Ionizing Radiation and Busulfan Induce Premature Senescence in Murine Bone Marrow Hematopoietic Cells

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ABSTRACT

Exposure of murine bone marrow (BM) cells to ionizing radiation (IR; 4 Gy) resulted in >95% inhibition of the frequency of various day types of cobblestone area-forming cells in association with the induction of apoptosis in hematopoietic stem cell alike cells (Lin-Scal- c-kit+cells); IR: 64.8 ± 0.4% versus control: 20.4 ± 0.5%; P < 0.001) and progenitors (Lin−Scal− c-kit+ cells; IR: 46.2 ± 1.4% versus control: 7.8 ± 0.5%; P < 0.001). Incubation of murine BM cells with busulfan (BU; 30 μM) for 6 h also inhibited the cobblestone area-forming cell frequency but failed to cause a significant increase in apoptosis in these two types of hematopoietic cells. After 5 weeks of long-term BM cell culture, 33% and 72% of hematopoietic cells survived IR- and BU-induced damage, respectively, as compared with control cells, but they could not form colony forming units-granulocyte macrophages. Moreover, these surviving cells expressed an increased level of senescence-associated β-galactosidase, p16INK4a, and p19ARF. These findings suggest that IR inhibits the function of hematopoietic stem cell alike cells and progenitors primarily by inducing apoptosis, whereas BU does so mainly by inducing premature senescence. In addition, induction of premature senescence in BM hematopoietic cells also contributes to IR-induced inhibition of their hematopoietic function. Interestingly, the induction of hematopoietic cell senescence by IR, but not by BU, was associated with an elevation in p53 and p21Cip1/Waf1 expression. This suggests that IR induces hematopoietic cell senescence in a p53-p21Cip1/Waf1-dependent manner, whereas the induction of senescence by BU bypasses the p53-p21Cip1/Waf1 pathway.

INTRODUCTION

Radiotherapy and chemotherapy are common therapeutic modalities for cancer, leukemia, and lymphoma. Unfortunately, these therapies are not tumor-specific. Normal tissues, particularly the BM, are extremely vulnerable to cytotoxicity caused by these therapies (1). An acute and transient myelosuppression is a common side effect of radiotherapy and chemotherapy, which primarily damage the rapidly proliferating hematopoietic progenitors and their more mature progeny. However, persistent myelosuppression or BM failure occurring in some patients after radiotherapy and chemotherapy is an indication of injury to HSCs. HSCs are largely a nonproliferating population that can self-renew and produce progeny of at least 8 different hematopoietic lineages (2). However, HSCs are relatively sensitive to IR and some chemotherapeutic agents such as BU. Despite the wide use of IR and BU for the treatment of certain types of cancer and leukemia, and for BMT preconditioning, the cellular mechanisms by which IR and BU affect HSCs have not been well established.

There is indirect evidence suggesting that IR may damage HSCs by inducing apoptosis. First, IR is a potent inducer of apoptosis in a variety of cells, including normal and malignant hematopoietic cells (3–7). Secondly, overexpression of an antiapoptotic or down-regulation of a proapoptotic protein confers a certain degree of protection against IR-induced myelosuppression (8–12). Similarly, it was reported that incubation of myeloid and lymphoid leukemia cells with BU induced apoptosis (13, 14). However, no increase in apoptotic cell death was observed in BM biopsies from chronic myelogenous leukemia patients undergoing chemotherapy with BU (15). Therefore, it is not known to what extent we can extrapolate the apoptotic response of malignant cells to normal HSCs in their response to BU treatment.

The damage to HSCs by IR and BU may not be limited to the induction of apoptosis, as exposure of human normal diploid fibroblasts and other types of cells to IR and various chemotherapeutic agents also causes clongenic cell deletion by induction of premature senescence (16–20). Cells undergoing premature senescence exhibit some of the same characteristics as those of replicative senescent cells, such as permanent cell cycle arrest, enlarged and flattened cell morphology, increased acidic or SA-β-gal activity, and elevated expression of the proteins encoded by the Ink4a-Arf locus (17, 18, 21). Two major pathways have been implicated in the induction of premature senescence. These include the p53-p21Cip1/Waf1 or p19ARF-Mdm2-p53-p21Cip1/Waf1 pathway, triggered by DNA damage, and the p16INK4a-Rb pathway, activated by the Ras-mitogen-activated protein kinase cascade (17, 18, 21). Activation of either pathway is sufficient to induce senescence. However, extensive cross-talk exists at multiple levels between these two pathways. Frequently, the two pathways work in concert to induce premature senescence.

Induction of either apoptosis or premature senescence, or both, in HSCs and progenitors can result in inhibition of their hematopoietic function. However, it is not known if IR and chemotherapeutic agents, such as BU, are capable of inducing premature senescence in BM hematopoietic cells, and to what degree that HSC and progenitor apoptosis and/or senescence may contribute to IR- and chemotherapy-induced myelosuppression. Therefore, the present study was designed to determine whether IR and BU induce apoptosis and/or premature senescence in murine BM hematopoietic cells.

MATERIALS AND METHODS

Reagents. PE-conjugated anti-Sca-1 (Clone E13–161.7; rat IgG2a), APC-conjugated anti-c-kit (Clone 2B8; rat IgG2b), biotin-conjugated anti-CD5 (Clone 53–7.3; rat IgG2a), anti-Gr-1 (Clone RB6–8C5; rat IgG2b), anti-Mac-1 (Clone M1/70; rat IgG2b), and anti-TER-119 (Clone Ter-119; rat IgG2b); purified rat anti-CD16/CD32 (Clone 2.4G2; Fcγ receptor blocker; rat IgG2b); and FITC-conjugated streptavidin were purchased from BD-PharMingen (San Diego, CA). Rabbit anti-p21Cip1/Waf1 (M-19), anti-p53 (C5), anti-p16INK4a, and anti-p19ARF antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA), Novocastra (Newcastle-upon-Tyne, United Kingdom), and Abcam (Cambridge, United Kingdom), respectively. Biotinylated goat anti-rabbit IgG (H+L) was purchased from Vector (Burlingame, CA). BU was obtained from Sigma (St. Louis, MO).

Mice. Male C57BL/6 mice were purchased from the National Cancer Institute and housed 4 to a cage at the Medical University of South Carolina.
Association for Assessment and Accreditation of Laboratory Animal Care certified animal facility. They received food and water ad libitum. All of the mice were used at approximately 8–10 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.

Isolation of BM-MNCs and Lin<sup>-</sup> Cells. The femora and tibiae were harvested from the mice immediately after they were euthanized with CO<sub>2</sub>. BM cells were flushed from the bones into HBSS containing 2% FCS using a 21-gauge needle and syringe. Cells from 3–10 mice were pooled and centrifuged through Histopaque 1083 (Sigma) to isolate BM-MNCs. For the isolation of Lin<sup>-</sup> cells (22), BM-MNCs were incubated with biotin-conjugated rat antibodies specific for murine CD5, Mac-1, CD45RB/B220, Ter-119, and Gr-1. The labeled mature lymphoid and myeloid cells were depleted twice by incubation with goat antirat IgG paramagnetic beads (Dynal Inc., Lake Success, NY) at a bead:cell ratio of ~4:1. Cells binding the paramagnetic beads were removed with a magnetic field. The negatively isolated Lin<sup>-</sup> cells were washed twice with 2% FCS/HBSS and resuspended in complete medium (RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, 10 μM HEPES buffer, 100 units/ml penicillin, and 100 μg/ml streptomycin) at 1 × 10<sup>6</sup>/ml.

Treatment of BM-MNCs or Lin<sup>-</sup> Cells with IR or BU. BM-MNCs or Lin<sup>-</sup> cells (1 × 10<sup>6</sup>/ml) suspended in complete medium were exposed to 4 Gy IR generated in a Mark IV 137 Cesium γ-iradiator (JL Shepherd, Glendale, CA) at a dose rate of 1.21 Gy/min, or incubated with BU (30 μM) or 0.2% DMSO (vehicle used as control). Cells were incubated in wells of a 24-well plate at 37°C, 5% CO<sub>2</sub>, and 100% humidity for 6 h.

After the incubation, the cells were washed once to remove BU and resus- pended in 8 ml of CAFC medium (StemCell Technologies). Nonadherent and adherent hematopoietic cells harvested from 2- or 5-week LTBMCs were cytospun onto slides. Cells were fixed in 2% (v/v) formaldehyde and 0.2% glutaraldehyde, and then incubated in SA-β-gal staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactosidase; 40 μM citric acid (pH 6.0), 40 mM sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride, and 2 mM magnesium chloride) at 37°C for 10 h. Senescent cells were identified as blue-stained cells by standard light microscopy, and a total of 1000 cells were counted in five random fields on a slide to determine the percentage of SA-β-gal-positive cells.

Immunolabeling for Fluorescence Microscopy. Cells were cytospun onto slides, air dried, and then fixed in ice-cold methanol for 5 min. They were permeabilized with 0.2% Triton X-100 and blocked with 5% goat serum before incubation with the anti-p53, anti-p21<sup>Waf1/Cip1</sup>, anti-p16<sup>INK4a</sup>, or anti-p19<sup>ARF</sup> antibodies. Cells were incubated with biotinylated-goat antirabbit antibody (Vector) and then with streptavidin-PE (PharMingen) after extensive washing between each staining. DNA was then labeled with Hoechst 33342 (Molecular Probes). Slides were finally mounted in Gel/Mount (Biomeda Corp., Foster, CA). Cells were viewed and photographed using an Axiosplan research microscope (Carl Zeiss Inc., Jena, Germany) equipped with a 100 W mercury light source and a 25× plan-neofluar na.13 objective. The images were captured with a Dage CCD100 integrating camera (Dage-MTI, MI) and a Flashpoint 128 capture board (Integral Technologies, IN). The captured images were processed using Image Pro Plus software (Media Cybernetics, MD) and displayed with Adobe Photoshop V6.0.

Semiquantitative RT-PCR. Total RNA was isolated from the hematopoietic cells harvested from 2- or 5-week LTBMCs using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. RNA yield and quality were determined by measuring absorbencies at 260 nm and 280 nm, respectively. First-strand cDNA was synthesized from 5 μg of total RNA using SuperScript II first-strand synthesis system (Invitrogen) according to the manufacturer’s manual. Two μl of cDNA was used for the PCR amplification using 2 units of Pfx DNA polymerase (Invitrogen) and 200 nmol of targeting primers. The housekeeping gene GAPDH cDNA was amplified simultaneously as an internal quantitative control, and all of the samples were normalized to the PCR signal of GAPDH. The sequences of primers used in this study were: p16<sup>INK4a</sup>, TCCGCGTGAGACAGACTGGCCAGC (sense) and CATCGCCGACATC-CAGCCGAGG (anti-sense); p19<sup>ARF</sup>, AAGAAGTCTGCGTCGGCGACG (sense) and AGTACCAGGGACATTTGAGCA (anti-sense); p21<sup>CIP1/WAF1</sup>, AACCTGCGTATGTTCCGACC (sense) and AAAGGTTCACCAGTTCCG (anti-sense); p53, CAGCTATCCTCCTCCCTCAA (sense) and GGCT-CATAAGTGACACACCAGCC (anti-sense); and GAPDH, TGAAGTGTGGT-GTAAGACGATTGGGC (sense) and CATTGAGGCACTGGTACACC (anti-sense). PCR amplification was carried out using an Eppendorf Mastercycler under the following conditions: 1 cycle of 10 min at 94°C for initial denaturation, followed by 30 cycles of 94°C for 30 s, 58 or 60°C for 40 s, and 72°C for 50 s. For amplification of GAPDH cDNA, the number of cycles was 23. The amplified PCR products were separated by 1.5% agarose gel electrophoresis at 100 V for 1 h, stained with ethidium bromide, visualized with UV light, and finally photographed to record the results.

Statistical Analysis. The data were analyzed by ANOVA. If ANOVA 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 \( P < 0.05 \).

**RESULTS**

**IR and BU Inhibit BM Cell Hematopoietic Function Via Apoptosis-dependent and -independent Mechanisms.** The effects of IR and BU on the hematopoietic function of HSC alike cells and progenitors were analyzed by CAFC assay (23). This assay provides an estimate of the hematopoietic function of a spectrum of CAFC day-types that correspond to various stages of progenitors (day-7 CAFC and day-14 CAFC to CFU-GM and day-12 CFU-spleen, respectively) and the primitive HSCs with long-term repopulating ability (day-28 and -35 CAFC; Ref. 23). As shown in Fig. 1A, exposure of BM-MNCs to IR (4 Gy) or incubation of the cells with BU (30 \( \mu \)M) for 6 h resulted in a significant reduction in CAFC frequency (\( P < 0.001 \) versus control). The survival fractions of all day-types of CAFC for irradiated cells were <5% of control, and the survival fraction was 83.6% of control for day-7 CAFC and progressively declined to 8.0% of control for day-35 CAFC after BU treatment. This result indicates that IR inhibits various stages of hematopoietic cells in a nonspecific manner, whereas BU selectively inhibits more primitive hematopoietic cells.

To determine whether IR and BU inhibit CAFC by induction of hematopoietic cell apoptosis, Lin\(^-\) cells were exposed to IR (4 Gy) or incubated with BU (30 \( \mu \)M) for 18 h, and apoptotic cell death was analyzed in HSC alike cells (Lin\(^-\) Sca\(^+\) c-kit\(^+\) cells) and progenitors (Lin\(^-\) Sca\(^+\) c-kit\(^-\) cells; Ref. 27). As shown in Fig. 1B, exposure of Lin\(^-\) cells to IR significantly increased the percentage of annexin V-positive cells or apoptotic cells in HSC alike cells (IR: 64.8% versus control: 20.4%) and progenitors (IR: 46.2% versus control: 7.8%; \( P < 0.001 \)), which resulted in a significant reduction in the numbers of these cells (\( P < 0.001 \); Fig. 1C). In contrast, incubation of Lin\(^-\) cells with BU failed to induce significant changes in the percentage of apoptotic cells in HSC alike cells and progenitors (\( P > 0.05 \); Fig. 1B). Moreover, BU treatment had no significant effect on the number of HSC alike cells (\( P > 0.05 \); Fig. 1C), whereas it only slightly decreased that of progenitors (14% reduction as compared with control; \( P < 0.05 \); Fig. 1C). These results suggest that IR inhibits CAFC primarily by induction of apoptosis in HSC alike cells and progenitors, whereas BU inhibits the hematopoietic function of HSC alike cells and progenitors via an apoptosis-independent mechanism.

**Effects of IR and BU on LTBMC.** After 5 weeks of LTBMC, an average of 0.93 \( \times 10^6 \) viable hematopoietic cells were recovered from control LTBMC, which represents \( \sim 3\% \) of the input test cell inoculum (3 \( \times 10^7 \) BM cells) for the culture. The number of the cells (0.32 \( \times 10^6 \)) recovered from the LTBMC for irradiated cells was significantly reduced (\( \sim 66\% \) reduction) as compared with control (\( P < 0.001 \)). In contrast, a moderate but still significant reduction (\( \sim 28\% \)) in the number of the cells (0.67 \( \times 10^6 \)) recovered from LTBMC for BU-treated cells was observed (\( P < 0.001 \) versus control). Next, the effects of IR and BU on the function of these hematopoietic cells harvested from a 5-week LTBMC were examined using a clonogenic assay for CFU-GM. As shown in Fig. 2B, the hematopoietic cells harvested from the 5-week LTBMC for control cells had the ability to form CFU-GM, whereas the cells from the LTBMC for both irradiated and BU-treated cells exhibited a diminished ability to form CFU-GM. This result suggests that although some BM hematopoietic cells might have survived IR- and BU-induced damage, these cells had been permanently arrested and probably became senescent.

**IR and BU Increase the Expression of SA-\( \beta \)-gal.** To determine whether IR and BU inhibit hematopoietic function by induction of BM hematopoietic cell senescence, the hematopoietic cells harvested from 2- and 5-week LTBMC were stained for SA-\( \beta \)-gal, a biomarker for senescent cells (28). No significant SA-\( \beta \)-gal staining was detected in the hematopoietic cells harvested from a 2-week LTBMC (data not shown). After 5 weeks of LTBMC, a significant increase in SA-\( \beta \)-gal staining was found in the hematopoietic cells harvested from the LTBMC for irradiated and BU-treated cells as compared with control cells (\( P < 0.05 \) and 0.01, respectively; Fig. 3). These findings confirm...
that IR and BU are capable of inducing senescence in BM hematopoietic cells.

IR but not BU Induces p53 Activation and p21<sup>Cip1/Waf1</sup> Expression. To determine whether IR and BU induce hematopoietic cell senescence via the p53-p21<sup>Cip1/Waf1</sup> pathway, the expression of p53 and p21<sup>Cip1/Waf1</sup> in the hematopoietic cells harvested from 2- and 5-week LTBMC were examined by immunostaining using p53- and p21<sup>Cip1/Waf1</sup>-specific antibodies. It was found that 10% of control and BU-treated cells showed p53 and p21<sup>Cip1/Waf1</sup> staining after 2- and 5-week LTBMC (Fig. 4; data not shown). In contrast, almost all of the irradiated cells (95%) harvested from a 2-week LTBMC were stained positive for p53 and p21<sup>Cip1/Waf1</sup> (Fig. 4). After 5-week LTBMC, the percentage of the irradiated cells stained positive for p53 and p21<sup>Cip1/Waf1</sup> expression declined to 13% and 26%, respectively. The increase in p53 expression in the irradiated cells is likely attributable to IR-induced increase in p53 stability, because no significant increase in the level of p53 mRNA was detected in these cells by a semiquantitative RT-PCR technique (Fig. 5). However, the increase in p21<sup>Cip1/Waf1</sup> expression appears to be regulated at the level of transcription, because an elevation in the level of p21<sup>Cip1/Waf1</sup> mRNA was detected in the irradiated cells (Fig. 5). These results suggest that IR-induced senescence in BM hematopoietic cells is associated with activation of p53 and induction of p21<sup>Cip1/Waf1</sup>, whereas BU induces hematopoietic cell senescence bypassing the p53-p21<sup>Cip1/Waf1</sup> pathway.

IR and BU Induce p16<sup>Ink4a</sup> and p19<sup>Arf</sup> Expression. Induction of p16<sup>Ink4a</sup> and/or p19<sup>Arf</sup> is also involved in the initiation and maintenance of cellular senescence after DNA damage or oncogenic stress in a species- and cell-type-dependent manner (17, 29–31). Therefore, the expression of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> in the hematopoietic cells recovered from LTBMC was examined as well.

After 2- and 5-week LTBMT, the expression of p16<sup>Ink4a</sup> in control cells was minimal (<6%). However, ~37.2% of the irradiated cells and 45.5% of the BU-treated cells harvested from the week-2 LTBMC became p16<sup>Ink4a</sup>-positive cells, respectively (Fig. 4). Correspondingly, the percentage of p19<sup>Arf</sup>-positive cells increased from ~3% to ~13% and ~26% in the irradiated cells harvested from 2- and 5-week LTBMC, respectively. These results suggest that IR-induced senescence in BM hematopoietic cells is associated with activation of p53 and induction of p21<sup>Cip1/Waf1</sup>, whereas BU induces hematopoietic cell senescence bypassing the p53-p21<sup>Cip1/Waf1</sup> pathway.

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higher dose of BU (up to 200 μM) for a longer period (48 or 72 h) did not induce hematopoietic cell apoptosis (data not shown). The inability of BU to induce apoptosis in HSC alike cells and their normal progeny is a novel and unexpected finding, because previous studies showed that incubation of myeloid and lymphoid leukemia cells with BU induced apoptosis (13, 14). Similarly, we found that treatment of MO7e cells, a growth factor-dependent myeloid leukemia cell line, with BU causes MO7e cell apoptosis (data not shown). These results suggest that normal and malignant hematopoietic cells may respond differently to BU treatment. However, the mechanisms instigating the differential response of normal and malignant hematopoietic cells to BU have yet to be elucidated. The lack of induction of apoptosis in HSC alike cells and their normal progeny by BU suggests that BU inhibits hematopoietic function via an apoptotic-independent mechanism. This suggestion is additionally supported by the finding that z-VAD had no effect on BU-induced inhibition of HSC alike cell and progenitor hematopoietic function.\(^4\) In addition, this suggestion is in agreement with the finding that no significant increase in apoptosis was detected in BM biopsies from chronic myelogenous leukemia patients receiving BU chemotherapy (15).

The finding that the majority (72% of control) of BM hematopoietic cells survived BU treatment whereas only a small fraction (34% of control) of the cells survived exposure to IR after 5 weeks of LTBMC provides additional evidence to support the supposition that IR and BU inhibit hematopoietic function via apoptosis-dependent and -independent mechanism, respectively. Interestingly, almost all of these surviving hematopoietic cells failed to form CFU-GM. This suggests that although some of these BM hematopoietic cells may have survived IR- and BU-induced damage, they lose their proliferative potential and hematopoietic function, probably because of induction of permanent growth arrest or senescence. This suggestion is supported by the findings that surviving hematopoietic cells had an increased SA-β-gal activity, a biomarker for senescent cells (28). In addition, surviving hematopoietic cells recovered from LTBMC with irradiated cells showed increases in the expression of p53, p21\(^{Cip1/Waf1}\), p16\(^{Ink4a}\), p19\(^{Arf}\), and GAPDH mRNA (Fig. 5). These results suggest that the induction of BM hematopoietic cell senescence by IR and BU is associated with an increased expression of p16\(^{Ink4a}\) and p19\(^{Arf}\) in a time-dependent manner.

**DISCUSSION**

We have found that exposure of BM-MNCs to IR inhibits the frequency of various day-types of CFU in association with the induction of apoptosis in HSC alike cells and progenitors. In addition, preincubation of the cells with z-VAD, a broad-spectrum caspase inhibitor, significantly attenuated IR-induced apoptosis in HSC alike cells and progenitors, and suppression of their hematopoietic function.\(^4\) These findings are in agreement with previous studies showing that overexpression of an antia apoptotic protein or down-regulation of a proapoptotic protein reduced IR-induced suppression of hematopoietic function, suggesting that IR causes myelosuppression primarily by inducing HSC and progenitor apoptosis (8–12).

Interestingly, incubation of BM-MNCs and/or Lin\(^-\) cells with BU, a potent chemotherapeutic agent widely used for the treatment of certain leukemia and BMT preconditioning, failed to induce apoptosis in HSC alike cells and progenitors, whereas it significantly inhibited their hematopoietic function. The lack of induction of HSC alike cell and progenitor apoptosis by BU was neither time- nor dose-dependent, because in a preliminary study, incubation of BM-MNCs with a

\(^{4}\) Unpublished observations.


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