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Mice Lacking RIP3 Kinase are not Protected from Acute Radiation Syndrome

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Abstract

Exposure to high doses of ionizing radiation can cause lethal injury to normal tissue, thus inducing acute radiation syndrome. Acute radiation syndrome is caused by depletion of bone marrow cells (hematopoietic syndrome) and irreparable damage to the epithelial cells in the gastrointestinal tract (gastrointestinal syndrome). Although radiation initiates apoptosis in the hematopoietic and gastrointestinal compartments within the first few hours after exposure, alternative mechanisms of cell death may contribute to injury in these radiosensitive tissues. In this study, we utilized mice lacking a critical regulator of necroptosis, receptor interacting protein 3 (RIP3) kinase, to characterize the role of RIP3 in normal tissue toxicity after irradiation. Our results suggest that RIP3-mediated signaling is not a critical driver of acute radiation syndrome.

INTRODUCTION

The increasing threat of nuclear accidents and radiological terrorism has created a need to understand the molecular mechanisms regulating acute radiation syndrome (ARS). Exposure to high doses of radiation induces ARS by injuring critical organ systems, including the hematopoietic system and the gastrointestinal tract (1, 2). Hematopoietic ARS, characterized by pancytopenia, is triggered by total-body irradiation (TBI) (6.5–9 Gy in mice) and can result in infection, hemorrhage and death within 30 days in mice (2, 3). Bone marrow transplants can prevent lethality after TBI in some patients, and the U.S. Food and Drug Administration (FDA) recently approved a granulocyte colony-stimulating factor for use after a radiological incident based on animal studies (4, 5). Higher doses of radiation (>12 Gy in mice) to the abdomen can cause gastrointestinal ARS by disrupting the intestinal mucosal barrier, thereby increasing dehydration and susceptibility to infection (2, 6, 7). Injury to the gastrointestinal tract can lead to death within 10 days of radiation exposure in mice and no mitigators have been approved by the FDA to treat gastrointestinal ARS (2).

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To facilitate the development of medical countermeasures that prevent ARS, it is essential to characterize the cell death pathways that mediate radiation injury in distinct organ systems. During the acute phase of hematological radiation toxicity, cell death of hematopoietic progenitor cells and differentiated progeny is mediated by apoptosis (3). Both p53 and its transcriptional target, p53 upregulated modulator of apoptosis (PUMA), activate the intrinsic pathway of apoptosis in the hematopoietic compartment and promote hematopoietic ARS (8–11). In addition to mediating acute cell death, hematological radiation injury also induces chronic defects in hematopoietic stem cells, which decreases the ability of these cells to thrive in cell competition assays and minimizes their long-term engraftment potential (12–14). The radiation-induced impairment in hematopoietic stem cell fitness is regulated in part through apoptosis-independent mechanisms, including cell senescence (12, 15, 16).

In the gastrointestinal tract, death of crypt epithelial cells compromises the integrity of the small intestine by preventing stem cell-driven tissue regeneration (17–19). Some gastrointestinal progenitor cells that reside near the base of the crypt in mice undergo apoptosis in response to low-dose radiation (1 Gy), which triggers the proliferation of clonogenic cells that reside at other positions in the crypt (19). These surviving clonogenic cells can be organized into tiers that have progressively decreased radiosensitivities, with the second tier being recruited at doses less than 9 Gy and the third tier at doses greater than 9 Gy (19). Although the gastrointestinal epithelium undergoes p53-mediated apoptosis, knockout mice lacking *p53* or mice with *p53* specifically deleted in the gastrointestinal epithelium are sensitized to the gastrointestinal ARS, suggesting that p53 plays a protective role in this tissue (9, 20–23). Furthermore, mice that have deletion of two pro-apoptotic genes, *Bak* and *Bax*, in the gastrointestinal epithelium exhibit significantly reduced apoptosis in the crypt, but these mice are not protected from gastrointestinal ARS (20). Collectively, these results suggest that death of relatively radioresistant clonogenic cells within the crypt, rather than radiosensitive progenitor cells, may regulate lethality from gastrointestinal ARS. Because most radioresistant crypt cells die at doses higher than 9 Gy by a mechanism independent of apoptosis, the intrinsic pathway of apoptosis is not the sole mediator of gastrointestinal ARS (17). Therefore, additional studies are required to fully elucidate the molecular mechanisms of cell death regulating ARS.

Recently published studies have highlighted potential roles for apoptosis-independent mechanisms of cell death after radiation exposure, including mitotic death and various forms of programmed necrosis, such as pyroptosis (caspase-dependent) and necroptosis (caspase-independent) (20, 24–26). For example, necroptosis, which is characterized by kinase activity of receptor interacting protein 3 (RIP3), can be modulated by drugs that target this signaling pathway (27). Several recently reported studies have concluded that pharmacological inhibition of necroptosis can mitigate death from ARS in mice (28–30). Since pharmacological inhibitors may have off-target effects, in this study, we utilized a genetic approach with *rip3* knockout mice to investigate the role of RIP3 in hematopoietic and gastrointestinal ARS. We show that mice lacking RIP3 are not protected from TBI, which induced the hematopoietic syndrome, or subtotal body irradiation (SBI), which induced the gastrointestinal syndrome.

MATERIALS AND METHODS

Animals

All animal procedures used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Duke University. The *rip3* knockout mouse strain was kindly provided with a C57BL/6N genetic background by Vishva M. Dixit (Genentech, San Francisco, CA). These mice were originally generated by targeting 129 R1 embryonic stem cells, which were injected into C57BL/6N blastocysts (31). Experiments were performed on 6-to-11-week-old male and female mice. Age-matched littermate controls were utilized to minimize the effect of subtle differences in genetic background on the radiation response.

Small Intestinal Epithelial Cell Isolation

Irradiated and nonirradiated mice were euthanized 0, 24 and 48 h after 14.6 Gy SBI. Approximately 12 cm of the jejunum was removed. Small intestines were flushed with cold phosphate buffered saline (PBS), cut longitudinally and washed again. Small intestines were rotated at 4°C for 1 h in 8 ml of epithelium isolation buffer (EIB), containing: PBS, 2 mM EDTA, 1 mM DTT, 50 mM gentamicin and 1× Antibiotic-Antimycotic (Thermo Fisher Scientific™ Inc., Rockford, IL). Small intestines were transferred to a new tube with 5 ml of cold EIB and epithelial cells were detached by vigorous shaking. The muscularis layers were removed and the epithelial cells were washed twice with cold PBS.

Immunoblotting

Mouse small intestinal epithelial cells were lysed in cold RIPA buffer supplemented with aprotinin, PhosSTOP™ tablet, cOmplete™ mini protease inhibitor cocktail tablet (all from Sigma-Aldrich® LLC, St. Louis, MO) and 1 mM PMSF. Soluble proteins were quantified and equal amounts of protein were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with specific antibodies for RIP3 (1:1,000; Novus Biologicals LLC, Littleton, CO), phosphorylated RIP3 (1:200; Abcam®, Cambridge, MA) or GAPDH (1:5,000; Proteintech™ Group Inc., Chicago, IL). Proteins were visualized using the infrared fluorophore labeled secondary antibodies, anti-rabbit IRDye® 800 and anti-mouse IRDye 680 (both from LI-COR® Biosciences, Lincoln, NE) and imaged with the Odyssey® Imaging System (LI-COR Biosciences).

Quantitative Reverse Transcription-PCR

Mouse small intestinal epithelial cells were pelleted and resuspended in 1 ml of TRI Reagent (Invitrogen™, Grand Island, NY). Total RNA was extracted and quantified. cDNA was generated from 1 µg of RNA using iScript™ cDNA Synthesis Kit (Bio-Rad® Laboratories Inc., Hercules, CA). Relative expression levels were determined using qPCR assays performed on the QuantStudio™ 6 Flex Real-Time PCR System with PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific) and specific primer sets (RIP3 primers: 5′-GGTAGATGCTGCTGTCTCCG-3′ and 5′-AGGGTTCCTCCAAATGCAGG-3′). Target gene quantification levels were normalized to the housekeeper gene, TATA-box binding protein (TBP) using a specific primer set (TBP primers: 5′-TGCACAGGAGCCAAGAGTGAA-3′ and 5′-CACATCACA GCTCCCCACCA-3′).

Irradiations

Mice were irradiated with an X-RAD 320 biological irradiator (Precision X-ray Inc., North Branford, CT). All animals were treated 50 cm from the source with 320 kVp, 12.5 mA X rays at a dose rate of 2.23 Gy/min, using a 2.5-mm aluminum and 0.1-mm copper filter. During TBI, unanesthetized animals were placed in a pie cage without restraints and with no shielding. During SBI, unanesthetized mice were restrained in jigs and placed under lead shielding to protect the head and front limbs from exposure, as described elsewhere (20). All mice were irradiated at approximately the same time, 10:30 a.m. (± 1 h), to minimize the effect of circadian rhythm on radiation response. Whole bone marrow cells were irradiated *in vitro* using a second X-RAD 320 biological irradiator (Precision X-ray Inc.). Cells were treated 50 cm from the source with 320 kVp, 10 mA X rays at a dose rate of 1.96 Gy/min, using a 2-mm aluminum filter. Dose rates were measured by the Duke University Radiation Safety Division staff using an ion chamber.

Hematopoietic Colony Formation Assay

Whole bone marrow cells were isolated from the femurs and tibias of mice ($n = 2-3$ per genotype) by grinding the bones in hematopoietic stem cell buffer [Hank's Balanced Salt Solution with Ca^{2+} and Mg^{2+} (Gibco®, Grand Island, NY), 5% fetal bovine serum and 2 mM EDTA]. ACK lysing buffer (Lonza, Morristown, NJ) was utilized to deplete red blood cells. After red blood cell lysis, the cells were counted using a Coulter counter (Beckman Coulter® Inc., Fullerton, CA) and plated in duplicate in MethoCult™ GF 3434 (STEMCELL™ Technologies Inc., Vancouver, Canada). Cells were irradiated 1 h after plating and colonies were counted 7 days later by a single blinded observer.

Histological Analysis

Tissue samples from the femur were fixed in 4% paraformaldehyde overnight, decalcified in Richard-Allan Scientific™ Decalcifying Solution (Rockford, IL) for 24–48 h, preserved in 70% ethanol and then embedded in paraffin. Tissue specimens from the jejunum were carefully rinsed with cold PBS, fixed in 10% neutrally buffered formalin for 24–48 h, preserved in 70% ethanol, bundled and embedded vertically in paraffin to facilitate the generation of transverse sections. Sections from these tissues were stained with hematoxylin and eosin (H&E) prior to analysis.

Crypt Survival Assay

A 12-cm segment of the jejunum was harvested from irradiated mice 96 h after SBI ($n = 2-5$ mice per genotype and experimental condition). BrdU (Sigma-Aldrich) was administered 1.5 h prior to euthanasia by intraperitoneal injection at 120 mg/kg. Immunohistochemistry was performed on tissue sections from the jejunum using anti-BrdU (1:100; Abcam). The number of surviving crypts per cross section was scored in approximately 7 circumferences per mouse by a single, blinded observer. To correct for the increased likelihood of counting a larger crypt, we multiplied the surviving number of crypts by a correction factor (mean width of crypts in nonirradiated *rip3^{+/+}* mice divided by mean width of irradiated crypts in each experimental group).

Study Design and Statistics

All data are presented as mean $\pm$ SEM. Student's *t* test (two-tailed) was used to compare the mean of two groups. Two-way analysis of variance (ANOVA) was used to examine the interaction between genotype and radiation or time point. For ARS studies, Kaplan-Meier analysis was performed followed by the log-rank test for statistical significance. Significance was considered at $P < 0.05$. With the exception of the 7.8 Gy TBI pilot experiment (Fig. 3A), all ARS studies were repeated in 2–3 individual mouse cohorts. The Kaplan-Meier curves include all data generated in these replicate cohorts. Prism 7 (GraphPad Software Inc., LaJolla, CA.) was used for the statistical analysis.

RESULTS

Radiation exposure can lead to the accumulation of RIP3 protein, thereby initiating necroptosis (28). In this study, C57BL/6N mice received 14.6 Gy SBI and gastrointestinal epithelial cells were harvested to confirm that exposure increased RIP3 messenger RNA (mRNA) and protein expression 24 h postirradiation. Although RIP3 mRNA levels remained elevated 48 h postirradiation, we detected a reduction in the protein concentration at this time point, suggesting that turnover of RIP3 protein occurs more quickly than turnover of mRNA through either translational or post-translational regulation (Fig. 1A and B). Nevertheless, RIP3 kinase was activated 48 h postirradiation as detected by phosphorylation of RIP3 through Western blot analysis (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR15001.1.S1>). To dissect the role of RIP3-mediated cell death in ARS, we acquired *rip3* knockout mice (31). We confirmed a decrease in mRNA and protein concentration in the *rip3*^{+/-} mice and an absence of detectable mRNA or protein in the *rip3*^{-/-} mice (Fig. 2A and B). The *rip3* knockout mice and littermate controls were then exposed to either TBI or SBI to induce hematopoietic or gastrointestinal syndrome, respectively.

To investigate whether mice lacking RIP3 were protected from radiation-induced hematopoietic syndrome, *rip3* knockout mice and littermate controls were total-body irradiated. A small cohort of mice received 7.8 Gy TBI, which resulted in hematopoietic ARS in 100% of mice within 30 days (LD_{100/30}). No significant difference was observed among the *rip3*^{+/+}, *rip3*^{+/-} and *rip3*^{-/-} mice (Fig. 3A). To determine whether RIP3 deficiency prevented hematopoietic syndrome at lower radiation doses, mice received either 7 Gy (LD_{80/30}) or 6.7 Gy (LD_{25/30}) TBI. Although we observed a trend toward enhanced radiosensitivity in the *rip3*^{-/-} mice, the status of *rip3* did not significantly affect the occurrence of hematopoietic syndrome (Fig. 3B and C). Similar trends were observed in both male and female mice. Furthermore, we detected no difference in the *in vitro* survival of hematopoietic progenitors from *rip3* knockout mice compared to *rip3*^{+/+} controls after 2, 4 or 6 Gy exposure using a colony formation assay in methylcellulose (Fig. 3D). Consistent with published guidelines for studying hematopoietic ARS (2), mice receiving 6.7 Gy TBI developed pancytopenia and depletion of cells within the bone marrow within 8 days postirradiation (Fig. 3E–I; Supplementary Fig. S2; <http://dx.doi.org/10.1667/RR15001.1.S1>). We did not observe a significant difference among the complete blood counts in the irradiated *rip3*^{+/+}, *rip3*^{+/-} and *rip3*^{-/-} mice. Together, these results suggest that RIP3 does not promote acute hematopoietic syndrome.

To assess the role of RIP3 in acute gastrointestinal syndrome, *rip3* knockout mice and littermate controls received SBI, in which approximately one-third of the bone marrow was shielded. A sublethal dose of 15.9 Gy (LD_{40/10}) induced gastrointestinal syndrome to a similar extent in *rip3*^{+/+}, *rip3*^{+/-} and *rip3*^{-/-} mice (Fig. 4A). Although this experiment does not utilize multiple radiation doses to generate a dose-effect curve, it suggests that the status of RIP3 does not alter survival from gastrointestinal syndrome. Similar results were obtained for both male and female mice. Furthermore, the status of RIP3 does not alter the number of regenerating crypts within the small intestine after irradiation, as determined by a crypt survival assay (Fig. 4B–D). These data indicate that RIP3-deficient mice are not protected from radiation-induced gastrointestinal syndrome.

DISCUSSION

Necroptosis is characterized by the programmed activation of RIP3 kinase and culminates in plasma membrane rupture (Fig. 5) (27). Activation of death receptors, Toll-like receptors or T-cell receptors can trigger the formation of a cytosolic complex that recruits and activates RIP3 (32). The recruitment of RIP3 converts the complex into a necrosome, which drives necroptosis through the auto-phosphorylation of RIP3 (33). Activated RIP3 recruits and phosphorylates mixed-lineage kinase domain-like protein (MLKL), which then translocates to the plasma membrane and increases membrane permeability (34–37). The receptor interacting protein 1 (RIP1) kinase is also a core component of the necrosome, playing a role in RIP3 recruitment and activation (38). However, RIP1 can engage in pro- or anti-necroptotic signaling pathways based predominantly on the cell death stimulus and the availability of RIP3 (38, 39). Since sufficient expression of RIP3 is required to initiate necroptosis, RIP3 is considered to be the primary regulator of the necroptotic program and in select cases necroptosis can occur in the absence of RIP1 (39–41).

Emerging evidence found in the literature on *in vitro* and *in vivo* studies demonstrate that radiation can induce necroptotic cell death (24, 28, 30). In the current study, we utilized mouse genetics to investigate the specific role of RIP3 kinase in ARS. Although we observed induction and phosphorylation of RIP3 after irradiation, mice lacking this critical kinase in the necroptosis pathway were not protected from hematopoietic or gastrointestinal ARS. Likewise, functional assays did not reveal an increase in stem- or progenitor cell survival in *rip3* knockout mice after irradiation.

In several recently reported studies, an inhibitor of necroptosis, Necrostatin-1, mitigated death from ARS in mice (28–30). Necrostatin-1 is an allosteric inhibitor of RIP1 kinase, but does not affect RIP3 activity (42). Since these two kinases play distinct roles in regulating necroptosis, it is conceivable that Necrostatin-1 improves survival from ARS in mice through RIP3-independent mechanisms. Additionally, Necrostatin-1 has also been characterized as an indoleamine 2,3-dioxygenase inhibitor capable of modulating inflammation and therefore has a role in RIP1-independent cell death (43, 44). Thus, the complexity of RIP1 signaling and the RIP1-independent effects of Necrostatin-1 may explain the different results obtained with genetic ablation of *rip3* versus pharmacological inhibition of RIP1.

Our results with mice permanently lacking RIP3 expression do not exclude the possibility that RIP3 may differentially regulate ARS at various time points postirradiation. Huang *et al.* recently reported that treatment with Necrostatin-1 could rescue mice from hematopoietic ARS when administered 24, 48 or 72 h after TBI (28). However, treatment with Necrostatin-1 immediately prior to irradiation did not prevent lethality, suggesting that modulating RIP1 activity is most effective in regulating ARS after the levels of pro-necroptotic inflammatory cytokines are elevated (28). It is therefore conceivable that RIP3 regulates survival from ARS only at specific time points postirradiation, which cannot be evaluated in knockout mice. Alternatively, permanent disruption of RIP3-mediated signaling could result in compensation by an alternative mechanism of cell death after radiation exposure.

While several recent studies suggest that Necrostatin-1 may improve survival from ARS, we did not identify a role for RIP3 in regulating survival after radiation exposure. Although Necrostatin-1 may be a useful countermeasure after a radiological disaster, particularly when used in combination with other cell death inhibitors (29), our studies of a whole animal knockout mouse model demonstrate that RIP3 may be dispensable for ARS and indicate that additional mechanistic studies are necessary to investigate how this compound is regulating cell death. Further characterization of ARS in the absence of RIP1 and MLKL may provide new insights to help design improved mitigators of ARS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIG. 1.

Radiation induces expression of RIP3 kinase, a critical mediator of necroptosis. Expression of RIP3 mRNA (panel A) and RIP3 protein (panel B) in gastrointestinal epithelial cells isolated from *rip3^{+/+}* mice at various time points after 14.6 Gy SBI. * $P < 0.05$ compared to nonirradiated control (Student's *t* test).

FIG. 2.

Verification of *rip3* knockout mouse model. Expression of RIP3 mRNA (panel A) and RIP3 protein (panel B) in gastrointestinal epithelial cells isolated from *rip3*^{+/+}, *rip3*^{+/-} and *rip3*^{-/-} mice. *P < 0.05 (Student's *t* test).

FIG. 3.

Mice lacking RIP3 kinase are not protected from radiation-induced hematopoietic syndrome. Panels A–C: Overall survival of *rip3^{+/+}*, *rip3^{+/-}* and *rip3^{-/-}* mice after 7.8 Gy, 7 Gy and 6.7 Gy TBI, respectively, which cause hematopoietic ARS. Log-rank test was used for statistical analysis. Panel D: Surviving fraction of hematopoietic progenitor cells from *rip3^{+/+}*, *rip3^{+/-}* and *rip3^{-/-}* mice after irradiation in a methylcellulose colony formation assay. Two-way ANOVA was performed to examine the interaction between genotype and radiation dose. Quantification of platelets and white blood cells (panels E and F, respectively) in the peripheral blood at various time points after 6.7 Gy TBI. Two-way ANOVA was performed to examine the interaction between genotype and time point. Panels G–I: Representative H&E stained sections of mouse bone marrow 8 days after 6.7 Gy TBI in *rip3^{+/+}*, *rip3^{+/-}* and *rip3^{-/-}* mice. Scale bar, 200 μm .

FIG. 4.

Mice lacking RIP3 kinase are not protected from radiation-induced gastrointestinal syndrome. Panel A: Overall survival of $rip3^{+/+}$, $rip3^{+/-}$ and $rip3^{-/-}$ mice after 15.9 Gy SBI, which causes gastrointestinal ARS. Log-rank test was used for statistical analysis. Panels B and C: Representative H&E- and BrdU-stained sections of mouse jejunum at 96 h after 13.4 or 16.8 Gy SBI. Scale bar, 100 μ m. Panel D: Quantification of the surviving BrdU-positive crypts per circumference within the jejunum 96 h after SBI in $rip3^{+/+}$, $rip3^{+/-}$ and $rip3^{-/-}$ mice. A correction factor was used to account for heterogeneity in crypt widths between the

treatment groups. Two-way ANOVA was performed to examine the interaction between genotype and radiation dose.

FIG. 5.

Schematic of RIP3-dependent necroptosis. Diagram of RIP3-driven necroptosis in response to a cell death stimulus, such as radiation. Upon release of pro-necrotic cytokines, such as tumor necrosis factor (TNF), various adaptor proteins associate with the cytokine receptor and recruit RIP1. Within this signaling complex, RIP1 is subject to polyubiquitylation, which promotes activation of the nuclear factor- κ B (NF- κ B) pathway. Deubiquitylation allows RIP1 to dissociate from the plasma membrane and interact with a cytosolic complex, where it recruits RIP3 to form the necrosome. Trans- and autophosphorylation of RIP1 and RIP3 initiate necroptosis by activating MLKL.