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Effects of the AT₁ Receptor Antagonist L-158,809 on Microglia and Neurogenesis after Fractionated Whole-Brain Irradiation

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Abstract

Cognitive dysfunction develops in approximately 50% of patients who receive fractionated whole-brain irradiation and survive 6 months or more. The mechanisms underlying these deficits are unknown. A recent study demonstrated that treatment with the angiotensin II type 1 receptor antagonist (AT₁RA) L-158,809 before, during and after fractionated whole-brain irradiation prevents or ameliorates radiation-induced cognitive deficits in adult rats. Given that (1) AT₁RA may function as anti-inflammatory drugs, (2) inflammation is thought to contribute to radiation injury, and (3) radiation-induced inflammation alters progenitor cell populations, we tested whether the cognitive benefits of L-158,809 treatment were associated with amelioration of the sustained neuroinflammation and changes in neurogenesis that are induced by fractionated whole-brain irradiation. In rats examined 28 and 54 weeks after irradiation, L-158,809 treatment did not alter the effects of radiation on the number and activation of microglia in the perirhinal cortex and hippocampus, nor did it prevent the radiation-induced decrease in proliferating cells and immature neurons in the hippocampus. These findings suggest that L-158,809 does not prevent or ameliorate radiation-induced cognitive deficits by modulation of chronic inflammatory mechanisms, but rather may reduce radiation-induced changes that occur earlier in the postirradiation period and that lead to cognitive dysfunction.

Introduction

Approximately 220,000 patients are diagnosed each year with primary or metastatic brain cancer (1–3). Partial- or whole-brain irradiation is an effective treatment for primary and/or metastatic brain tumors and is also used prophylactically to prevent metastases to the brain, a common site of metastatic cancer (4). Whole-brain irradiation has proven efficacy in eliminating neoplasms; however, approximately 50% of long-term cancer survivors who receive whole-brain irradiation develop progressive cognitive dysfunction attributed to normal

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tissue damage (5–7). The cellular and molecular bases of cognitive dysfunction induced by whole-brain irradiation have yet to be fully elucidated.

Neuroinflammation is a significant component of the brain's response to radiation (8,9) and is manifested as an increase in the number of activated (e.g. CD68-expressing) microglia. Radiation-induced neuroinflammation likely affects a variety of neural processes; among the most widely studied effects are decreased hippocampal proliferation and neurogenesis, which have been associated with hippocampal-dependent cognitive deficits that commonly develop after whole-brain irradiation (10,11). Interventions that modulate inflammation may provide protection against the normal tissue damage that is hypothesized to lead to radiation-induced cognitive deficits.

Pharmacological blockade of the renin-angiotensin system (RAS) is an attractive therapeutic target against radiation-induced brain injury. Although the systemic RAS has classically been viewed as a hormonal system that regulates blood pressure and fluid balance (12,13), several organ-specific systems, including one in the brain, exist and function independently from the systemic RAS (14,15). Angiotensin II (Ang II) is the best characterized of the biologically active RAS peptides and signals through Ang II type 1 and Ang II type 2 receptors (AT₁R and AT₂R). Ang II is involved in inflammatory responses and neuronal function in the brain (16–18). RAS blockade by angiotensin-converting enzyme inhibitors (ACEis) or AT₁R antagonists (AT₁RAs) ameliorates radiation-induced injury in the lung, kidney and optic nerve (19–21). Therefore, blockade of the brain RAS may ameliorate radiation-induced neuroinflammation and/or restore neurogenesis in the brain. AT₁RAs are as effective as ACEis in preventing radiation damage to the lung and kidney (22) and provide advantages in experimental studies since the ability of ACE to cleave biologically active peptides that are not related to the RAS [e.g. bradykinin and opioid peptides (23)], complicates interpretation of experimental effects of ACEis.

A robust and tractable animal model of radiation-induced cognitive dysfunction greatly facilitates the development and testing of novel therapies such as RAS inhibition. We have demonstrated that young adult male rats exposed to a 40-Gy fractionated whole-brain radiation regimen develop deficits in multiple cognitive domains. Deficits in the hippocampal-independent novel object recognition (NOR) task and in the hippocampal-dependent radial arm maze and Morris water maze developed with similar time courses (24–26), suggesting that radiation damage occurs throughout the brain, not just in regions of ongoing neurogenesis like the dentate gyrus (DG) of the hippocampus. Importantly, treatment with the AT₁RA L-158,809 has been demonstrated to prevent or ameliorate radiation-induced cognitive deficits in the NOR task, indicating that RAS blockade may be effective in preventing delayed radiation-induced cognitive dysfunction.

In the present study we tested the hypothesis that L-158,809 ameliorates radiation-induced cognitive dysfunction through chronic modulation of neuroinflammation and/or by protecting ongoing neurogenesis. Brains were obtained from rats that were characterized behaviorally at 26 and 52 weeks after completion of fractionated whole-brain irradiation and exhibited radiation-induced deficits in the NOR task that were ameliorated by treatment with L-158,809 (26). The number of microglia and the number and percentage of activated microglia served as regional indices of the inflammatory response. We assessed microglial density and activation in the perirhinal cortex (PRh), since the NOR task is perirhinal dependent. We also quantified the number and activation state of microglia in the granule cell layer and hilus (GCL/hilus) of the DG and in the CA3 region of the dorsal hippocampus since radiation-induced inflammatory processes likely act widely in the brain to produce a range of cognitive deficits and since decreased performance in hippocampal-dependent tasks after whole-brain irradiation has been reported by several laboratories [e.g. (10,11,27,28)]. Finally, given evidence that (1) sustained

deficits in neurogenesis result from radiation-induced inflammation and contribute to cognitive deficits (11,29,30) and (2) activity of the RAS influences cell proliferation and differentiation (17), we evaluated the densities of proliferating cells and immature neurons in the subgranular zone (SGZ) of the DG, a region of the brain where neurogenesis continues throughout adulthood (31).

Methods and Materials

Animals and Irradiation Procedures

Adult (10–12 week old) male Fischer 344 × Brown Norway (F344xBN) rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and housed in pairs on a 12:12-h light-dark schedule with food and water available *ad libitum*. The animal facility at Wake Forest University Health Sciences is accredited by the American Association for Accreditation of Laboratory Animal Care and complies with all Public Health Service-National Institutes of Health and institutional policies and standards for laboratory animal care. All protocols described here were approved by the Institutional Animal Care and Use Committee.

After a 2-week acclimation, rats were randomized to four experimental groups: (1) sham-irradiated without drug treatment (sham/water), (2) fractionated whole-brain irradiation without drug treatment (radiation/water), (3) sham-irradiated plus the AT₁RA, L-158,809 (Merck & Co., Inc., Rahway, NJ, sham/AT₁RA), and (4) fractionated whole-brain irradiation plus L-158,809 (radiation/AT₁RA). Fractionated whole-brain irradiation (40 Gy in eight fractions of 5 Gy, twice/week for 4 weeks at 4.41 Gy/min) was performed in a self-shielded ¹³⁷Cs irradiator with collimating devices for delivery to the whole brain and lead shielding to protect the body and eyes as described in detail previously (26,32). All rats, including shams, were anesthetized using a ketamine/xylazine mixture (75/7 mg/kg body weight, i.p.) before each irradiation session. The twice-weekly radiation dose was administered to alternate sides of the head on alternate days to ensure that each animal received the same midline dose over the course of the treatment. L-158,809 treatment (20 mg/liter in the drinking water) began 3 days before the start of irradiation and continued until subjects were euthanized either 28 weeks or 54 weeks after the final radiation fraction. Fresh drinking water with or without L-158,809 was provided every other day; animals were weighed weekly and the volume of water consumed was recorded and used to monitor drug dose over the course of the experiment (averaging 2 mg/kg per day).

Cognitive Testing

A detailed description of the cognitive testing of the animals in this study has been published previously (26,33). Cognitive function was assessed in each animal 2 weeks before their euthanization at 28 or 54 weeks after the completion of irradiation using the NOR, a robust measure of recognition memory in rodents (34,35). In brief, each rat first explored two identical objects during a 3-min sample phase in the test chamber and then was returned to the home cage for a 1-min delay period. One of the objects in the test chamber was replaced with a novel object and the rat then was returned to the chamber for a 3-min test period. The discrimination ratio, calculated as the difference between the time spent exploring the novel object and the time spent exploring the familiar object during the test period, divided by the total time spent exploring the two objects, was used as a measure of the rat's recognition memory (26). Thus a decrease in the discrimination ratio indicates a deficit in recognition memory.

Tissue Processing

Rats were deeply anesthetized with sodium pentobarbital (150 mg/kg body weight) and decapitated 28 or 54 weeks after the completion of fractionated or sham whole-brain irradiation. The 2-week period between completion of cognitive testing and tissue procurement was

included to minimize any effects of the behavioral testing procedures (which involved only a few minutes of interaction in the testing chamber) on the neurobiological variables of interest. Each brain was extracted rapidly and hemisected at the midline; the right hemisphere was flash frozen in liquid nitrogen and stored for other analyses while the left hemisphere was immersion-fixed in phosphate-buffered 4% paraformaldehyde (pH 7.4) for 24 h at 4°C. The fixed hemispheres were cryoprotected in sucrose, embedded in Tissue Freezing Medium (TFM, Triangle Biomedical Sciences, Inc., Durham, NC), and stored at -80°C until sectioned. Serial coronal sections through the entire hippocampal formation [bregma -1.8 to -6.8 (36)] were cut at 60 µm on a cryostat, collected in antifreeze solution (1:1:2 ethylene glycol, glycerol and 0.1 M sodium phosphate buffer, pH 7.4), and stored at -20°C until processed for immunohistochemistry or immunofluorescence.

Immunohistochemistry and Immunofluorescence

Systematically random series were selected for immunolabeling. For analysis of the hippocampal regions 1-in-12 (GCL/hilus) and 1-in-6 (dorsal CA3) series of sections representing the entire anterior-to-posterior extent of the hippocampus were labeled and analyzed. Given the difficulty of defining absolutely the borders of the PRh, we did not attempt to quantify absolutely the number of activated microglia in the region but rather assessed the density in a well-defined region of PRh (see below) in three sections per animal from the series used for analysis of GCL/hilus. Sections from equal numbers of rats representing the four treatment groups were processed and analyzed in cohorts. Material from each group, 28 weeks or 54 weeks after irradiation ($n = 6$ rats/condition), was processed and evaluated independently.

The antifreeze solution was washed from the sections using 0.1 M Tris-buffered saline (TBS), pH 7.5, and the endogenous peroxidase activity was reduced by incubating sections for 30 min in 1% hydrogen peroxide (H₂O₂) in TBS. Sections were incubated for 1 h at room temperature in TBS containing 5% normal serum and 0.3% Triton X-100 prior to incubation overnight with primary antibody in the same solution at 4°C. The primary antibodies used were: rabbit monoclonal anti-Ki67 [clone SP6; a marker for proliferating cells (37), AbCam, Cambridge, MA, 1:200], rabbit polyclonal anti-ionized calcium-binding adaptor molecule 1 [Iba1; labels all macrophages/microglia (38), Wako, Richmond, VA, 0.083 µg/ml], mouse monoclonal anti-CD68 [clone ED1; a lysosomal label for phagocytic macrophages/microglia (39), AbD Serotec, Raleigh, NC, 2.5 µg/ml]. For sections immunolabeled with Ki67, antigen retrieval by incubation in 10 mM sodium citrate, pH 6, at 90°C for 10 min was performed prior to the H₂O₂ incubation (described above). Primary antibodies were detected with biotinylated secondary antibodies (1:300), amplified using a peroxidase-conjugated avidin-biotin complex (Vectastain ABC Elite kit) and visualized using Vector SG (Ki67), diaminobenzidine (Iba1), or nickel-enhanced diaminobenzidine (CD68/ED1) peroxidase substrates (Vector Laboratories, Inc., Burlingame, CA). Ki67 and Iba1 were labeled in the same series of sections. Labeling and visualization of Ki67 were performed as described; then sections were treated with 1% H₂O₂ in TBS for 30 min (room temperature) prior to labeling of Iba1. All sections were counterstained for 20 min with 100 µM of the nucleic acid stain Sytox Green (Invitrogen-Molecular Probes, Carlsbad, CA) to permit identification of the cell layers in the hippocampal formation and facilitate contour drawing for stereological analyses (described below). Sections were mounted from TBS onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), blotted of residual buffer, air-dried for 30 min, dehydrated using a graded series of ethanol, cleared in xylene, and covered with cover slips using Cytoseal 60 permanent mounting medium (VWR International, LLC, West Chester, PA).

Labeling of neuroblasts and immature neurons in the dorso-medial SGZ was evaluated using goat polyclonal anti-doublecortin antibody (DCX, Santa Cruz, Santa Cruz, CA, 1 µg/ml) (40). Sections were washed with TBS, underwent antigen retrieval via incubation in 10 mM

sodium citrate, pH 6, at 90°C for 15 min, and were incubated for 1 h in TBS containing 5% normal donkey serum and 0.3% Triton X-100 and then incubated overnight at 4°C with addition of primary antibody. DCX was visualized using Cy-5-conjugated, highly cross-adsorbed donkey anti-goat secondary antibody (Jackson ImmunoResearch, West Grove, PA, 7.5 µg/ml). Sections were counterstained with Sytox Green as described above and then mounted from TBS onto Superfrost Plus slides, blotted of residual buffer and covered with cover slips using Biomedica Gel Mount (Fisher Scientific, Pittsburgh, PA). Slides were sealed using Cytoseal 60 to prevent dehydration and were stored in the dark at 4°C to reduce photobleaching.

Quantitative Analyses

All analyses were performed blinded to the experimental condition using coded slides in cohorts that sampled equally across the four experimental groups. Identical analyses were performed for material from both 28 and 54 weeks after irradiation. The animals in this study were characterized behaviorally using a PRh-dependent version of the NOR task (26); therefore, the density of microglia and the density and percentage of activated microglia in PRh were assessed. Microglial and other changes also were analyzed in the hippocampus since that region has been the focus of much recent analysis of radiation-induced normal tissue injury in the brain and since radiation-induced deficits in hippocampal-dependent tasks have been demonstrated repeatedly [e.g., refs. (10,11)].

The PRh lacks readily identifiable anatomical borders and is not well suited to stereological analysis, so the densities of microglia (Iba1⁺) and activated microglia (ED1⁺) were quantified in a well-defined region of PRh using the NeuroLucida system (MBF Bioscience, Williston, VT) and a modification of the optical dissector (41) described previously (42,43). The PRh region of interest (ROI) was defined as a 400-µm-wide area centered on the rhinal fissure and extending from the pial surface to the white matter in sections located between bregma -3.5 and -5.5 (Fig. 1). To analyze the density of microglia, Iba1⁺ cells were counted using a 40× objective in eight random 100-µm × 100-µm counting frames within a rectangular lattice projected over the PRh ROI in each of five systematically random sections (preliminary analyses established that greater sampling did not reduce intra- or interanimal variance). Labeled cells in focus on the top surface of the section and those touching the bottom and left borders of each counting frame were not counted. Given their less distinctive labeling and heterogeneous distribution, counting ED1⁺ microglia required a different approach. Labeled cells were visualized using a planapochromatic 60× oil-immersion objective (NA 1.42) and were counted exhaustively in the entire ROI in each of three systematically random sections (preliminary analyses indicated greater sampling did not reduce variance), as described previously for other neural regions (43).

The discrete borders of hippocampal regions facilitated stereological analysis of the number of labeled cells in the ROIs. For analysis of the hippocampus, 1-in-12 (GCL/hilus) and 1-in-6 (dorsal CA3) series of sections representing the entire anterior-to-posterior extent of the hippocampus were analyzed. Total numbers of microglia (Iba1⁺) and of activated, phagocytic microglia (ED1⁺) were estimated stereologically in the GCL/hilus and dorsal CA3 using the optical fractionator technique (41,44) and the Stereo Investigator system (MBF Bioscience, Williston, VT) on an Olympus BX51 microscope (Olympus America, Inc., Center Valley, PA). Contours defining hippocampal ROIs were drawn using a 10× objective and then immunohistochemically labeled cells within each optical disector were counted using the 60× oil immersion objective. The percentage of total microglia (Iba1⁺) that were activated (ED1⁺) was computed from analyses performed on adjacent sections from each animal and compared across experimental conditions. The GCL/hilus ROI was defined as the area containing the GCL and hilus/polymorph layer of the DG (Fig. 1). In more anterior sections in which the dorsal and ventral GCL were not fused into a closed contour, a line connecting

the suprapyramidal and infrapyramidal blades of the GCL was drawn to create a closed contour. The dorsal CA3 region of interest included all strata of CA3. A closed contour was constructed at the border of CA3 and the DG by connecting the two blades of the DG. The pyramidal layer of CA2 was included within the ROI to determine the dorsal border between CA3/CA2 and CA1. Analyses of CA3 were restricted to the dorsal CA3 (bregma -2.8 through -4.0) (45) given its established role in cognitive functions demonstrated to be sensitive to radiation (46, 47). For all optical fractionator analyses, a 12- μm disector height, 2- μm guard zones, and 100- $\mu\text{m} \times 100\text{-}\mu\text{m}$ counting frame were used; the coefficient of error (CE) (48) for each hippocampal subregion investigated ranged from 0.05 to 0.075.

The SGZ, located along the border between the cell body dense GCL and the cell body sparse hilus of the DG, is one of two regions in the brain where continual neurogenesis occurs throughout life and is very sensitive to radiation. The density of proliferating cells (Ki67⁺ cells) within the SGZ (defined as the region extending 25 μm either side of the boundary between the hilus and the GCL) was quantified using a modification of the optical disector as described previously (42,43) and was expressed as the density of Ki67⁺ cells per millimeter of SGZ examined. Counts were performed using the Neurolucida system (MBF Bioscience, Williston, VT) and a 60 $\times$ oil immersion objective. Sections were labeled sequentially for Ki67 and Iba1 to permit analyzing specifically the proliferation of microglia, but qualitative examination revealed that at 28 and 54 weeks postirradiation proliferating microglia constituted a minor (<1.5%) component of the proliferating cells in the SGZ and of the microglial population in PRh, the GCL/hilus or CA3. Therefore, Ki67⁺/Iba1⁺ cells were not quantified separately.

Immature neurons (DCX⁺ cells) were quantified as a measure of the production of new neurons. DCX⁺ cells typically occurred in clusters and could not be analyzed reliably in thick sections using wide-field microscopy. Therefore, DCX⁺ cells were counted using a Leica TCS SP2 confocal microscope with a 63 $\times$ oil immersion objective (NA1.4, Leica Microsystems, Brannockburn, IL) moving field by field along the extent of the SGZ. Counting the labeled cells in a single hemi-section required imaging z-stacks (1- μm steps) through the depth of the section in approximately 25 fields. Preliminary analyses established that the relative densities of labeled cells (comparing individuals or groups) were equivalent whether one section or a series of several sections were analyzed for each animal, indicating that a single section provided a reliable estimate of the density of DCX-labeled cells in the SGZ. Thus a single, equivalent coronal section from each animal was used to estimate the production of new neurons in the dorso-medial DG, expressed as the density of DCX⁺ cells per millimeter of SGZ. For both Ki67 and DCX-immunolabeled cells within the SGZ, all cells were counted except those in the top focal plane of each section to avoid overestimation.

Statistical Analysis

Results are given as means $\pm$ SEM. All data were analyzed with SigmaStat (SYSTAT Software, San Jose, CA). Two-way analysis of variance (ANOVA) was used to test for main effects of irradiation status (sham-irradiated or irradiated) and drug status (water or AT₁RA) and for interactions. Since handling of animals and processing of tissue were performed separately for the two times, ANOVAs were run separately for each time. For significant main effects, pairwise multiple comparisons were performed using the Holm-Sidak test. A *P* value of ≤ 0.05 was considered the threshold for significance. In addition to testing by ANOVA, we used regression analysis to test for correlations at the level of individual animals between the discrimination ratio (as a measure of cognitive function) and each neurobiological measure (e.g., number of microglia or percentage of activated microglia in the PRh).

Results

Previous studies showed that (1) fractionated whole-brain irradiation of young adult male F344xBN rats leads to a chronic, progressive decrease in cognitive function, demonstrated by deficits in performance in the NOR task, and (2) treatment with the AT₁RA L-158,809 prevents or ameliorates these radiation-induced cognitive changes (26). Given the evidence that the brain RAS may act in part through inflammatory mechanisms, we used tissue from behaviorally characterized animals to test whether the cognitive benefits of AT₁RA treatment in irradiated rats are associated with amelioration of radiation-induced changes in markers of radiation-induced neuroinflammation or cell turnover, neurobiological changes that are thought to contribute to radiation-induced cognitive dysfunction.

Effects of Whole-Brain Irradiation and AT₁R Antagonism on Performance in the NOR

The brains analyzed in the current study were obtained from rats tested in a study published previously (26). Given the time- and labor-intensive nature of quantitative immunohistological analyses, six animals from each experimental group were selected for analysis of neurobiological changes in the PRh and hippocampus. At 26 weeks postirradiation, the discrimination ratio for irradiated rats without drug treatment (radiation/water) averaged 0.11 ± 0.07 compared to 0.53 ± 0.09 , 0.62 ± 0.07 and 0.51 ± 0.12 for the sham/water, sham/AT₁RA, and radiation/AT₁RA groups, respectively. Thus recognition memory was impaired in irradiated rats without drug treatment, but drug treatment ameliorated the deficit. Similarly, at 52 weeks postirradiation, the discrimination ratio for irradiated rats without drug treatment averaged -0.05 ± 0.05 compared to 0.70 ± 0.04 , 0.57 ± 0.05 and 0.51 ± 0.05 for the sham/water, sham/AT₁RA, and radiation/AT₁RA groups. Thus the subset of animals from which neurobiological data were obtained represented well the radiation-induced decrease in cognitive function and the amelioration of that deficit by L-158,809 that was evident in the larger study (26).

Effects of Whole-Brain Irradiation and AT₁R Antagonism on Microglial Number and on Number and Percentage of Activated Microglia

The total number of microglia (Iba1⁺ cells), the number of activated microglia (ED1⁺ cells), and the proportion of microglia that were activated (percentage of ED1⁺ cells) were analyzed to assess changes in microglial population after fractionated whole-brain irradiation. We hypothesized that L-158,809 might affect cognition by modulating the size of the microglial population and/or reducing microglial activation. Iba1, ionized calcium-binding adaptor molecule 1, is located in the cytosol of all microglia and infiltrating monocytes regardless of activation state, although its expression is increased with activation (38). Iba1⁺ cells are referred to here as microglia for convenience but with the recognition that the population may include some macrophages recruited from the periphery. Photomicrographs in Fig. 2A and B show the distribution and appearance of microglia in the PRh. Qualitative examination revealed that most of the Iba1⁺ cells exhibited a ramified morphology and that large, macrophage-like cells were rare. No attempt was made to quantify morphological differences, but it appeared that in irradiated rats in the 28-week group and in all rats in the 54-week group the intensity of Iba1 labeling was increased and that many labeled cells had shorter, larger-diameter processes, as described previously for moderately activated microglia (49–51). There was no apparent effect of treatment with L-158,809 on morphology or the intensity of Iba1 labeling. The ED1 antibody recognizes the rat homologue of human CD68, a lysosomal/endosomal-associated membrane glycoprotein that is involved in phagocytosis and expressed only or at significantly higher levels in activated microglia and macrophages (39). Representative photomicrographs of ED1 labeling are shown in Fig. 2C and D. Consistent with the expected lysosomal location of CD68, ED1 labeling typically appeared as individual or small groups of puncta near the nucleus of labeled cells.

Quantitative analysis demonstrated that fractionated whole-brain irradiation decreased the density of microglia in the PRh by 20–25% at 28 weeks postirradiation [Fig. 3A; $F(1,20) = 36.006$, $P < 0.001$]. This radiation-induced decrease was no longer apparent at 54 weeks [$F(1,20) = 0.009$, $P = 0.92$]. As expected, fractionated whole-brain irradiation increased the density and percentage of activated microglia in the PRh substantially (Fig. 3B and C); the twofold or greater increase that was evident at 28 weeks postirradiation [density ED1⁺: $F(1,20) = 35.226$, $P < 0.001$; percentage ED1⁺: $F(1,20) = 45.575$, $P < 0.001$] was not sustained at 54 weeks [percentage density ED1⁺: $F(1,20) = 0.693$, $P = 0.42$; %ED1⁺: $F(1,20) = 0.488$, $P = 0.49$]. L-158,809 treatment had no effect in irradiated or sham-irradiated rats at either time.

The effects of radiation on microglial number and activation were more modest in the hippocampal regions than in the PRh (Table 1). Neither radiation nor AT₁RA treatment significantly affected total microglial numbers in the GCL/hilus or in the dorsal CA3 at 28 weeks after whole-brain irradiation. At 54 weeks the total number of microglia in the GCL/hilus (but not in CA3) was reduced by 17% in irradiated animals (compared to shams) in the absence of AT₁RA treatment; no reduction was evident in drug-treated animals. Radiation increased the number (GCL/hilus only) and percentage (GCL/hilus and CA3) of activated microglia at 28 weeks but not at 54 weeks postirradiation. In the GCL/hilus there was a significant increase in the percentage of ED1⁺ microglia in irradiated compared to sham-irradiated animals without drug treatment but not in rats receiving L-158,809; in CA3 the radiation effect was significant in drug-treated rats but not in those without L-158,809.

The experimental design of this study did not permit direct statistical comparisons between the two survival periods, but microglial activation was consistently greater in the 54-week group than in the 28-week group. In PRh, where robust radiation-induced increases in activation were apparent at 28 but not at 54 weeks, it appeared that the “loss” of the radiation effect by the later time was due to the number and proportion of activated microglia increasing in sham animals rather than the levels decreasing in irradiated animals to the level in the younger shams. Similarly, in the hippocampal regions the number and density of ED1⁺ cells was greater at 54 than at 28 weeks postirradiation in all groups and reached a common level.

Effects of Whole-Brain Irradiation and AT₁R Antagonism on Proliferation and Production of Immature Neurons in the SGZ

In addition to examining the microglial population in the PRh and hippocampus, we quantified effects of radiation and L-158,809 treatment on proliferation and neurogenesis in the DG. In agreement with previous studies, we saw a significant radiation-induced reduction in the density of proliferating cells in the SGZ at 28 [$F(1,20) = 81.641$, $P < 0.001$] and 54 weeks [$F(1,20) = 17.413$, $P < 0.001$] after completion of fractionated whole-brain irradiation (Fig. 4A). Fractionated whole-brain irradiation resulted in a 76% reduction in the density of proliferating cells at 28 weeks and a 54% decrease at 54 weeks after irradiation. Treatment with L-158,809 had no significant effect at either time [28 weeks: $F(1,20) < 0.000$, $P > 0.99$; 54 weeks: $F(1,20) = 1.040$, $P = 0.32$].

Immature neurons (DCX⁺ cells, Fig. 2E and F) were quantified within the SGZ as an indicator of neurogenesis (indirect since not all newborn neurons are incorporated into functional networks) (31). By 28 weeks postirradiation, fractionated whole-brain irradiation reduced the density of DCX⁺ cells by 60 to 70% [$F(1,20) = 49.505$, $P < 0.001$]. L-158,809 treatment also had a significant effect on the density of DCX⁺ cells at 28 weeks [$F(1,20) = 4.794$, $P < 0.05$]; the drug reduced newborn neurons by 35% in sham-irradiated but not irradiated rats (Fig. 4B). Neither radiation [$F(1,20) = 2.067$, $P = 0.17$] nor AT₁RA treatment [$F(1,20) = 1.090$, $P = 0.31$] produced significant changes in the density of immature neurons at 54 weeks postirradiation.

Correlation of Cognitive Function and Neurobiological Variables

We tested whether the cognitive function of individual animals (measured by the discrimination ratio) correlated with any of the measured microglial and neurobiological indices, since individual variation in the response to whole-brain irradiation and/or drug treatment might result in failure to detect ameliorative effects of L-158,809 when analyzing the group data by ANOVA. The discrimination ratio did not co-vary significantly with the density of Iba1⁺ or ED1⁺ cells (data not shown) or with the percentage of ED1⁺ cells in the PRh in either the 28-week or the 54-week group when all of the animals in each group were considered together (Fig. 5A, B). Testing for correlations separately within the water and L-158,809 groups, however, revealed a significant negative correlation at 28 weeks between the discrimination ratio and either the density of ED1⁺ cells ($r = 20.60$, $P = 0.04$) or the percentage of ED1⁺ cells (Fig. 5C) that was present only in rats that did not receive L-158,809, not in those that were treated with AT₁RA (Fig. 5D). Similar covariance, present in the water but not the AT₁RA groups, was apparent when regressing the discrimination ratio against the percentage of ED1⁺ cells in the GCL/hilus, the number of Ki-67⁺ cells in the SGZ, or the number of DCX⁺ cells in the SGZ (data not shown).

Discussion

Microglial Response after Whole-Brain Irradiation and AT₁R Antagonism

Inhibitors of the brain RAS are attractive candidates for treatment of radiation-induced normal tissue injury given that (1) increased inflammation, as indicated by changes in the microglial population, occurs after whole-brain irradiation (8,11,29,30,52), (2) AT₁R activation activates microglia in several injury models and *in vitro* (18,53–55), and (3) AT₁R blockade reduces inflammation and ameliorates injury after brain ischemia (56,57). Moreover, treatment with the AT₁RA L-158,809 before, during and after irradiation preserves or ameliorates cognitive function in our rodent model of radiation-induced brain injury (26). The animals in the current study clearly had radiation-induced deficits and AT₁RA-dependent benefits in the non-hippocampal-dependent NOR task and thus provided an opportunity to test for inflammatory and neurobiological changes that might underlie the beneficial effects of L-158,809 after whole-brain irradiation. Based on the evidence that inflammatory mechanisms underlie radiation-induced brain injury (11,29,30,58) and previous demonstrations of anti-inflammatory effects of AT₁RA (59,60), we hypothesized that the beneficial effects of L-158,809 on cognition after whole-brain irradiation involve chronic modulation of microglial activation, and possibly also protection of cell proliferation and neurogenesis in the hippocampus.

In contrast to expectations, quantitative analysis of the PRh, the cortical region most closely associated with performance in the NOR task, revealed no effects of L-158,809 treatment after fractionated whole-brain irradiation on the size of the microglial population in the PRh or on the proportion of microglia exhibiting an activated immunophenotype (ED1⁺). Neither the radiation-induced decrease in microglial number nor the increase in ED1⁺ microglia differed between animals with and without L-158,809 treatment. In the hippocampus, where radiation-induced inflammatory changes are well characterized, two observations might be taken as evidence for modest modulatory effects of L-158,809 on radiation-induced changes. First, at 54 weeks postirradiation, the number of microglia in the GCL/hilus was decreased relative to sham controls in irradiated rats without AT₁R blockade but not in those receiving L-158,809. Second, the percentage of ED1⁺ microglia in the GCL/hilus was increased at 28 weeks in rats without drug treatment but not in those receiving the antagonist. Such radiation-induced changes in rats without drug treatment but not in those receiving the AT₁RA may suggest a modest effect on radiation-induced changes in these measures of microglial function, but in neither case was there a significant difference between irradiated rats with and without drug

treatment. Moreover, analysis of the covariance (at the level of individual animals) of cognitive function with the microglial markers (Fig. 5) demonstrated that, although the discrimination ratio was significantly correlated with several inflammatory and neurobiological indices in rats that did not receive L-158,809, the correlation was completely eliminated in the rats that received the drug. Drug treatment ameliorated the cognitive deficit but had no effect on the microglial markers or on proliferation in the DG. Taken together, the analysis of group data and the animal-by-animal correlations indicate that L-158,809 does not ameliorate cognitive dysfunction through anti-inflammatory activity, at least not at the postirradiation times when cognitive deficits appear.

Among the critical questions for interpreting these findings are whether the drug as delivered here (20 mg/liter in the drinking water) crosses the blood-brain barrier (BBB) and acts within the CNS and whether the drug has anti-inflammatory actions (as one would expect given the pro-inflammatory activity of the RAS). Although no direct assessment of its transport across the BBB is available, L-158,809 is more lipophilic and is therefore more likely to cross the BBB than losartan, a more widely studied drug of the same class (57,61). The observation that the drug, as administered to the rats in the present study, ameliorates radiation-induced cognitive deficits (26) but does not affect blood pressure (62,63) is consistent with a CNS-mediated action, as is the demonstration in the present study that the drug altered neurogenesis in sham-irradiated rats at 28 weeks (Fig. 4B). Thus it appears that L-158,809 does act within the brain. Among the possible mechanisms of action within the CNS, anti-inflammatory effects are likely since L-158,809 and similar drugs are effective at reducing inflammatory responses in other organs [e.g. refs. (21,64–66)].

The evaluation of the microglial response in the present study assessed the number of microglia and their immunophenotype using ED1 labeling of CD68. The latter is well established as a marker of microglial activation after whole-brain irradiation and other pro-inflammatory challenges. Previous studies showed that robust radiation-related increases in CD68 expression occur in rodents examined at time from a few days to 3 months after a single dose of radiation (11,30,43,58). In the current study, fractionated whole-brain irradiation resulted in significant increases in the numbers and proportions of ED1⁺ cells at 28 weeks but not at 54 weeks after irradiation. The magnitude of the increase in activated microglia in irradiated compared to sham animals at 28 weeks was smaller than that seen at the more acute times investigated in previous single-dose studies and was not statistically significant in all regions under all conditions. The smaller relative increase at 28 weeks after irradiation (compared to shorter survival periods in previous studies) and the absence of a significant irradiation effect at 54 weeks were not due to decreased activation in irradiated animals with longer survival periods but rather to a robust aging-related increase in microglial activation [see also ref. (43)]. Thus microglial activation, manifested as CD68 expression, appears to be sustained chronically after fractionated whole-brain irradiation, although aging-related changes eventually produce comparable levels of activation in nonirradiated animals.

Neither radiation- nor aging-related increases in CD68 expression appeared to be influenced by AT₁R blockade, but the breadth and diversity of functional changes in the microglial population are only poorly understood. Radiation and the brain RAS may modulate other responses that were not evident with our analyses. For example, microglia, even in an activated state, can release trophic factors that influence neuronal function and survival after injury (67,68). Microglia in irradiated animals treated with L-158,809 may produce anti-inflammatory signals and/or trophic factors that modulate information processing in nearby neurons and thereby influence neural function.

A simple hypothesis relating cognitive benefits to suppression of a chronic inflammatory response is not supported by the present study, since no changes in established inflammatory

markers were seen in these animals at postirradiation times at which amelioration of cognitive deficits is apparent. Beneficial effects of AT₁R antagonism after whole-brain irradiation may, however, involve anti-inflammatory mechanisms acting earlier in the postirradiation period, the cognitive effects of which become apparent only after several months. This possibility is consistent with the observation that L-158,809 treatment for only 5 weeks after irradiation partially ameliorated the cognitive deficit from radiation in the NOR task measured at 26 weeks (26). Additional studies examining inflammatory and other changes in the weeks prior to the development of cognitive deficits will be required to investigate that possibility.

SGZ Proliferation and Neurogenesis after Whole-Brain Irradiation and AT₁R Antagonism

Numerous studies of radiation-induced brain injury have established that whole-brain irradiation results in a sustained decrease in neurogenesis due to both death of progenitor cells and ongoing changes in the microenvironment that are regulated at least in part by inflammatory signals (10,11,29,58,69). Thus hippocampal neurogenesis was an important component of the present study. Even in the absence of demonstrable effects on neuroinflammation, assessing effects of AT₁R blockade on proliferation and neurogenesis was critical since the brain RAS may influence proliferation and neurogenesis through other mechanisms. In this study, L-158,809 treatment did not ameliorate radiation-induced changes in proliferation or the density of DCX⁺ cells in the SGZ but decreased DCX⁺ cells in nonirradiated rats (28-week group). The experimental design did not permit assessment of effects of radiation or AT₁RA treatment on cell survival, but a recent study demonstrated that the same radiation regimen used in this study had no effect on the number of granule neurons or the volume of the DG when assessed 1 year after irradiation (70), consistent with the conclusion that neurogenesis is not greatly affected at the late time. The absence of an ameliorative effect on radiation-induced changes in the present study is consistent with the lack of effects on direct measures of inflammation. The decrease in differentiating neurons in sham animals treated with L-158,809 indicates that AT₁RAs influence neurogenesis by suppressing production of new neurons or, alternatively, by speeding up their maturation such that new neurons spend less time in the DCX-expressing stage of neurogenesis (31). The latter is consistent with previous studies that demonstrated that, in contrast to AT₁R stimulation, which promotes cell proliferation, antagonists to the AT₁R increase AT₂R activation, which promotes neuronal differentiation and maturation (15,17).

Other Possible Targets of AT₁R Regulation

In the absence of evidence that L-158,809 ameliorates cognitive dysfunction through chronic suppression of inflammation, there are several other possible mechanisms, including direct effects at the synaptic level. First, Ang II is localized to synaptic vesicles and can modulate both pre- and postsynaptic transmission. Several laboratories have demonstrated AT₁R-mediated inhibition of long-term potentiation, a cellular model of learning and memory (16, 71–74). Second, the RAS regulates extracellular matrix (ECM) turnover and alters cell-cell and cell-ECM interactions that affect synaptic formation and strength. For example, AT₁RAs suppress AT₁R-induced expression of TGF- β , a fibrogenic cytokine involved in extracellular matrix deposition (59). Such suppression could increase synaptic plasticity. Third, AT₁R antagonism may improve cognitive function after fractionated irradiation through effects on the vasculature, since radiation causes vascular changes that produce ischemic/hypoxic conditions (75). Beneficial cognitive effects of AT₁RAs could be mediated by increasing cerebral blood flow and alleviating local, radiation-induced hypoxic conditions (17).

Conclusions

In summary, L-158,809 treatment before, during and after fractionated whole-brain irradiation prevents or ameliorates the progressive cognitive deficits that arise long after the completion

of radiotherapy. The findings of this study suggest that these cognitive benefits occur without inhibition of microglial activation or protection of neurogenesis, mechanisms that have been the focus of many studies of radiation-induced brain injury. Given the clinical promise of AT₁RAs, studies of radiation-induced cognitive dysfunction and its modulation by RAS inhibitors must continue to explore different mechanisms that may contribute to the drugs' beneficial effects.

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FIG. 1. Region of interest contours for analyses in PRh, GCL/hilus (SGZ within DG indicated by dashed line), and dorsal CA3 [adapted from ref. (36) with permission].

FIG. 2.

Markers of microglia, activated microglia, proliferating cells and immature neurons in F344 $\times$ Brown Norway rats. Panels A and B: Iba1 labeling of microglia in the PRh of sham (panel A) and irradiated (panel B) rats at 28 weeks postirradiation. Most Iba1⁺ cells in both conditions have extensive processes, but many cells in irradiated rats exhibit increased labeling intensity and shorter, thicker processes. Panels C and D: ED1 labeling in the PRh of sham (panel C) and irradiated rats (panel D, 28 weeks postirradiation). In sham animals, the density of ED1⁺ cells is lower, and most of the labeled cells contain only small regions of light labeling (short arrows). In irradiated animals, the density of ED1⁺ cells is increased, and the majority of the labeled cells contain both more intense and more extensive labeling (long arrows), often with two or

more immunolabeled regions adjacent to the nucleus. Panel E: Single immature neuron in the SGZ of the DG labeled for DCX (arrow). Panel F: Sytox green labeling of all nuclei demonstrates the organization of the GCL. Scale bars = 50 μm , 25 μm (inset).

FIG. 3.

Quantitative analysis of microglial number and activation in the PRh. Whole-brain irradiation (fWBI) significantly decreased the total microglial population (panel A) in the PRh at 28 but not at 54 weeks after irradiation (** $P < 0.001$, * $P < 0.01$ compared to sham); treatment with the AT₁RA L-158,809 had no effect in sham animals and did not modify the radiation-induced reduction in Iba1⁺ cells. Radiation increased the numbers (panel B) and percentages (panel C) of activated microglia in the PRh at 28 but not at 54 weeks postirradiation, regardless of drug treatment (** $P < 0.001$, * $P < 0.01$ compared to sham).

FIG. 4.

Effects of fractionated whole-brain irradiation and AT₁R antagonism on proliferation and neurogenesis in the SGZ. Panel A: Radiation (fWBI) decreased the density of proliferating cells in the SGZ at both 28 and 54 weeks postirradiation. L-158,809 had no effect on proliferation at either time (* $P < 0.001$, ** $P < 0.01$ compared to sham controls). The experimental design did not permit statistical comparisons between the two survival periods, but the density of proliferating cells in sham rats in the 54-week group was less than half that in the 28-week group, presumably due to an aging-related decline. Panel B: Significant radiation- and AT₁RA-induced reductions in the density of immature neurons were evident at 28 weeks after irradiation (* $P < 0.001$ compared to sham controls; # $P < 0.01$ compared to sham

without L-158,809 treatment), but the density of immature neurons was equivalent across all groups at 54 weeks. The effects of radiation and drug treatment were not additive; AT₁R blockade in irradiated rats did not reduce the density of DCX⁺ cells below that seen in irradiated rats without drug treatment.

FIG. 5.

Analysis of covariance of cognitive function and microglial activation. When considering all rats in all groups at 28 (panel A) or 54 (panel B) weeks postirradiation, there was no significant correlation between performance in the NOR task (lower discrimination ratio indicates poorer recognition memory) and the percentage of microglia in the PRh that were ED1⁺. Separating the rats without drug treatment (panel C) from those receiving L-158,809 (panel D) at the earlier time revealed that cognitive performance and microglial activation were significantly correlated, but only in animals without AT₁R blockade.

TABLE 1

Microglial Measures in the Hippocampus

	Time (weeks)	Sham/water	Radiation/water	Sham/AT1RA	Radiation/AT1RA
<i>GCL/hilus of the DG</i>					
No. of Iba1 ⁺ cells $\times 10^{-3}$	28	48.7 (3.2)	45.2 (3.0)	48.0 (3.7)	48.0 (3.0)
	54 ^a	57.0 (2.5)	47.1 (4.7) ^e	54.1 (3.7)	50.9 (3.7)
No. of ED1 ⁺ cells $\times 10^{-3}$	28 ^b	20.4 (1.6)	25.0 (2.0)	19.3 (2.0)	23.2 (1.6)
	54	37.6 (1.5)	33.9 (2.0)	33.7 (2.1)	37.2 (1.0)
Percentage of microglia ED1 ⁺	28 ^c	42.0 (3.2)	55.3 (3.0) ^f	40.0 (3.7)	48.8 (3.0)
	54	66.3 (2.5)	72.5 (4.7)	62.5 (3.7)	70.5 (3.7)
<i>CA3</i>					
No. of Iba1 ⁺ cells $\times 10^{-3}$	28	28.2 (2.5)	24.3 (1.2)	24.9 (1.6)	23.1 (1.3)
	54	30.4 (1.1)	28.3 (3.1)	29.9 (1.7)	26.0 (1.4)
No. of ED1 ⁺ cells $\times 10^{-3}$	28	10.1 (0.6)	9.8 (0.4)	9.8 (1.0)	11.1 (0.8)
	54	19.4 (0.5)	18.2 (1.4)	18.0 (0.6)	17.7 (1.1)
Percentage of microglia ED1 ⁺	28 ^d	36.6 (2.4)	43.3 (2.0)	39.2 (3.1)	48.3 (3.6) ^g
	54	64.0 (2.7)	65.8 (4.6)	60.9 (2.4)	68.5 (4.0)

Note. Values given are means (SEM).

^a $F((1,20)) = 6.225, P = 0.02$.

^b $F((1,20)) = 5.625, P = 0.03$.

^c $F((1,20)) = 11.628, P = 0.003$.

^d $F((1,20)) = 7.701, P = 0.01$.

^e $P = 0.02$ compared to sham/water.

^f $P = 0.009$ compared to sham/water.

^g $P = 0.04$ compared to sham/AT1RA.