a receptor-mediated mechanism. The enhanced immunoreactivity of inflammatory proteins COX-2 and GFAP observed 7 h post irradiation as compared to control cells was also blocked by pretreatment with Ang-(1-7). Similarly, the D-Ala-Ang-(1-7) effectively prevented the Ang-(1-7) mediated inhibition of elevated COX-2 or GFAP by radiation (Figures 3C and 3D). Sham irradiated primary astrocytes treated with Ang-(1-7), D-Ala-Ang-(1-7) or combination of the two treatments showed no changes in the regulation of these inflammatory cytokines or proteins.

Pretreatment with Ang-(1-7) 1 h prior to irradiation also prevented radiation-induced increases in MAP kinase signaling. Western blot hybridization analyses showed that radiation increased the total levels of PKC α in primary cultures of rat astrocytes by approximately 2-fold at 30 min after irradiation (Figure 3E). The effect was attenuated by incubation of the cells with 100 nM Ang-(1-7) 1 h prior to radiation treatment. Radiation also increased phosphorylation of MEK and ERK in the astrocytes (Figures 3F and 3G), an effect that was blocked by incubation with Ang-(1-7) 1 h prior to irradiation of the cells. The AT₍₁₋₇₎ receptor antagonist blocked Ang-(1-7) prevention of radiation-induced increases in PKC α and p-ERK. However, treatment with D-Ala-Ang-(1-7) alone caused an increase in p-MEK in sham irradiated cells, which precludes the ability to confirm that the Ang-(1-7) decrease in p-MEK is mediated through the Mas receptor. The increase in downstream transcription factors AP-1 and NF- κ B 30 min post irradiation was prevented by Ang-(1-7) treatment and the effect was inhibited by addition of D-Ala-Ang-(1-7), suggesting that Ang-(1-7) counter-regulates the activation of the MAP kinase pathway by radiation through the AT₍₁₋₇₎ receptor-mediated mechanism (Supplementary Figure 2, Panels A and B).

Ang-(1-7) increases dual specificity phosphatase-1 (DUSP1) to inhibit radiation-induced activation of MAP kinase signaling and inflammation in primary astrocytes

Na₃VO₄, an inhibitor of phosphatase activity, was used to determine if activation of phosphatase activity is a mechanism for Ang-(1-7) inhibition of radiation-induced MAP kinase signaling and inflammation in primary astrocytes. Pretreatment with phosphatase inhibitor Na₃VO₄ inhibited Ang-(1-7) attenuation of radiation-induced phosphorylation of MEK and ERK (Figure 4A and 4B). Furthermore, COX-2 enhanced immunoreactivity by radiation was not prevented by Ang-(1-7) in the presence of Na₃VO₄ (Figure 4C). These data indicate that Ang-(1-7) modulation of MAP kinase activation and inflammation by radiation may occur through increasing a phosphatase.

Ang-(1-7) modulates MAP kinase signaling via increases in DUSP1 in models of breast cancer fibrosis[34] and cardiac remodeling[42], suggesting that the heptapeptide hormone may modulate DUSP1 in MAP kinase-mediated inflammation. Pretreatment with Ang-(1-7) increased immunoreactivity of DUSP1 in primary astrocytes and combination of pretreatment with Ang-(1-7) and radiation increased total levels of DUSP1 by 3 fold (Figure 4D). Radiation alone had no significant effect on DUSP1 concentrations in astrocytes as compared to the levels in control cells. Interestingly, pretreatment with Na₃VO₄ prevented these Ang-(1-7) mediated effects on DUSP1, suggesting that changes in total levels of DUSP1 are partially regulated by phosphatase activity. Thus, these data suggest that Ang-(1-7) may mediate radiation-induced MAP kinase signaling and inflammation through increases in DUSP1.

Discussion

Preclinical studies have identified radiation-induced neuroinflammation as a potential mechanism for the development of radiation-induced brain injury, including progressive cognitive impairment [3, 24]. While the brain microenvironment consists of different cell types including microglia, oligodendrocytes, neurons, endothelial cells, and astrocytes, *in vivo* and *in vitro* studies have primarily focused on the radiation-induced neuroinflammation in microglia. Radiation initiates inflammation through MAP kinase signaling in immortalized murine microglia BV-2 cells[39, 40] and activates microglia (CD68/ED1 marker) 2 months to 1 year following fWBI in rodent models[21, 25]. Radiation studies of mixed glial cultures (microglia and astrocytes) suggest that microglia are the primary mediators of radiation-induced neuroinflammation and are necessary to activate astrocytes[12, 13]. However, previous studies with RAS blockers demonstrate that preventing activation of microglia may not be sufficient to protect against radiation-induced cognitive decline. Treatment with the angiotensin receptor blocker (ARB), L-158,809, or the angiotensin converting enzyme inhibitor (ACEI), ramipril, inhibits radiation-induced cognitive impairment, but only ramipril prevents microglial activation in the hippocampus[21, 25, 26]. Thus, it is likely that other brain cells play a role in the pathogenesis of radiation-induced brain injury and are affected by the RAS blockers.

This is the first study to demonstrate a radiation-induced MAP kinase inflammatory signaling mechanism in astrocytes. Astrocytes constitute approximately 50% of the total glial cell population within the brain and represent a heterogeneous class of cells which perform diverse functions including modulation of synaptic transmission and secretion of neurotrophic factors[43]. In response to injury, astrocytes become reactive, proliferate and increase expression of glial fibrillary acidic protein (GFAP)[43, 44]. Irradiation of the rat and mouse brain causes gliosis (enlargement and proliferation of astrocytes) and increases expression of GFAP[17]. Radiation triggers astrocytes to express COX-2 and intercellular adhesion molecule (ICAM-1) that are likely to contribute to breakdown of the blood-brain barrier and subsequent infiltration of leukocytes[13]. In the study reported here, radiation activates MAP kinase signaling to induce increases in inflammatory cytokines IL-6 and IL-1 β and inflammatory mediators COX-2, GFAP, AP-1 and NF- κ B in primary astrocytes. Furthermore, treatment with Ang-(1-7), an anti-inflammatory peptide of the RAS, prior to irradiation inhibits radiation-induced inflammation in astrocytes, likely through MAP kinase inhibition. Ang-(1-7) prevents radiation-induced increases in PKC α , p-MEK and p-ERK. Ang-(1-7) also increases DUSP1, a phosphatase of ERK. These data suggest that astrocytes play an inflammatory role in radiation-induced brain injury and RAS blockers are likely to function as potential therapeutics for prevention of radiation-induced brain injury, via MAP kinase inhibition.

RAS blockers may prevent radiation-induced cognitive impairment by not only attenuating the function of Ang II, but also by increasing Ang-(1-7)[22]. Treatment with RAS blockers increases systemic levels of anti-inflammatory, anti-proliferative, and anti-fibrotic Ang-(1-7) [26]. Ang-(1-7) has been shown to modulate several phosphatases in different disease models. Ang-(1-7) inhibits proliferation of cardiac fibroblasts in a rodent model of myocardial infarction[42, 45] and reduces fibrosis in orthotopic models of breast cancer through a reduction in MAP kinase activation and an increase in DUSP1[34]. LPS-stimulated inflammation in macrophages is also inhibited by treatment with Ang-(1-7)[29]. In the study reported here, Ang-(1-7) inhibits radiation-induced ERK phosphorylation and Ang-(1-7) treatment alone and in combination with radiation, significantly increases total levels of DUSP1, suggesting that reduction in MAP kinase signaling through an increase in DUSP1 may serve as a potential mechanism for the prevention of radiation-induced inflammation in primary astrocytes. This study also identifies p-MEK as a target for Ang-

(1-7) modulation of MAP kinase signaling. Ang-(1-7) inhibits radiation-induced increases in p-MEK in primary astrocytes. However, this study does not identify the specific mechanism for how Ang-(1-7) decreases p-MEK because Ang-(1-7) regulates multiple phosphatases[36, 46, 47]. Consistent with this, the phosphatase inhibitor Na_3VO_4 inhibited Ang-(1-7) from preventing radiation-induced increases in p-MEK and p-ERK. Together these data suggest that Ang-(1-7) increases phosphatases to inhibit radiation-induced inflammation and MAP kinase signaling in primary astrocytes.

Based on the findings described above, Figure 5 outlines a proposed model for radiation-induced inflammation in primary astrocytes and modulation of this radiation response by Ang-(1-7). Radiation activates MAP kinase signaling by increasing total levels of PKC α and phosphorylating MEK and ERK. MAP kinase activation induces increases in activation of transcription factors AP-1 and NF- κ B. Activation of these transcription factors increases expression of inflammatory cytokines IL-1 β and IL-6 and inflammatory mediator COX-2, generating an inflammatory phenotype and increased expression of GFAP in astrocytes. Ang-(1-7) inhibits this radiation response in astrocytes by reducing MAP kinase activation. Ang-(1-7) binds to the AT₍₁₋₇₎ receptor and increases DUSP1, potentially attenuating radiation-induced ERK phosphorylation. This effect is amplified by Ang-(1-7) also directly decreasing total levels of PKC α and inhibiting phosphorylation of MEK. The MEK inhibitor U-0126 also prevented radiation-induced increases in PKC α , suggesting that downstream mediators of MAP kinases may contribute to radiation-induced changes in PKC α and p-MEK. Inhibition of MAP kinase signaling prevented radiation-induced increases in transcription factors AP-1 and NF- κ B and induction of inflammatory factors IL-1 β , IL-6, and COX-2. Phosphatase inhibition by Na_3VO_4 prevented Ang-(1-7) from inhibiting radiation-induced MAP kinase activation, suggesting that Ang-(1-7) alters phosphatase activity as a mechanism for preventing radiation-induced inflammation in primary astrocytes. Taken together, this study indicates that Ang-(1-7) signaling can prevent radiation-induced MAP kinase activation and inflammation in primary astrocytes and is a likely therapeutic for preventing radiation-induced brain injury, including a progressive cognitive impairment.

Radiation-induced brain injury *in vivo* is a multicellular process that consists of a chronic and persistent inflammatory response, which is associated with decreased neurogenesis and impaired neuronal function[3, 48]. It is unlikely that RAS blocker inhibition of radiation-induced cognitive impairment is solely due to the reduction in astrocyte inflammation by Ang-(1-7). The AT₍₁₋₇₎ receptor is ubiquitously expressed throughout the brain[49] and it is likely that Ang-(1-7) blocks radiation-induced inflammation in multiple cell types. This study indicates that Ang-(1-7) inhibits radiation-induced inflammation by MAP kinase inhibition in astrocytes. Of note, MAP kinase signaling is a proposed mechanism for radiation-induced inflammation in other brain cell types including microglia[12, 13, 39, 40] and treatment with PPAR agonists can decrease p-ERK in irradiated rat brain and inhibit radiation-induced cognitive impairment[50]. Thus Ang-(1-7) may also inhibit radiation-induced inflammation in multiple brain cell types *in vitro* and *in vivo*, and its efficacy should be tested in other cells types and in an animal model.

There is currently no clear standard of care for preventing radiation-induced brain injury, including a progressive cognitive impairment[3]. Several current clinical trials of potential pharmacological mediators of cognitive impairment are being developed based on preclinical data suggesting that anti-inflammatory agents can prevent MAP kinase activation and radiation-induced brain injury[21, 26, 40, 51-53]. With the caveat that acute results in tissue culture may not directly extrapolate to the late effects in brain, our data suggests that RAS blockers may prevent radiation-induced brain injury via Ang-(1-7) inhibition of MAP kinase mediated astrocyte inflammation. Furthermore, Ang-(1-7) possesses anti-tumorigenic

properties in preclinical[34, 54, 55] and clinical cancer studies[56, 57]. Thus RAS blockers and/or Ang-(1-7) therapies may serve a dual purpose, targeting tumor proliferation and protecting normal tissue from radiation-induced inflammation. Consequently, these studies indicate the importance of MAP kinase signaling in radiation-induced brain injury and suggest that targeting this pathway may, in part, ameliorate radiation-induced cognitive impairment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Radiation induces inflammation through MAP kinase activation in primary astrocytes
- Ang-(1-7) blocks radiation-induced inflammation in primary astrocytes
- Ang-(1-7) inhibits radiation-induced MAP kinase activation and increases DUSP1

Figure 1. Radiation induces inflammation and activation of MAP kinase signaling in primary rat astrocytes

Primary rat astrocytes were subjected to sham irradiation or a single dose of 10 Gy of ^{137}Cs γ ray irradiation and analyzed as indicated. **Panel A:** IL-1 β and IL-6 mRNAs levels were determined by Taq-man real time PCR on mRNAs isolated 1h or 7h post-irradiation, respectively, and normalized to 18S mRNA. **Panel B:** COX-2 and GFAP protein were analyzed by Western blot hybridization of lysates isolated 7h post-irradiation; β -actin was used as loading control. **Panel C:** Lysates isolated 30 min post-irradiation were analyzed by Western blot for total PKC α , p-MEK, and p-ERK; β -actin was used as a loading control. **Panel D:** Nuclear lysates isolated 30 min post-irradiation were subjected to EMSA using AP-1 and NF- κ B consensus oligonucleotides. Data is expressed as fold changes of Mean $\pm$ S.E.M from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 3$

Figure 2. The MEK inhibitor, U-0126, prevents radiation-induced MAP kinase activation and inflammation

Primary rat astrocytes were pretreated with 1 μ M U-0126 or vehicle control for 1 h, then irradiated with a single dose of 10 Gy of ^{137}Cs γ rays or sham irradiated and analyzed as indicated. **Panel A:** p-ERK, **Panel B:** PKC α , and **Panel C:** COX-2 were analyzed by Western blot hybridization at 30 min (A, B) or 7h (C) post-irradiation; β -actin was used as loading control as compared to sham and untreated controls. **Panel D:** IL-1 β (1h) mRNA and **Panel E:** IL-6 (7h) mRNA levels were analyzed using Taq-man Real-time PCR and normalized to 18S mRNA. Data was expressed as fold changes of Mean $\pm$ S.E.M from three independent samples of cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ##, $p < 0.01$ U-0126 response; $n = 3$

Figure 3. Ang-(1-7) inhibits radiation-induced MAP kinase activation and inflammation through the AT₍₁₋₇₎ receptor

Primary rat astrocytes were pretreated with 100 nM Ang-(1-7) for 1 h, 1 μ M D-Ala-Ang-(1-7) for 30 min or the combination of 1 μ M D-Ala-Ang-(1-7) for 30 min and then 100 nM of Ang-(1-7) for 1 h prior to a single dose of 10 Gy of ^{137}Cs γ irradiation and analyzed as indicated. **Panel A:** IL-1 β and IL-6 mRNA levels were measured with Taq-man real time PCR at 1h or 7h post-irradiation, respectively, and normalized to 18S mRNA. **Panel B:** COX-2 and GFAP protein and **Panel C:** PKC α , p-MEK, and p-ERK protein were analyzed by Western blot hybridization at 7h (B) or 30 min (C) post-irradiation; β -actin used as a loading control. Data was expressed as fold changes of Mean $\pm$ S.E.M from three

independent samples of cells. * $p < 0.05$ radiation response, ** $p < 0.01$ radiation response, *** $p < 0.005$ radiation response; ##, $p = 0.01$ Ang-(1-7) response; ###, $p = 0.001$ Ang-(1-7) response; ††, $p = 0.01$ D-Ala-Ang-(1-7); †††, $p = 0.001$ D-Ala-Ang-(1-7) response; $n = 3$

Figure 4. Sodium Vanadate (Na₃VO₄), a phosphatase inhibitor, prevents Ang-(1-7) inhibition of radiation-induced increases in MAP kinase activation and COX-2

Primary rat astrocytes were pretreated with 1 μ M Na₃VO₄ for 1 h, 100 nM of Ang-(1-7) for 1 h or the combination of 1 μ M Na₃VO₄ and 100 nM of Ang-(1-7) for 1 h prior to a single dose of 10 Gy of ¹³⁷Cs γ irradiation and analyzed as indicated. **Panel A:** p-MEK, **Panel B:** p-ERK, **Panel C:** COX-2 and **Panel D:** DUSP1 protein were analyzed 30 min (A, B, D) or 7h (C) post-irradiation by Western blot hybridization and compared to untreated controls, β -actin was used as a loading control. Data was expressed as fold changes of Mean $\pm$ S.E.M from three independent samples of cells. **p<0.01 radiation response, ***p<0.005 radiation response; ##, p 0.01 Ang-(1-7) response; ###, p 0.001 Ang-(1-7) response; †††, p 0.001 Na₃VO₄ response; n=3

Figure 5. Proposed model outlining the role of Ang-(1-7) in the modulation of the radiation-induced inflammatory response in primary astrocytes

Irradiation of primary astrocytes leads to an increase in MAP kinase signaling and activation of the transcription factors AP-1 and NF- κ B to enhance the expression of IL-1 β , IL-6 and COX-2. Ang-(1-7) inhibits radiation-induced MAP kinase activation by binding to the AT₍₁₋₇₎ receptor and inhibiting radiation-induced increases in PKC α , and MAP kinase activation. Ang-(1-7) may inhibit radiation-induced inflammation by increasing DUSP1 and other phosphatases.