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NFκB and Survivin-Mediated Radio-Adaptive Response

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A survivin-mediated radio-adaptive response was induced in SA-NH murine sarcoma cells following activation of nuclear transcription factor κB (NFκB) by very low doses of ionizing radiation of 5, 20 or 100 mGy. SA-NH cells and a clone stably transfected with a plasmid containing a mutated IκBα gene that prevents the activation of NFκB (SA-NH+mIκBα1) were used to investigate the role of NFκB activation in the development and expression of the survivin-mediated radio-adaptive response. Tumor cells were exposed to very low doses of radiation 30 min prior to or at times ranging from 30 min to 6 h after the first of two 2 Gy doses separated by 24 h under *in vitro* conditions. Evidence of very low dose radiation induced a radio-adaptive response only in SA-NH but not SA-NH+mIκBα1 cells was shown by both an increase in SA-NH cell survival of 20–40% using a standard colony forming assay and reduced apoptosis frequencies of 20–40% as determined by the TUNEL assay. Changes in survivin protein levels as a function of irradiation conditions were monitored by Western blot. A 100 mGy exposure 30 min prior to a 2 Gy dose resulted in an elevation in total survivin protein 24 h later in SA-NH but not SA-NH+mIκBα1 cells. Transfection of cells with survivin siRNA inhibited elevation of survivin protein by very low dose radiation and the subsequent radio-adaptive response in SA-NH cells. These data suggest that the survivin-mediated radio-adaptive response is dependent upon the ability of cells to activate NFκB.

INTRODUCTION

Radio-adaptive responses induced by exposure of biological systems to very low radiation doses prior to their exposure to relatively high radiation doses have been extensively investigated using the endpoints of DNA damage and repair, chromosomal damage, micronuclei formation, mutagenesis and transformation, and cell killing and carcinogenesis (*1–7*). The radio-adaptive response is induced by a very small priming dose administered in the range of 1–200 mGy followed a short time later by a larger dose in the range of 1–10 Gy (*8*). The underlying mechanisms of action driving these adaptive responses are varied and generally are linked to specific exposure conditions. Just as the phenomena of sub-lethal and potentially lethal damage repair have been identified and defined operationally by radiation biologists to account for changes in radiation resistance due to differences in treatment conditions, so too can very low dose radiation-induced adaptive responses develop via different underlying mechanisms of action influenced by the conditions of radiation exposure. The exposure of normal cells to very low doses of ionizing radiation ($\leq$0.1 Gy) has been reported to induce a metabolic shift from oxidative phosphorylation to aerobic glycolysis due to metabolic reprogramming of HIF-1α resulting in increased radiation resistance to a subsequent exposure to a high dose administered 12 h later (*9*). Very low doses in the range of 5–100 mGy have also been reported to induce elevated manganese superoxide dismutase (SOD2) gene expression via TNF signaling and NFκB activation resulting in enhanced radiation resistance to a second higher challenge dose administered to cells 24 h later, which is the time interval during which intracellular SOD2 levels reach maximum elevation (*6, 7*). We recently discovered and reported on an additional adaptive response paradigm requiring the use of very low dose radiation (VLDR) administered during a multi-high dose protocol (*10*). This radio-adaptive response did not involve TNF signaling or elevation of SOD2, but rather was identified as being mediated through the action of the anti-apoptotic protein survivin (BIRC5) (*10*). Survivin is overexpressed primarily in malignant tissues, and this overexpression has been correlated with enhanced resistance to both ionizing radiation- and chemotherapy-induced cell killing and is accompanied by reduced frequencies of apoptosis (*11–13*). Survivin is known to participate in a complex functional mechanism affected by myriad signaling pathways. These include Wnt/β-catenin signaling, HIF-1α, HSP90, PI3K/AKT, mTOR, ERKs, MAPK, STAT3, p53, PTEN, ras,

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EGFR and VEGF (*14*). A signaling pathway affected by NFκB activation that leads to changes in survivin expression involves NFκB → PDK1 → AKT → GSK3β → Wnt/β-catenin (*15, 16*). Because NFκB activation has been demonstrated to play an integral role in the expression of the SOD2-mediated adaptive response, we evaluated its potential role in the expression of the survivin-mediated adaptive response characterized in our earlier report (*10*). While survivin levels are characteristically elevated in malignant cells, exposure of tumor cells to very low doses of ionizing radiation can induce a further transient increase. This results in an incremental increase in tumor cell radiation resistance to subsequent exposures to higher doses in a multi-dosing protocol. In this study, we investigated the survivin-mediated radio-adaptive response to assess the effectiveness of VLDR exposures of 5, 20 or 100 mGy given prior to or after the first of two 2 Gy doses separated by 24 h as a function of NFκB activation status in SA-NH murine sarcoma cells. Changes in tumor cell survival and apoptosis frequency were monitored using a standard *in vitro* colony forming assay and the TUNEL assay, respectively.

MATERIALS AND METHODS

Cells and Culture Conditions

SA-NH cells derived from an SA-NH murine sarcoma tumor and adapted for *in vitro* growth were supplied by Dr. Luka Milas, the University of Texas MD Anderson Cancer Institute. Cells were cultured in McCoy's 5A medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Invitrogen Life Technologies). Cell cultures were maintained at 37°C in a humidified environment containing 5% CO_2 and were grown to confluence in preparation for injection into mice (*17, 18*). SA-NH cells stably transfected with a pcDNA3 plasmid containing a mutant IκBα gene under the control of a CMV promoter in which serines 32 and 36 are mutated and a neomycin resistance gene to allow the selection of stable transfectants using G-418 and designated SA-NH+mIκBα1 were developed as described in detail elsewhere (*19*) and were used to assess the role of NFκB activation in the expression of the survivin-mediated radio-adaptive response. Growth conditions for SA-NH+mIκBα1 cells were identical to those identified for SA-NH cells.

Irradiation Conditions

Tumor cells grown to confluence were irradiated at room temperature using a Philips RT250 X-ray generator operated at 250 kVp and 15 mA as described in detail elsewhere (*10*). For the 2 Gy exposures, the dose rate was 1.33 Gy/min; for 100 mGy exposures, the dose rate was 0.3 Gy/min, and for 5 and 20 mGy exposures, the dose rate was 0.05 Gy/min.

Survivin siRNA Transfection

SA-NH and SA-NH+mIκBα1 cells were grown to confluence and transfected with 100 n*M* survivin (BIRC5) or negative control (NC) short interfering RNA (siRNA) (Ambion by Life Technologies, Foster City, CA) using Lipofectamine 2000 reagent (Invitrogen Life Technologies) as described elsewhere (*10*). The sequence of the mouse survivin siRNA was 5′-UGUCUGUCCAGUUUCAAGAat-3′. siRNA oligomer and Lipofectamine 2000 were diluted in serum-free media and incubated for 5 min at room temperature. The siRNA oligomer and Lipofectamine 2000 were then combined and incubated for 20 min. Growth medium was aspirated from the dishes and cells were washed with phosphate-buffered saline (PBS, Invitrogen Life Technologies) at 37°C. siRNA Lipofectamine 2000 complexes were added to the dishes and incubated for 24 h with cells under their normal growth conditions. The medium was then aspirated, the cells washed with PBS at 37°C and fresh complete medium at 37°C added to the dishes.

Western Blotting

Total protein was prepared from both SA-NH and SA-NH+mIκBα1 cells using a method described elsewhere (*10*). Cells were washed with cold PBS, harvested on ice, transferred to 50 ml tubes and pelleted at 1,000 rpm for 5 min at 4°C. Each cell pellet was resuspended in 350 μl 50 m*M* potassium phosphate buffer, pH 7.8 and sonicated on ice three times for 15 s each (*18*). Protein was quantified by the Bradford method and adjusted to 2 μg/μl with potassium phosphate buffer (*20*). Survivin (Cell Signaling Technology, Danvers, MA), and α-tubulin (Cell Signaling Technology) protein levels were assessed using the WesternBreeze Chemiluminescent Western Blotting Immunodetection System (Invitrogen Life Technologies). Protein (10 μg) was electrophoresed on a 12% Tris Gylcine gel and transferred onto PVDF membranes. The blots were blocked for 1 h with 10 ml blocking solution, washed twice with 20 ml ddH_2O for 5 min each, and then incubated with primary antibody (1:1,000 dilution of rabbit anti-survivin; 1:1,000 dilution of rabbit anti-α-tubulin) for 1 h at room temperature. The blots were then washed 4× for 5 min each with antibody wash solution and incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase for 30 min at room temperature. The blots were again washed 4× for 5 min each with antibody wash solution followed by three 2 min washes with ddH_2O. The protein bands were visualized by applying 2.5 ml chemiluminescent substrate to the membranes for 5 min. Membranes were exposed to BioMax XAR film (Kodak, Rochester, NY), scanned using an HP ScanJet 8200 (Houston, TX) and band intensities quantified using NIH ImageJ 1.49b software.

TUNEL Apoptosis Assay

Apoptosis was monitored using the TACS 2 TdT-Blue Label in Situ Apoptosis Detection Kit from Trevigen following the manufacturer's instructions and described in detail elsewhere (*10*). Cells were grown to confluence, trypsinized and pelleted by centrifugation at 1,000 rpm for 5 min at 4°C. Cells, fixed in 70% ethanol, were stored at 4°C overnight. Cells, dropped onto treated glass microscope slides and air-dried overnight, were sequentially immersed in 100%, 95% and then 70% ethanol for 5 min each. After rehydration, the slides were immersed in PBS for 10 min followed by addition of 50 μl of Cytonin for 30 min at room temperature, washed twice and immersed in quenching solution for 4.5 min, washed once in PBS for 1 min at room temperature and then immersed in TdT labeling buffer for 5 min at room temperature. Labeling reaction mixture (50 μl) was applied to each cell sample, incubated at 37°C for 1 h, then immersed in TdT stop buffer for 5 min and washed twice in PBS, 5 min, at room temperature. Strep-HRP solution (50 μl) was added for 10 min at 37°C. Slides were washed 2× in PBS for 5 min and immersed in TACS-Blue label solution, washed 3× in apoptosis grade water for 2 min, immersed in Nuclear Fast Red and then sequentially washed 10× in: apoptosis grade water (2×), 70% ethanol (2×), 95% ethanol (2×), 100% ethanol (2×) and o-xylene (2×). Slides were placed in a light-tight container, mounting medium was added and allowed to harden overnight at room temperature and apoptotic cells then scored using an Axioplan fluorescence microscope, 63x oil immersion objective, using GFP and Texas Red filters. At least 1,000 cells were scored and

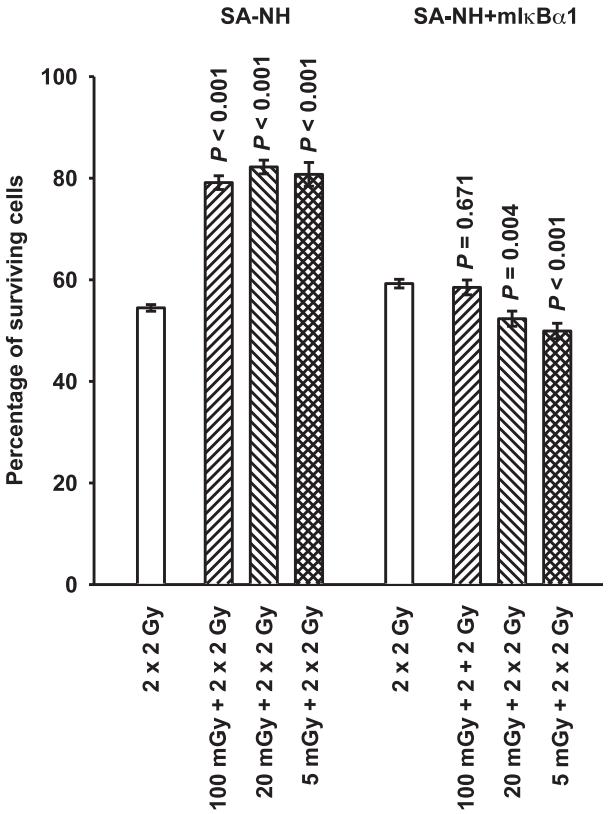

FIG. 1. The effect of very low dose radiation (VLDR) administered before high-dose irradiation on cell survival as a function of NFκB activation. The effects of VLDR exposures ranging from 5–100 mGy administered 30 min prior to the first of two 2 Gy exposures separated by 24 h on the induction of a radio-adaptive response *in vitro* on SA-NH wild-type and SA-NH+mIκBα1 cells deficient in the ability to activate NFκB were determined for the end points of changes in cell survival. *P* values were determined by comparing the survival of cells after two 2 Gy doses with those exposed to two 2 Gy doses along with the various VLDR using a two-tailed Student *t* test with values ≤ 0.05 identified as significant. Each experiment was repeated 3 times and error bars represent the standard error of the mean (SEM).

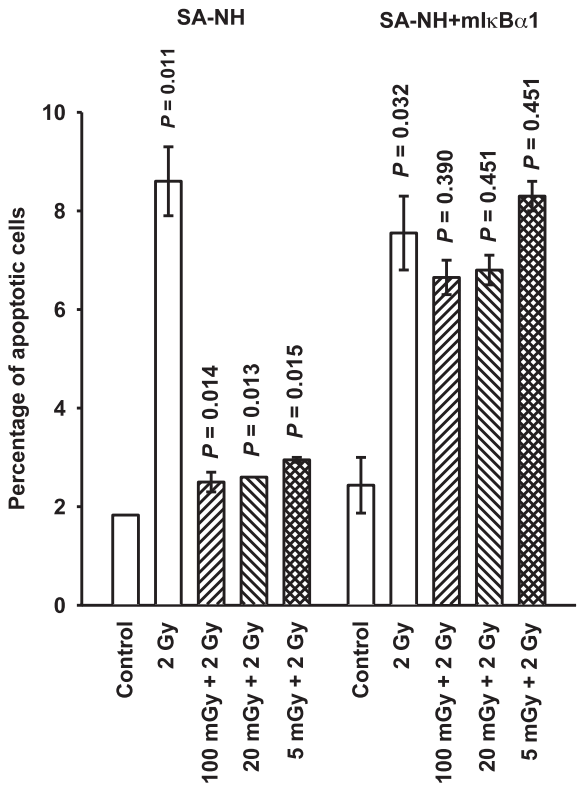

FIG. 2. The effect of VLDR administered before high-dose irradiation on induction of apoptosis as a function of NFκB activation. The effects of VLDR exposures of 5, 20 and 100 mGy administered 30 min prior to the first of two 2 Gy exposures separated by 24 h on the induction of a radio-adaptive response *in vitro* on SA-NH wild-type and SA-NH+mIκBα1cells deficient in the ability to activate NFκB were determined for the end points of changes in apoptosis frequency. *P* values were determined by comparing the apoptosis frequency of cells after two 2 Gy doses with those exposed to two 2 Gy doses along with VLDR using a two-tailed Student *t* test with values ≤ 0.05 identified as significant. Each experiment was repeated 3 times and error bars represent the SEM.

apoptosis frequency calculated as the ratio of the number of apoptotic cells relative to the total number counted.

Statistical Analysis

Means and standard errors were calculated for all data points from at least three independent experiments. Pairwise comparisons of cell survival and apoptosis frequencies between each of the experimental conditions were performed using a Student's two-tailed *t* test (SigmaPlot software 11.0, SPSS, Chicago, IL).

RESULTS

NFκB has been identified as an important factor in the development and expression of a radio-adaptive response mediated by SOD2 (*6, 7, 19*) as well as in survivin signaling pathways (*15, 16*). For these reasons we investigated its potential role in the survivin-mediated adaptive response that was originally described in our earlier study (*10*). The effects of 5, 20 or 100 mGy radiation doses administered 30 min prior to the first of two 2 Gy doses, each separated by 24 h, on cell survival in SA-NH and SA-NH+mIκBα1 cells is shown in Fig. 1 for comparison. While SA-NH cells expressed a robust adaptive response resulting in a 20% increase in cell survival, SA-NH+mIκBα1 cells were unaffected by exposure to VLDR and exhibited no change in cell survival. The lack of an adaptive response in these cells was also reflected by the lack of an effect on apoptosis frequencies measured 24 h later after their exposure to 2 Gy with or without pre-exposure to VLDR (see Fig. 2). In contrast, under similar treatment conditions, apoptosis was significantly inhibited in SA-NH cells. These responses remained the same even if VLDR was administered 30 min, 3 or 6 h after the initial 2 Gy dose (see Fig. 3).

It was previously reported that VLDR exposure of SA-NH cells 30 min after the first of two 2 Gy doses separated by 24 h resulted in a 1.7-fold elevation in survivin protein as determined by Western blotting (*10*). This increase in survivin was significantly reduced to 50% of control levels

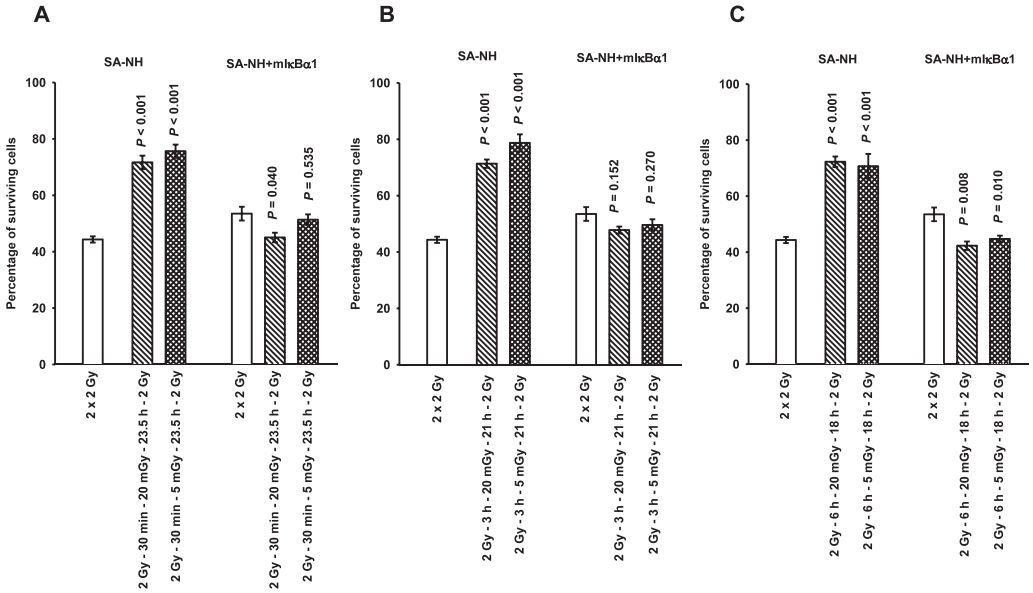

FIG. 3. The effect of VLDR administered after high-dose irradiation on the expression of the radio-adaptive response as a function of NFκB activation. The effects of VLDR exposures of 5 and 20 mGy administered 30 min (panel A), 3 h (panel B) or 6 h (panel C) after the first of two 2 Gy exposures separated by 24 h on the induction of a radio-adaptive response *in vitro* on SA-NH wild type and SA-NH+mIκBα1 were determined for changes in cell survival. *P* values were determined by comparing the survival of cells after two 2 Gy doses with those exposed to 2 Gy doses along with 5 or 20 mGy using a two-tailed Student *t* test with values ≤ 0.05 identified as significant. Each experiment was repeated 3 times and error bars represent the SEM.

in SA-NH cells transfected with survivin siRNA (*10*). We have extended and contrasted these observations to also include an evaluation of the effects of VLDR exposures on SA-NH+mIκBα1 cells. As shown in representative Western blots in Fig. 4, a 100 mGy exposure had no effect on survivin protein levels in SA-NH+mIκBα1 compared to SA-NH cells, while transfection with survivin siRNA resulted in a reduction in intracellular survivin protein in both cell lines.

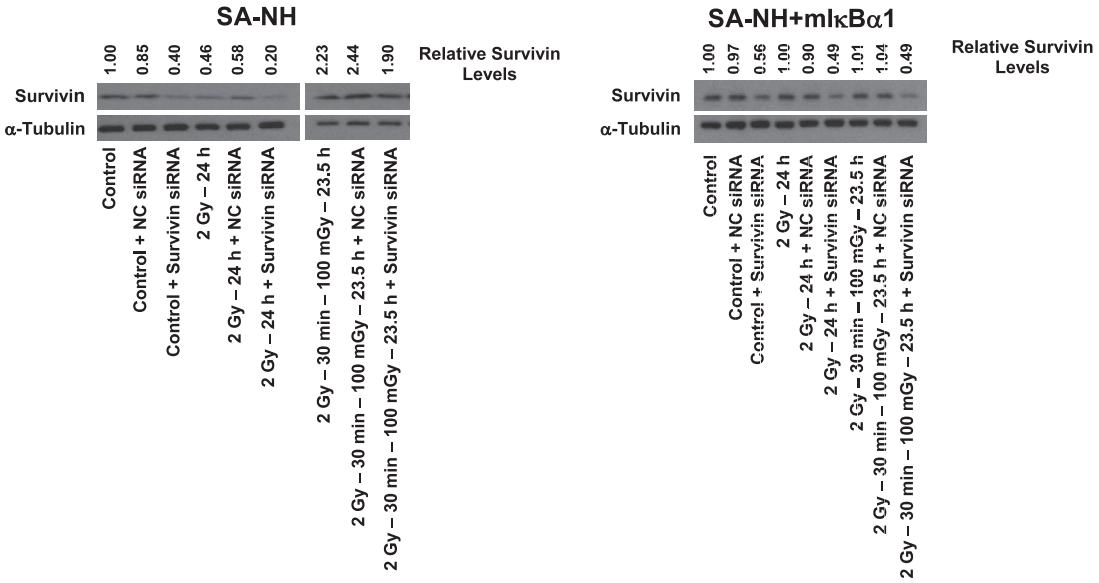

FIG. 4. Western Blot depicting relative survivin protein levels in SA-NH and SA-NH+mIκBα1 cells as a function of treatment. Representative Western blots from 3 separate experiments describe and compare the changes in survivin protein 24 h after a 2 Gy exposure with or without 100 mGy in SA-NH and SA-NH+mIκBα1 cells. Irradiation with 100 mGy induced over a twofold elevation of survivin in SA-NH cells. In contrast, 2 Gy alone or with 100 mGy administered 30 min after failed to induce any change in survivin protein as compared to nonirradiated control cells. Transfection of SA-NH and SA-NH+mIκBα1 cells with survivin siRNA reduced survivin protein in both cell lines irrespective of radiation treatment. Transfection with negative control (NC) siRNA had no effect on survivin protein. Relative survivin levels were determined through densitometry measurements of survivin and corresponding α-tubulin loading control band densities and normalized to nonirradiated control values.

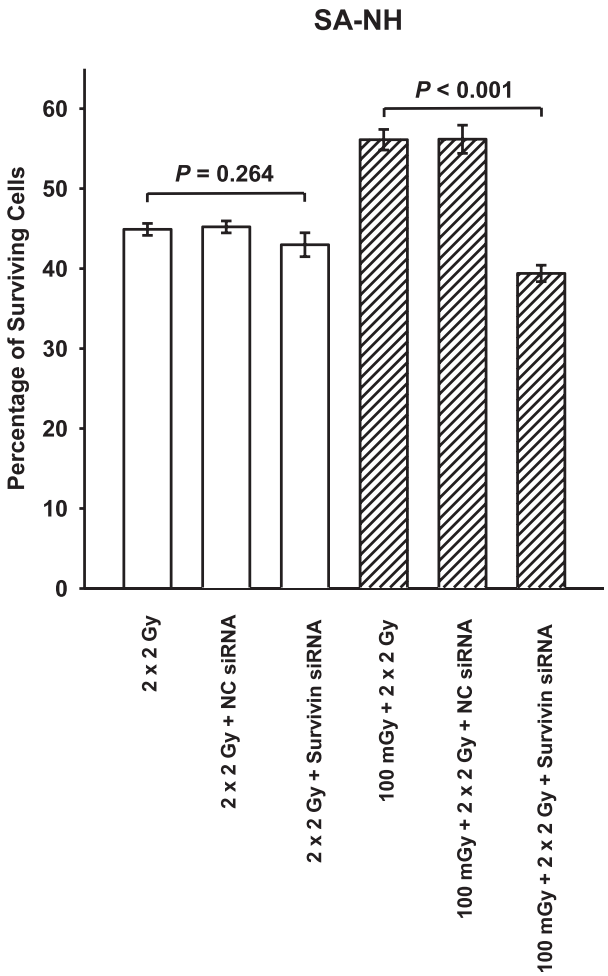

FIG. 5. The effect of survivin siRNA transfection of SA-NH cells on the expression of the radio-adaptive response. SA-NH cells, with or without survivin siRNA transfection, were evaluated for the expression of a radio-adaptive response as a function of exposure to 100 mGy administered 30 min prior to the first of two 2 Gy doses separated by 24 h. Transfection of SA-NH cells with survivin siRNA completely inhibited the expression of a 100 mGy-induced radio-adaptive response. Transfection with NC *siRNA* had no effect on the expression of the radio-adaptive response. Comparisons between survivin siRNA transfected and nontransfected SA-NH cells were performed using a two-tailed Student *t* test with values ≤ 0.05 identified as significant. Each experiment was repeated 3 times and error bars represent the SEM.

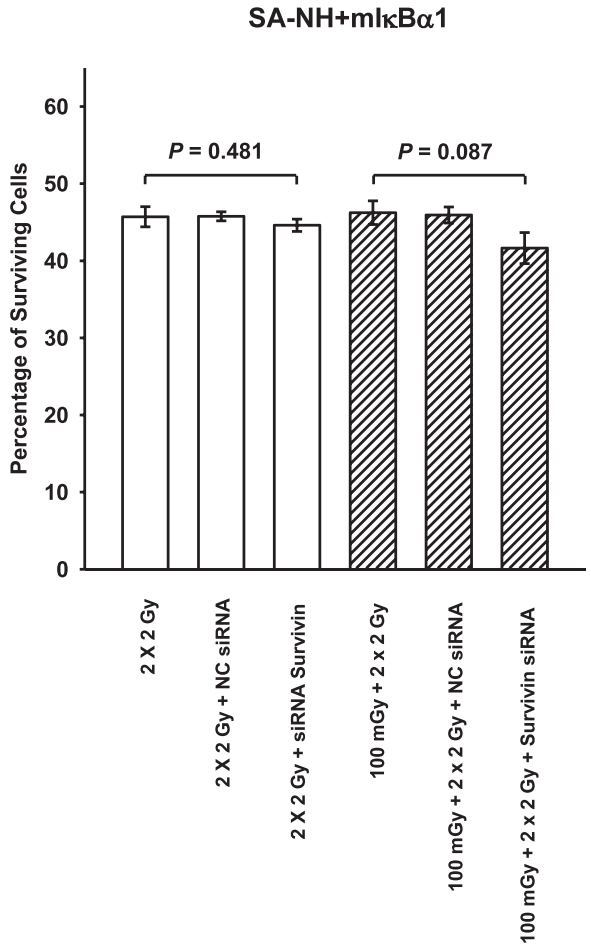

FIG. 6. The effect of survivin siRNA transfection of SA-NH+mIκBα1 cells on the expression of the radio-adaptive response. SA-NH+mIκBα1 cells, with or without transfection with survivin siRNA, failed to exhibit a radio-adaptive response after exposure to 100 mGy prior to exposure to two 2 Gy doses separated by 24 h. Transfection with NC siRNA also had no effect on the expression of the radio-adaptive response. Comparisons between survivin siRNA transfected and nontransfected SA-NH+mIκBα1 cells were performed using a two-tailed Student *t* test with values ≤ 0.05 identified as significant. Each experiment was repeated 3 times and error bars represent the SEM.

As described in Fig. 5, transfection of SA-NH cells with survivin siRNA resulted in the complete inhibition of the radio-adaptive response ($P < 0.001$) induced by exposure to 100 mGy administered 30 min prior to the first of two 2 Gy doses separated by 24 h. Consistent with the observations in Fig. 1 that SA-NH+mIκBα1 cells do not exhibit this radio-adaptive response, transfection with survivin siRNA had no significant effect on their response to this irradiation protocol ($P = 0.087$). These results are shown in Fig. 6 for comparison.

DISCUSSION

Very low dose radiation (VLDR)-induced adaptive responses can be considered in a general sense as stress-induced transient phenomena characterized by an enhanced cellular capability for responding to the deleterious effects of subsequent high dose radiation exposures. While the endpoints routinely associated with the expression of these radio-adaptive responses are enhanced cell survival and repair of damage accompanied by reduced genomic instability, DNA damage, chromosomal damage, mutagenesis, and carcinogenesis, the underlying mechanisms of action are varied and are dependent upon the experimental conditions employed. As an example, a single VLDR exposure administered prior to a single high challenge dose of radiation has been observed to induce a radio-adaptive response mediated through the action of SOD2. This process is dependent upon TNF signaling to activate NFκB and stimulate SOD2 gene expression (*6, 7*). This form of radio-adaptive response is completely inhibited in cells

transfected with *Sod2 siRNA* and/or defective in TNF signaling and NFκB activation (*6, 7, 18*). In contrast, under a multi-radiation dose protocol where VLDR is administered prior to or after each of several large challenge doses, a radio-adaptive response is also expressed but it is independent of TNF signaling and changes in SOD2 gene expression. Instead, this adaptive response is mediated by the transient elevation of the inhibitor of apoptosis (IAP) protein survivin (*10*). Because of the similarity between this experimental protocol and standard tumor imaging procedures coupled with multi-fractionated doses of ionizing radiation routinely used in Image-Guided Radiotherapy (IGRT), there is a clinical relevance for the risk of development of a radio-adaptive response capable of increasing radiation resistance in tumors resulting in adverse effects in clinical outcomes for patients treated with IGRT.

While an intact TNF signaling pathway is not required for the expression of the survivin-mediated radio-adaptive response (*10*), data presented in Figs. 1–4 show the requirement for NFκB activation in this process. The potential role of NFκB in the survivin-mediated radio-adaptive response was investigated by transfecting SA-NH cells with a pcDNA3 plasmid containing a mutant IκBα gene under the control of a CMV promotor in which serines 32 and 36 were mutated. These mutations abolish the inducible phosphorylation of these residues, preventing the subsequent ligand-induced degradation that leads to activation of NFκB. As described in Figs. 1, 2 and 4, SA-NH stably transfected with mutant IκBα, designated SA-NH+mIκBα1, failed to exhibit an adaptive response as compared to wild-type SA-NH when exposed to 5, 20 or 100 mGy 30 min prior to or 30 min, 3 h, or 6 h after the first of two 2 Gy doses. There was no effect on either cell survival using a colony forming assay or apoptosis as measured by TUNEL assay demonstrating the required activation of NFκB in this radio-adaptive response paradigm. While transfection of SA-NH cells with survivin siRNA inhibited the expression of the VLDR adaptive response, the lack of a radio-adaptive response mediated by survivin in SA-NH+mIκBα1 cells correlated with the lack of an effect on survivin protein levels as a function of VLDR exposure (see Fig. 4).

Figures 1–3 demonstrate that the adaptive response can be induced by VLDR exposures administered either before or after each challenge dose. It is hypothesized that this initial signal is driven via a reactive oxygen species (ROS) mechanism since survivin has been demonstrated to mediate self-protection through a ROS-initiated signaling pathway (*21*). However, the underlying mechanism driving this process is not clearly understood. It has been calculated that on average a 1 Gy dose will give rise to 2,000 primary ionization events per cell (*22*). A 5 mGy dose will therefore give rise to approximately 10 ionization events per cell. In contrast, about 10^9 ROS are calculated to escape mitochondria into the cytoplasm per average cell per day generating about 11,600 ROS per second per average cell via a mitochondrial escape mechanism (*23*). So while the adaptive response is thought to be the result of an induced signaling process, the role of ROS in this process is complex requiring a robust intracellular augmentation process(es) that is as yet undefined.

The radio-adaptive response induced by VLDR is a phenomenon that can have a significant impact on the expected outcomes of patients treated with IGRT. While all cancers treated with radiation therapy have unique complexities associated with their treatment, some because of anatomical location and potential for organ movement during exposure require longer set up times. In the original description of this VLDR-induced survivin-mediated radio-adaptive response, human colon carcinoma RKO36, transformed mouse embryo fibroblast, and SA-NH murine sarcoma cells each exhibited this phenomenon when exposed to 2 Gy with VLDR resulting in elevated surviving fractions ranging from 20–40% (*10*). If, for example, the survivin-mediated radio-adaptive response occurs multiple times during a conventional 30 fraction IGRT treatment regimen, the potential for adversely affecting expected outcomes can become a reality. Central to both the survivin-mediated and the SOD2-driven radio-adaptive responses is the requirement for NFκB activation. These adaptive responses should be recognized not simply as academic curiosities, but as potential confounders to effective cancer therapies and worthy of further study.

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