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Activation of Nuclear Factor κB *In vivo* Selectively Protects the Murine Small Intestine against Ionizing Radiation-Induced Damage

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ABSTRACT

Exposure of mice to total body irradiation induces nuclear factor κB (NFκB) activation in a tissue-specific manner. In addition to the spleen, lymph nodes, and bone marrow, the tissues that exhibit NFκB activation now include the newly identified site of the intestinal epithelial cells. NFκB activated by total body irradiation mainly consists of NFκB p50/RelA heterodimers, and genetically targeted disruption of the NFκB *p50* gene in mice significantly decreased the activation. By comparing tissue damage and lethality in wild-type and NFκB p50 knockout ($p50^{-/-}$) mice after they were exposed to increasing doses of total body irradiation, we additionally examined the role of NFκB activation in total body irradiation-induced tissue damage. The results show that $p50^{-/-}$ mice are more sensitive to total body irradiation-induced lethality than wild-type mice ($LD_{50/Day\ 7}$: wild-type = 13.12 Gy *versus* $p50^{-/-}$ = 7.75 Gy and $LD_{50/Day\ 30}$: wild-type = 9.31 Gy *versus* $p50^{-/-}$ = 7.81 Gy). The increased radiosensitivity of $p50^{-/-}$ mice was associated with an elevated level of apoptosis in intestinal epithelial cells and decreased survival of the small intestinal crypts compared with wild-type mice ($P < 0.01$). In addition, RelA/TNFR1-deficient (RelA/$TNFR1^{-/-}$) mice also exhibited a significant increase in intestinal epithelial cell apoptosis after they were exposed to total body irradiation as compared with TNFR1-deficient ($TNFR1^{-/-}$) mice ($P < 0.01$). In contrast, no significant increase in total body irradiation-induced apoptosis or tissue injury was observed in bone marrow cells, spleen lymphocytes, and the liver, heart, lung, and kidney of $p50^{-/-}$ mice in comparison with wild-type mice. These findings indicate that activation of NFκB selectively protects the small intestine against ionizing radiation-induced damage.

INTRODUCTION

Nuclear factor κB (NFκB) is a dimeric DNA binding protein consisting of members of the NFκB/Rel family, which includes the subunits of NFκB1 (p50), NFκB2 (p52), RelA, RelB, and c-Rel (1–3). Its expression is ubiquitous in mammalian cells. Normally, NFκB resides in the cytoplasm in an inactive form in association with inhibitory proteins. These inhibitory proteins, which belong to a family of proteins named inhibitor of NFκB (4), prevent NFκB nuclear translocation by masking the NFκB nuclear localization signal and thus, inhibit NFκB DNA binding and transactivational function (1–3). Various stimuli activate a large number of distinct signaling pathways that eventually result in the phosphorylation of inhibitor of NFκB and its subsequent degradation by the proteasome or its dissociation from NFκB without additional degradation (1–3). The released NFκB then translocates to the nucleus and binds to κB or κB-like DNA motifs to initiate gene transcription. The putative target genes of NFκB are mainly involved in immune and inflammatory responses (1–3). These genes encode a variety of inflammatory molecules, including various inflammatory cytokines and adhesion molecules. In addition, NFκB also regulates the expression of many genes of which the products are involved in the control of cell proliferation and cell death (4–7).

Tumor cells usually express high levels of constitutive NFκB activity (8, 9). In addition, exposure of these cells to various cytotoxic agents including ionizing radiation increases NFκB activity (7–13). The role of NFκB in tumorigenesis and cellular resistance to tumor therapy has been extensively studied. The majority of reported studies have demonstrated that NFκB activation may give transformed cells a growth and survival advantage and additionally may render tumor cells resistant to ionizing radiation and a variety of cytotoxic agents by induction of antiapoptotic proteins (7–13). Therefore, molecularly targeted inhibition of NFκB has been actively pursued as a potential and novel adjuvant treatment for cancer in conjunction with radiotherapy and chemotherapy (7–13).

The purpose of using NFκB inhibitors as an adjuvant therapy for cancer is to increase the therapeutic index of radiotherapy and chemotherapy. The success of this approach relies on its ability to promote tumor cell killing by ionizing radiation or chemotherapy but to spare normal tissues from enhanced damage. Therefore, it is critical to determine the effects of NFκB inhibition on normal tissue function in response to ionizing radiation, because activation of NFκB by ionizing radiation has been documented not only in various tumor cells but also in different types of cultured normal cells *in vitro* (14–25). Previously using a mouse model, we investigated the tissue specificity of ionizing radiation-induced NFκB activation *in vivo* and found that total body irradiation induces NFκB activation in a tissue-specific manner (26). The activation was observed in the bone marrow and the peripheral lymphoid tissues of the spleen and mesenteric lymph nodes shortly after mice were exposed to a lethal dose of total body irradiation but was absent in all of the other tissues examined, including the liver, lung, colon, thymus, and brain (26). Now, we have discovered that exposure of mice to total body irradiation also activates NFκB in intestinal epithelial cells of the small intestine, a prime target of ionizing radiation damage. The NFκB activated by total body irradiation in various tissues mainly consists of NFκB p50/RelA heterodimers and genetically targeted disruption of the NFκB *p50* gene in mice significantly decreased the activation. Therefore, in the present study we compared the tissue damage and lethality in wild-type and NFκB p50 knockout ($p50^{-/-}$) mice after they were exposed to increasing doses of total body irradiation to determine the role of NFκB activation in ionizing radiation-induced normal tissue damage. The results of this study have important clinical implications in cancer therapy using NFκB inhibitors as an adjuvant therapeutic agent in conjunction with ionizing radiation.

MATERIALS AND METHODS

Mice. Normal male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Inbred B6,129PF2 (p50 wild-type or wild-type), B6,129P-Nfkb1 (p50 knockout or $p50^{-/-}$), RelA/TNFR1-deficient (RelA/$TNFR1^{-/-}$), and TNFR1-deficient ($TNFR1^{-/-}$) mice were bred at the Medical

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University of South Carolina, the Association for the Assessment and Accreditation of Laboratory Animal Care-certified animal facility. The homozygous breeding pairs of wild-type and $p50^{-/-}$ mice were obtained from the Jackson Laboratory and those of $RelA/TNFR1^{-/-}$ and $TNFR1^{-/-}$ mice were kindly provided by Dr. David Baltimore (California Institute of Technology, Pasadena, CA). All of the mice received food and water *ad libitum* and were maintained in pathogen-free microisolator cages (4 per cage). They were used at approximately 8–12 weeks of age. The Institutional Animal Care and Use Committee of Medical University of South Carolina approved all of the experimental procedures used in this study.

Ionizing Radiation. Mice were exposed to various doses of ionizing radiation in a JL Shepherd Model 143 ^{137}Cesium γ-irradiator (JL Shepherd, Glendale, CA) at a rate of 2.4 Gy/min. Mice were irradiated on a rotating platform.

Tissue Collection. After exposure to different doses of ionizing radiation, mice were euthanized at various times as indicated in individual experiments by CO_2 suffocation followed by cervical dislocation. A group of unirradiated mice was euthanized similarly as control. The spleen, liver, lung, kidney, and heart were harvested, weighed, and fixed with 10% neutral-buffered formalin for histopathological examination. The small intestines were collected. Some of them were processed as described later for epithelial cell apoptosis and crypt survival assays. The others were used for the preparation of nuclear extracts. In addition, the femoral bones were isolated for the preparation of bone marrow-mononuclear cells as reported previously (27, 28).

Preparation of Nuclear Extracts. About 20-cm length of the proximal jejunum was immediately removed after mice were euthanized and flushed with ice-cold PBS. After the jejunum was cut open on a ice-chilled Petri dish, intestinal mucosa was scraped in ice-cold PBS with 1 mol/L dithiothreitol as reported elsewhere (29). The intestinal epithelial cells were collected by centrifugation. The nuclear extracts of intestinal epithelial cells were prepared using a method as published previously (26, 27, 30), and the protein concentrations of the nuclear extracts were accurately quantified using the Bio-Rad Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Analysis of NFκB Activities by Gel Shift and Supershift Assay. The double-stranded oligonucleotides containing a consensus κB sequence (5′-AGTTGAGGGACTTTCCCAGGC-3′; Integrated DNA Technologies, Inc., Coralville, IA) were labeled using the Biotin 3′ End DNA Labeling kit (Pierce, Rockford, IL) according to the manufacturer's protocol. The gel shift assay was performed using the LightShift Chemiluminescent electrophoretic mobility shift analysis kit (Pierce) following the manufacturer's instructions. Briefly, an aliquot of nuclear extracts containing 5 μg of protein was incubated with 2 μl of electrophoretic mobility shift analysis binding buffer, 20 fmoles of biotin-labeled NFκB probe and 1 μl of poly(deoxyinosinic-deoxycytidylic acid) in a total volume of 20 μl for 20 min at room temperature. The reaction mixtures were separated on a 6% native polyacrylamide gel by electrophoresis and then transferred to Biodyne B Nylon Membrane (Pierce). After the membrane was incubated with LightShift Stabilized Streptavidin-Horseradish Peroxidase Conjugate and the Luminol/Enhancer and Stable Peroxide Solution, the NFκB DNA complexes were detected by exposure of the membrane to X-ray film. The relative nuclear NFκB DNA binding activities were quantified by scanning densitometry. The specificity of the identified NFκB DNA binding activity in the nuclear extracts was confirmed by using 200-fold excess of unlabeled NFκB, mutated NFκB (Santa Cruz Biotechnology, Santa Cruz, CA), or activator protein-1 (Promega, Madison, WI) oligonucleotides. The addition of the excess unlabeled NFκB oligonucleotides into the gel shift reaction resulted in elimination of the relative NFκB DNA binding activities, whereas that of the excess unlabeled mutated NFκB or activator protein-1 oligonucleotides did not affect the assay (Fig. 1*C*). For gel supershift analysis, extracted nuclear proteins (5 μg) were incubated with 2 μg of the polyclonal antibodies specifically against the p50, p52, RelA, and/or Erg-2 proteins (Santa Cruz Biotechnology) for 20 min before their incubations with biotin-labeled NFκB probe in the gel shift assay described above.

Analysis of Bone Marrow-Mononuclear Cell Apoptosis and the Hematopoietic Function of Bone Marrow Stem Cells and Progenitors. Bone marrow-mononuclear cell apoptosis was determined by measuring sub-$G_{0/1}$ cells after DNA staining with propidium iodide and the hematopoietic function of bone marrow stem cells and progenitors was measured by cobblestone area-forming cell assay. These assays were performed as described previously (27, 28). The frequencies of cobblestone area-forming cell were determined at

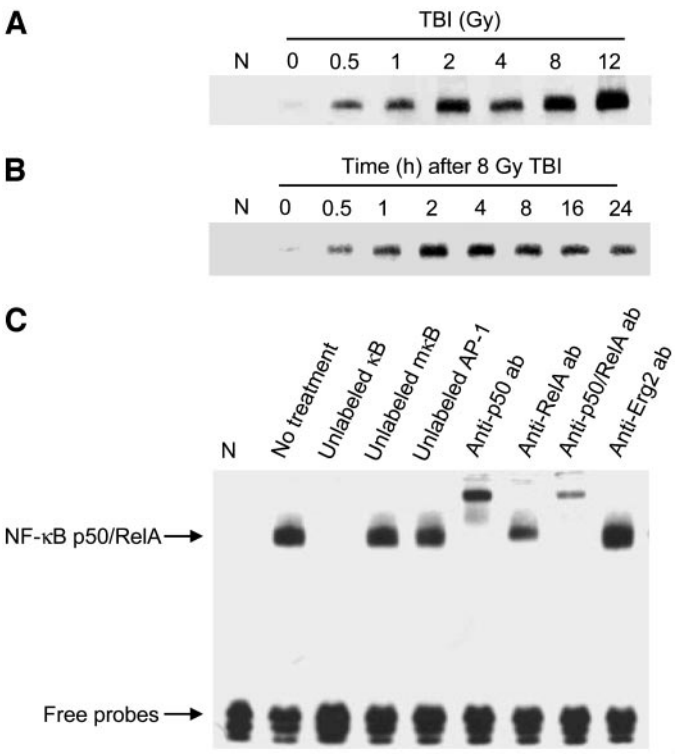

Fig. 1. Ionizing radiation induces NFκB activation in intestinal epithelial cells in a dose- and time-dependent manner. Wild-type mice were euthanized 2 hours after receiving 0.5, 1, 2, 4, 8, or 12 Gy total body ionizing radiation (*A*) or euthanized at 0.5, 1, 2, 4, 8, 16 or 24 hours after 8 Gy total body irradiation (*B*). Control mice (*0*) were euthanized without exposure to total body irradiation. Nuclear proteins were extracted from isolated intestinal epithelial cells and analyzed for NFκB DNA binding activity using gel shift assay. The molecular composition of ionizing radiation-activated NFκB in intestinal epithelial cells from wild-type mice was determined by gel supershift assay (*C*). *N*, no sample; *TBI*, total body irradiation; *ab*, antibody.

weekly intervals (on days 7, 14, 21, 28, and 35). Wells were scored positive if at least one dark-phase hematopoietic clone (or cobblestone area containing ≥5 cells) was seen. The frequency of cobblestone area-forming cell was then calculated by using Poisson statistics as described previously (31, 32). This assay provides an estimate of the hematopoietic function of a spectrum of cobblestone area-forming cell day-types that correspond to various stages of stem cells and progenitors. Specifically, day-7 cobblestone area-forming cell and day-14 cobblestone area-forming cell correspond to colony forming unit-granulocytes and monocytes and day-12 colony forming unit-spleen, respectively. The primitive stem cells with long-term repopulating ability correspond to day-28 and -35 cobblestone area-forming cell (32).

Intestinal Apoptosis Assay. The small intestine was rapidly removed from the abdomen after mice were euthanized at various times as described in individual experimental plans. The intestinal contents were removed, and then the intestinal tube was flushed with saline and cut into ~10 1-cm lengths, which were bundled together with micropore tape before fixation with 10% neutral-buffered formalin overnight. The tissues were embedded in paraffin, and sections (5 μm) were cut perpendicular to the long axis of the intestine and stained with hematoxylin and eosin. The number of apoptotic cells per crypt was assessed by morphological criteria in a blind fashion as described previously by Potten *et al.* (33). Only well-oriented crypts (50 crypts/animal) in longitudinal sections containing Paneth cells, a crypt lumen, and an uninterrupted column of epithelial cells extending to the crypt-villus junction were scored. In addition, ionizing radiation-induced intestinal epithelial cell apoptosis was examined by immunohistochemistry of active caspase 3 and terminal deoxynucleotidyl transferase-mediated nick end labeling assay (34). For active caspase 3 immunohistochemistry, intestinal tissue sections were deparaffinized and rehydrated. After blocking endogenous peroxidase with 0.3% hydrogen peroxide solution for 15 min, the sections were boiled in 10 mM citrate buffer (pH 6.0) for 10 min and then cooled for 20 min to enhance antigen exposure. Nonspecific binding was blocked by incubation of the sections in 10% normal

goat serum for 30 min. The sections were incubated with 1:500 rabbit polyclonal antiactive caspase 3 antibody (R&D Systems, Minneapolis, MN) for 18 h at 4°C, extensively washed, and then incubated with 1:200 biotinylated goat antirabbit secondary antibody for 45 min at room temperature. The immunostaining was developed using Vectastain ABC reagents (Vector Laboratories, Inc., Burlingame, CA), 3,3′-diaminobenzidine, and hydrogen peroxide. The sections were counterstained with hematoxylin. Terminal deoxynucleotidyl transferase-mediated nick end labeling assay was performed using the ApopTag fluorescent *in situ* apoptosis detection kit (Serologicals, Norcross, GA). Briefly, deparaffinized and rehydrated intestinal tissue sections were permeabilized with proteinase K (20 μg/ml) for 20 min, washed, and then incubated with digoxygenin-deoxynucleotide triphosphates and terminal deoxynucleotidyl transferase at 37°C for 1 h. The sections were immersed in stop/wash buffer for 10 min to terminate the reaction and then incubated with fluorescent-conjugated antidigoxigenin antibody for 30 min. Antifade mounting medium was used for fluorescence coverslipping.

Crypt Survival Assay. Three days after exposure to ionizing radiation (12 Gy), each mouse received i.p. injection of 120 mg/kg BrdUrd (Sigma, St. Louis, MO) and 12 mg/kg 5-fluoro-2′-deoxyuridine (Sigma) to label the S-phase cells. Two hours after the injection, mice were euthanized, and their proximal jejunum were collected, prepared, fixed, embedded, and sectioned at 5 μm as described above. Cells incorporating bromodeoxyuridine were detected by mouse antibromodeoxyuridine antibody and visualized by immunofluorescence using Texas red-labeled goat antimouse IgG (red) and Hoechst 33342 (blue, for nuclear counter staining). A surviving crypt is defined as one containing ≥5 bromodeoxyuridine-positive cells as described previously (35). The number of surviving crypts per cross-section was determined for each mouse by scoring the number of surviving crypts in 10 complete, well-oriented cross-sections in a blind manner and dividing the total by the number of cross-sections scored.

Statistical Analysis. The data were analyzed by analysis of variance. If analysis of variance justified post hoc comparisons between group means, these were conducted using the Student-Newman-Keuls test for multiple comparisons. For experiments in which only single experimental and control groups were used, group differences were examined by unpaired Student's *t* test. Differences were considered significant at $P < 0.05$. All of these analyses were done using GraphPad Prism from GraphPad Software, Inc. (San Diego, CA).

RESULTS

Ionizing Radiation Induces NFκB Activation in Intestinal Epithelial Cells in a Dose- and Time-Dependent Manner. We demonstrated previously that mice responded to total body irradiation with increased NFκB activity in a tissue-specific manner (26). Specifically, the increase in NFκB activity was found in the bone marrow, spleen, and lymph node, but was not seen in the liver, lung, colon, thymus, and brain (26). Now, we demonstrate that exposure of mice to total body irradiation also activated NFκB in intestinal epithelial cells in a dose-dependent manner after mice were exposed to increasing doses of total body irradiation (from 0.5 to 12 Gy; Fig. 1*A*). In addition, the increase in NFκB activity in intestinal epithelial cells was time dependent, because the increase occurred within 30 minutes, peaked at 2 hours, and then gradually declined thereafter but remained elevated for up to 24 hours after exposure to 8 Gy total body irradiation (Fig. 1*B*).

The molecular composition of the NFκB activated by ionizing radiation in intestinal epithelial cells was determined by gel supershift assay. As shown in Fig. 1*C*, a single retarded band that represented the specific NFκB DNA binding activity in the nuclear extracts of irradiated intestinal epithelial cells was abrogated by the addition of excess unlabeled NFκB oligonucleotides but was not affected by that of unlabeled activator protein-1 or mutated NFκB oligonucleotides, demonstrating the specificity of the assay. The retarded band was supershifted by the addition of anti-p50 and/or anti-RelA antibodies, but was not changed by the addition of anti-Erg-1 antibody, indicating that ionizing radiation-activated NFκB in intestinal epithelial cells mainly consisted of p50/RelA heterodimers. A similar molecular composition of ionizing radiation-activated NFκB was also found in the spleen, lymph node, and bone marrow in our studies reported previously (26, 27, 30).

Targeted Disruption of the *p50* Gene in Mice Attenuated Ionizing Radiation-Induced NFκB Activation in Intestinal Epithelial Cells. Because the NFκB complex activated by ionizing radiation in murine intestinal epithelial cells mainly consists of p50/RelA heterodimers, we examined whether the gene-targeted disruption of the *p50* gene could attenuate ionizing radiation-induced NFκB activation in intestinal epithelial cells. As shown in Fig. 2, intestinal epithelial cells from both unirradiated wild-type and $p50^{-/-}$ mice expressed a barely detectable level of NFκB activity. Exposure of wild-type mice to 8 Gy total body irradiation resulted in a 219-fold increase in intestinal epithelial NFκB activity, whereas only a 43-fold increase in NFκB activity was observed in the intestinal epithelium of $p50^{-/-}$ mice after the same dose of total body irradiation ($P < 0.001$). The residual NFκB activity activated by ionizing radiation in $p50^{-/-}$ intestinal epithelium mainly consists of p52/RelA heterodimers (Fig.

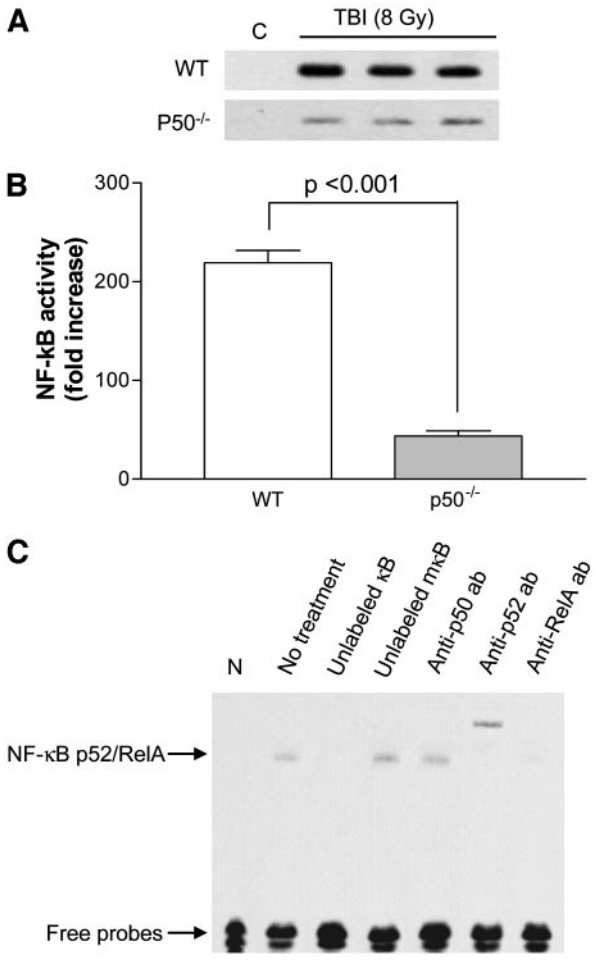

Fig. 2. Targeted disruption of the *p50* gene attenuated ionizing radiation-induced NFκB activation in intestinal epithelial cells. Wild-type (*WT*) and $p50^{-/-}$ mice were exposed to 8 Gy total body irradiation or unirradiated (*C*). Two hours after total body irradiation, mice were euthanized, and nuclear proteins were extracted from isolated intestinal epithelial cells and analyzed for NFκB DNA binding activity using gel shift assay (*A*). The increase in NFκB activity in irradiated intestinal epithelial cells from individual animals was quantified by densitometry and expressed as mean ($n = 3$) of fold increase from the baseline value obtained from unirradiated intestinal epithelial cells (*B*); *bars*, ±SE. The molecular composition of ionizing radiation-activated NFκB in intestinal epithelial cells from $p50^{-/-}$ mice was determined by gel supershift assay (*C*). *N*, no sample; *TBI*, total body irradiation; *ab*, antibody.

2*C*). This finding is in agreement with our previous observations in the spleen, lymph node, and bone marrow (27, 30), demonstrating that the p50 NFκB subunit is an essential component of the NFκB complexes activated by ionizing radiation *in vivo* and that the lack of p50 cannot be fully replaced by the other members of the NFκB/Rel family.

Activation of NFκB Selectively Protects Intestinal Epithelial Cells of the Small Intestinal Crypts from Ionizing Radiation-Induced Damage. NFκB can induce the expression of many pro-apoptotic and antiapoptotic proteins that regulate cell survival, and its activation has been implicated in inhibition or promotion of apoptosis in a cell type- and stimulus-dependent manner (4–7). The role of NFκB activation in ionizing radiation-induced normal tissue damage has not been well established. However, our recent studies have showed that activation of NFκB by ionizing radiation has no significant effect on ionizing radiation-induced splenic lymphocyte apoptosis (27). More recently, Egan *et al.* (36) have also reported that activation NFκB protects intestinal epithelial cells from ionizing radiation-induced apoptosis. Therefore, we additionally examined the role of NFκB in ionizing radiation-induced normal tissue damage in various nonlymphoid tissues. We compared total body irradiation-induced tissue damage and apoptosis in wild-type *versus* $p50^{-/-}$ mice, because $p50^{-/-}$ mice exhibited a significant reduction in ionizing radiation-induced NFκB activation (Fig. 2; refs. 27, 30).

As shown in Fig. 3*A*, exposure of wild-type and $p50^{-/-}$ mice to total body irradiation induced bone marrow-mononuclear cell apoptosis in a dose- and time-dependent manner. Similarly, total body irradiation (4 Gy) also significantly decreased the frequency of various day types of cobblestone area-forming cell, probably due to the induction of apoptosis (Fig. 3*B*; refs. 28, 37). However, there was no significant difference between wild-type animals and $p50^{-/-}$ mice in their response to ionizing radiation-induced bone marrow-mononuclear cell apoptosis and in their decrease in cobblestone area-forming cell frequency ($P > 0.05$), suggesting that activation of NFκB by ionizing radiation had no significant effect on ionizing radiation-induced bone marrow toxicity.

Exposure of wild-type and $p50^{-/-}$ mice to total body irradiation (8 Gy) also significantly increased intestinal epithelial cell apoptosis in the small intestinal crypts at 6 and 24 hours after ionizing radiation as compared with the baseline levels of apoptosis in unirradiated mice ($P < 0.01$; Fig. 4*A* and Fig. 5*A*). The increase in intestinal epithelial cell apoptosis then declined to insignificant levels at 72 hours after total body irradiation ($P > 0.05$; Fig. 4*A*). Although the levels of intestinal epithelial cell apoptosis were not significantly different between wild-type and $p50^{-/-}$ mice at 6 hours after ionizing radiation (apoptotic cell/crypt: wild-type 4.3 ± 0.7 *versus* $p50^{-/-}$ 3.8 ± 0.4, $P > 0.05$), the level of intestinal epithelial cell apoptosis in $p50^{-/-}$ mice was 1.4-fold higher than that of wild-type at 24 hours after total body irradiation (apoptotic cell/crypt: wild-type 4.1 ± 0.2 *versus* $p50^{-/-}$ 5.8 ± 0.5, $P < 0.01$). The increase in ionizing radiation-induced intestinal epithelial cell apoptosis in $p50^{-/-}$ mice at 24 hours after total body irradiation was associated with an enhanced activation of caspase 3 and DNA fragmentation (terminal deoxynucleotidyl transferase-mediated nick end labeling assay; Fig. 5*A*). Similarly, RelA/TNFR1$^{-/-}$ mice also showed a 1.5-fold increase in intestinal epithelial cell apoptosis at 24 hours after total body irradiation (8 Gy) as compared with TNFR1$^{-/-}$ mice (apoptotic cell/crypt: TNFR1$^{-/-}$ 3.5 ± 0.2 *versus* RelA/TNFR1$^{-/-}$ 5.3 ± 0.5, $P < 0.01$; Fig. 4*B*). In addition, $p50^{-/-}$ mice exhibited a diminished survival of the intestinal crypts at 3 days after exposure to 12 Gy total body irradiation when compared with the same dose-irradiated wild-type mice (Fig. 4*C* and Fig. 5*B*). The number of surviving crypts was significantly lower in irradiated $p50^{-/-}$ mice than that of irradiated wild-type mice

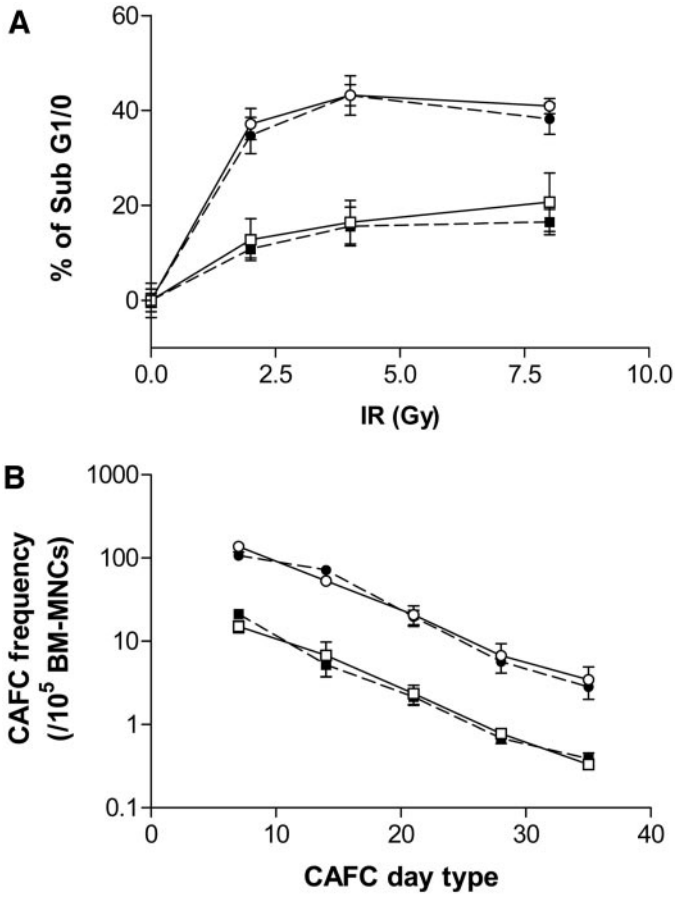

Fig. 3. Targeted disruption of the *p50* gene fails to affect ionizing radiation-induced apoptosis in bone marrow-mononuclear cells and suppression of hematopoietic function of bone marrow stem cells and progenitors. Six or 24 hours after receiving 0, 2, 4, or 8 Gy of total body irradiation, bone marrow-mononuclear cells were harvested from individual wild-type and $p50^{-/-}$ mice ($n = 5$). Apoptosis of these cells was measured by quantification of sub-$G_{0/1}$ population (*A*). In addition, the hematopoietic function of bone marrow stem cells and progenitors were measured by cobblestone area-forming cell assay after mice ($n = 3$) were exposed to 4 Gy total body irradiation (*B*). The day 7 cobblestone area-forming cell and day-14 cobblestone area-forming cell correspond to progenitors that produce colony forming unit-granulocytes and monocytes and day 12 colony forming unit spleen, respectively. The primitive bone marrow stem cells with long-term repopulating ability correspond to day 28 and 35 cobblestone area-forming cell. The data presented are mean; *bars*, ±SE. *A*, □, wild-type 6 hours; ■, p50−/− 6 hours; ○, wild-type 24 hours; ●, p50−/− 24 hours; *B*, ○, wild-type control; ●, p50−/− control; □, wild-type 4 Gy; ■, p50−/− 4 Gy.

(surviving crypts/cross-section: wild-type 28.3 ± 2.3 *versus* $p50^{-/-}$ 20.3 ± 0.9, $P = 0.012$; Fig. 4*C*). In contrast, no significant tissue damage was observed in the liver, heart, lung, and kidney after $p50^{-/-}$ and wild-type mice were exposed to 8 Gy total body irradiation (data not shown). Together, the data presented above suggest that activation of NFκB selectively protects the small intestine from ionizing radiation-induced damage but has no significant effect on ionizing radiation-induced lymphoid and hematopoietic toxicity.

Targeted Disruption of the *p50* Gene Sensitizes Mice to Total Body Irradiation-Induced Lethality. To determine whether targeted disruption of the *p50* gene not only increases ionizing radiation-induced intestinal epithelial cell damage but more importantly sensitizes mice to total body irradiation-induced lethality, wild-type and $p50^{-/-}$ mice were exposed to increasing doses of total body irradiation, and their survival was monitored for 30 days. The LD_{50} values for day 7 ($LD_{50/7}$) and day 30 ($LD_{50/30}$) were calculated for both strains of mice and are presented in Table 1. As shown in Table 1, $p50^{-/-}$ mice had much lower $LD_{50/7}$ and $LD_{50/30}$ values than those of wild-type mice ($LD_{50/7}$: $p50^{-/-}$ = 7.75 Gy *versus* wild-type = 13.12

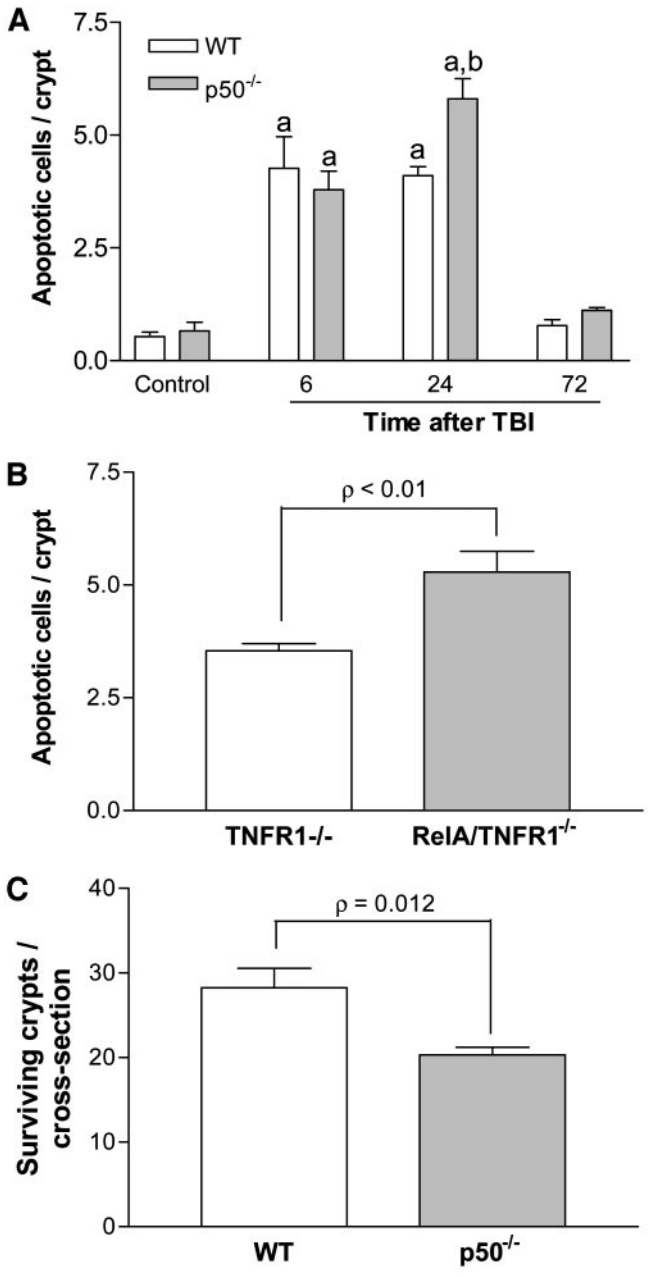

Fig. 4. Lack of *p50* or *RelA* sensitizes intestinal epithelial cells to ionizing radiation-induced damage. *A*, wild-type (*WT*) and $p50^{-/-}$ mice were exposed to 8 Gy total body irradiation or unirradiated (*control*). Apoptotic intestinal epithelial cells in the small intestinal crypts were determined at 6 ($n = 3$), 24 ($n = 7$), and 72 hours ($n = 3$) after total body irradiation (*TBI*). The results are expressed as mean; *bars*, ±SE. *a*, $P < 0.01$ *versus* their respective unirradiated controls ($n = 3$); *b*, $P < 0.01$ *versus* 24 hours after irradiated wild-type mice. *B*, $TNFR1^{-/-}$ and $RelA/TNFR1^{-/-}$ mice ($n = 4$) were exposed to 8 Gy total body irradiation. Apoptotic intestinal epithelial cells in the small intestinal crypts were determined at 24 hours after total body irradiation. The results are expressed as mean; *bars*, ±SE. *C*, wild-type and $p50^{-/-}$ mice were exposed to 12 Gy total body irradiation. The number of surviving crypts in the small intestine was determined at 3 days after total body irradiation. The results are expressed as mean; *bars*, ± SE ($n = 7$).

Gy; $LD_{50/30}$: $p50^{-/-}$ = 7.81 Gy *versus* wild-type = 9.31 Gy), indicating that $p50^{-/-}$ mice were more sensitive to total body irradiation-induced death than wild-type mice. However, the $LD_{50/30}$ value of $p50^{-/-}$ mice was similar to their $LD_{50/7}$ value. This suggests that the increased susceptibility of $p50^{-/-}$ mice to total body irradiation-induced lethality is mainly attributable to an enhanced intestinal damage, because it has been well established that the $LD_{50/7}$ value indicates that ionizing radiation caused intestinal toxicity and $LD_{50/30}$ value represents ionizing radiation-induced hematopoietic syndrome (38). This suggestion is in agreement with the observations that $p50^{-/-}$ mice exhibited an increase in intestinal epithelial cell apoptosis and decrease in crypt survival after they were exposed to total body irradiation .

DISCUSSION

Molecularly targeted inhibition of NFκB has the potential to be developed as a novel cancer therapy. Particularly, treatment with a combination of NFκB inhibitors and conventional ionizing radiation or chemotherapeutic agents may dramatically improve the antitumor

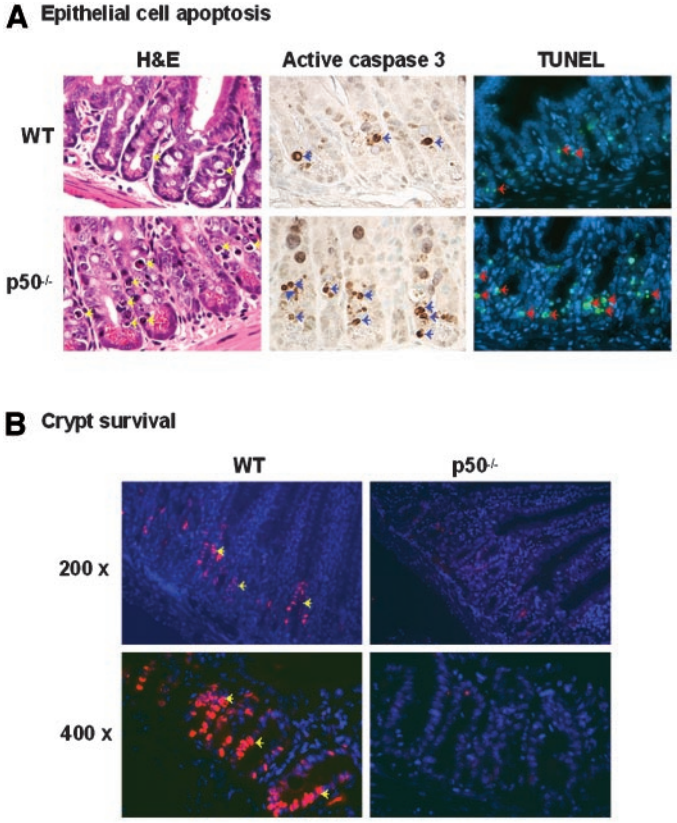

Fig. 5. Representative photomicrographs of irradiated small intestinal crypts. *A*, apoptotic cells (*arrow-pointed*) in the small intestinal crypts of wild-type (*WT*) and $p50^{-/-}$ mice at 24 hours after exposure to 8 Gy total body irradiation were identified by hematoxylin and eosin (*H&E*) staining, active caspase 3 immunostaining, and terminal deoxynucleotidyl transferase-mediated nick end labeling (*TUNEL*) assay (×400). *B*, surviving crypts (*arrow-pointed*) in wild-type and $p50^{-/-}$ mouse intestines at 3 days after exposure to 12 Gy total body irradiation. The S-phase cells incorporating bromodeoxyuridine were stained *red* by mouse antibromodeoxyuridine antibody, and Texas red-labeled goat antimouse IgG and nuclei were stained *blue* with Hoechst 33342.

Table 1 *LD_{50} values of TBI in WT and $p50^{-/-}$ mice*

	$LD_{50/Day\ 7}$ (Gy)	$LD_{50/Day\ 30}$ (Gy)
WT	13.12 (12.50–13.75)*	9.31 (8.39–10.24)
$p50^{-/-}$	7.75 (7.17–8.32)	7.81 (7.23–8.39)
DMF†	1.69	1.19

NOTE. Groups of WT mice (15–34 per group) were exposed to 8–15 Gy TBI, and groups of $p50^{-/-}$ mice (15–35 per group) were exposed to 6–9.5 Gy TBI. The survival of these mice was recorded during a 30-day observation period after TBI.

Abbreviations: WT, wild-type; DMF, dose modification factor; TBI, total body irradiation.

* 95% confidence intervals.

† DMF = $LD_{50\text{-}WT}/LD_{50\text{-}p50^{-/-}}$.

response. However, the effect of NFκB inhibition on the normal tissue response to ionizing radiation- and chemotherapy-induced injury must be evaluated before the clinical application of NFκB inhibition. Therefore, using a mouse model we examined the tissue specificity of ionizing radiation-activated NFκB and the role of NFκB in ionizing radiation-induced normal tissue damage. In addition to the spleen, lymph node, and bone marrow identified in our previous studies (26, 27, 30), we now discovered that exposure of mice to a low dose (≤2 Gy) of total body irradiation also activated NFκB in intestinal epithelial cells of the small intestine. The activation of NFκB in these tissues was in a dose-dependent fashion when mice were exposed to increasing doses (up to 12 Gy) of total body irradiation. In contrast, no significant activation of NFκB was observed in the liver, lung, colon, and brain after mice were exposed to total body irradiation in the same dose range (26, 27, 30). These results suggest that when mice are exposed to a clinically relevant dose (≤2 Gy) of total body irradiation, ionizing radiation activates NFκB *in vivo* in a tissue-specific manner.

However, when mice were exposed to a super lethal dose (20 Gy) of total body irradiation, a significant activation of NFκB was found in the liver and kidney (39). In addition, a delayed activation of NFκB was also observed in irradiated rat lung after 20 Gy pulmonary irradiation (40). These findings demonstrate that the tissues that are prone to ionizing radiation toxicity, such as the spleen, lymph node, bone marrow, and small intestine, are more sensitive to ionizing radiation-induced activation of NFκB, whereas radioresistant tissues are less responsive to ionizing radiation for NFκB activation. Thus, it appears that different normal tissues possess diverse sensitivity to ionizing radiation-induced activation of NFκB, which correlates to their susceptibility to ionizing radiation-induced tissue damage.

Such a correlation implies that NFκB may function as a sensor that can detect ionizing radiation-induced tissue damage. In turn, activated NFκB can modulate tissue responses to ionizing radiation damage by stimulating the expression of various genes that are involved in regulation of cell survival, cell proliferation, and tissue inflammation (1–3). Depending on the particular tissue involved, activation of NFκB may confer tissue protection or contribute to tissue injury in response to ionizing radiation (27, 36, 40). In agreement with this hypothesis, we found that down-regulation of NFκB activation by the targeted disruption of the *p50* or *RelA* gene in mice sensitized intestinal epithelial cells to ionizing radiation-induced damage. A similar finding was reported recently by Egan *et al.* (36) using mice that had genetically targeted ablation of inhibitor of NFκB kinase-β expression in intestinal epithelial cells to specifically block NFκB activation in these cells. These findings indicate that activation of NFκB is radioprotective in the small intestine. Correspondingly, $p50^{-/-}$ mice exhibited a higher sensitivity to ionizing radiation-induced intestinal syndrome and had a much lower $LD_{50/7}$ value than wild-type animals after they were exposed to increasing doses of total body irradiation. However, the inhibition of NFκB activation by targeted disruption of the *p50* gene had no significant effects on ionizing radiation-induced lymphoid and hematopoietic cell damage nor did it affect the responses of the liver, lung, heart, and kidney to ionizing radiation (27). This suggests that ionizing radiation not only activates NFκB *in vivo* in a tissue-specific manner, but more importantly the activation also confers tissue-specific protection against ionizing radiation-induced normal tissue damage.

The mechanisms underlying the tissue-specific protection against ionizing radiation by NFκB activation are not clear at the present. It may relate to cross-talk among different transcriptional factors activated by ionizing radiation. For example, it is well known that exposure of mice to total body irradiation also induces tissue-specific activation of p53 (41–43). Transcriptional induction of certain apoptosis proteins by p53 contributes to ionizing radiation-induced apoptosis in various tissues (41–45). NFκB and p53 share the transcriptional coactivator proteins CREB-binding protein and p300 (46–48). Competition between NFκB and p53 for binding to these transcriptional coactivator proteins may dictate the outcome of the responses in different tissues to ionizing radiation and has yet to be investigated.

The discovery that mice respond to total body irradiation with tissue-specific activation of NFκB and that this activation can selectively protect the intestine from ionizing radiation-induced damage is intriguing. It suggests that additional investigations are required for a better understanding of the role of NFκB in radiation biology and cancer therapy to provide guidance to the development of targeted NFκB inhibition as a novel adjuvant therapy for cancer in combination with radiotherapy. Particularly, it has yet to be determined if activation of NFκB by a clinical relevant dose of fractionated irradiation confers a similar protection against ionizing radiation-induced normal tissue damage. These investigations will be important to determine whether irradiation therapy and NFκB inhibition should or should not overlap in sensitive normal tissues to avoid an augmented normal tissue injury. On the basis of our finding, we hypothesize that a combination therapy using a NFκB inhibitor plus ionizing radiation will be useful in treating tumors that are not in close proximity to the small intestine. However, it may carry a significant risk if it is used to treat abdominal tumors, because NFκB inhibition may also increase ionizing radiation-induced intestinal damage. Likewise, these studies suggest that combination of NFκB inhibition with chemotherapy may also carry a substantial risk of normal tissue damage due the overlap of the two therapies within the body, which has yet to be determined. This underscores the importance of evaluating the potential clinical complications using ionizing radiation and chemotherapy in combination with NFκB inhibitors in cancer treatment.

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