Survivin As a Therapeutic Target for Radiation Sensitization in Lung Cancer

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ABSTRACT

Expression of survivin is elevated in most malignancies, especially in radiation-resistant cell lines. In this study, we investigated how radiation affects survivin expression in primary endothelial cells as well as in malignant cell lines. We found that 3 Gy significantly reduced survivin protein level in human umbilical vein endothelial cells (HUVECs) but not in tumor cell lines. Flow cytometry studies suggest that the down-regulation of survivin is independent of cell cycle. In addition, survivin mRNA level was also down-regulatable by irradiation. However, it was abrogated by actinomycin D-mediated inhibition of gene transcription. Luciferase reporter gene assays suggest that irradiation suppressed the survivin promoter. p53 overexpression reduced survivin expression, but overexpression of a p53 mutant failed to abolish the radiation-induced down-regulation in HUVECs. Alteration of p53 status in Val138 lung cancer cell line also failed to restore the radiation-inducible down-regulation. Over-expression of survivin in 293 cells prevented apoptosis induced by irradiation and increased cell viability after irradiation. The inhibition of survivin using antisense oligonucleotides caused a significant decrease in cell viability of irradiated H460 lung cancer cells. These data suggest that radiation transcriptionally down-regulates survivin in HUVECs. This regulatory mechanism is defective in malignancies and is not mediated by p53. Survivin overexpression may lead to resistance to radiotherapy by inhibiting apoptosis and enhancing cell viability. The inhibition of survivin results in sensitization of H460 lung cancer cells to radiation. These studies suggest that survivin may be a target for cancer therapy.

INTRODUCTION

Inhibitor of apoptosis protein belongs to a family of proteins that regulate cell death (1). Survivin is the smallest member of the mammalian inhibitor of apoptosis family. It inhibits caspase 9 and blocks apoptotic pathway (2). The most notable feature of survivin expression is its absence in most terminally differentiated normal tissues, but it is highly expressed in malignancies and embryonic tissues (3–4). Survivin overexpression in malignancies was shown to be independent of mitotic indices or Ki67 reactivity (5). Survivin expression is up-regulated in all phases of cell cycle, and the cancer-specific activity of survivin promoter was detected both in vitro and in vivo (6). p53 is one of the regulators, which transcriptionally down-regulates survivin through binding a bipartite p53-responsive element in the survivin promoter (7).

Survivin is also highly expressed in neoangiogenesis (3–4). Survivin expression in endothelial cells was necessary to sustain the antiapoptotic effect of vascular endothelial growth factor (8–11) and angiopoietin-1 (12). Clinically, high levels of survivin have been associated with decreased overall survival, increased recurrences, and resistance to therapy (4). Therefore, we examined the effects of irradiation on both protein and mRNA levels of survivin in cultured human umbilical vein endothelial cells (HUVECs) and tumor cell lines. We found that survivin is transcriptionally down-regulated by irradiation in HUVECs, but not in malignant cell lines. This transcriptional suppression is not mediated by p53. Furthermore, we found that overexpression of survivin led to radiation resistance, whereas the inhibition of survivin resulted in the radiosensitization of H460 lung cancer cells.

MATERIALS AND METHODS

Cell Culture, Adenoviral Vectors, and Chemicals. HUVECs were obtained from Clonetics and were maintained in endothelial basal medium-2 (EBM-2) medium supplemented with endothelial growth medium (EGM-2) single mV aliquots (BioWhittaker). Various cancer cell lines were obtained from American Type Culture Collection and cultured in their required media. Val138 cell (a gift from Dr. Maureen Murphy, Fox Chase Cancer Center, Philadelphia, PA) originates from human lung adenocarcinoma cell line H1299 stably transfected with temperature-sensitive p53 mutant. Val138 cells were cultured in DMEM (DMEM, Invitrogen) plus 10% fetal bovine serum, 100 units/ml penicillin and streptomycin, and 0.8 mg/ml Geneticin. HEK 293 cells (American Type Culture Collection) transfected with pCDNAhis-survivin or pCDNAhas vector were selected in DMEM with 1% FCS and 0.5 mg/ml G418 (Invitrogen). Single cell clones overexpressing survivin or neomycin control were confirmed by immunoblotting. Actinomycin D (Sigma) was used at a final concentration of 5 µg/ml. Irradiation (3 Gy) was given 1 h after the drug was added, by use of a Colbalt-60 radioactive source. Adenoviral vectors overexpressing LacZ and p53 were gifts from Dr. Shuang Huang, The Scripps Research Institute (San Diego, CA).

Western Immunoblots. Cells were treated with 3 Gy and various drugs and collected at various time points. The cells were counted and then were washed with ice-cold PBS twice before the addition of lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and leupeptin). Protein concentration was quantified by the Bio-Rad method. Equal amounts of protein were loaded into each well and separated by 14% SDS-PAGE gel, followed by transfer onto nitrocellulose membranes. Membranes were blocked by use of 10% nonfat dry milk in PBS for 2 h at room temperature. The blots were then incubated with the rabbit-antihuman [survivin (R&D Systems), p53 and phospho-p53-serine 15 (Cell Signal), cleaved caspase 3 (Cell Signal)] antibodies overnight at 4°C. Donkey antirabbit IgG secondary antibody (1:1000; Amersham) was incubated for 1 h at room temperature. Immunoblots were developed by using the enhanced chemiluminescence (ECL) detection system (Amersham) according to the manufacturer’s protocol and autoradiography.

Flow Cytometry. Cells were trypsinized, rinsed once, resuspended in PBS, and fixed with ice-cold 70% ethanol for at least 20 min. Fixed cells were then rinsed again with PBS and resuspended in 50 µg/ml propidium iodide (Sigma) with 40 kilounits (KU)/ml of DNase-free RNAse (Stratagene, La Jolla, CA). Cells were then run on a Becton Dickinson FACScan flow cytometer, and the percentage of cells in each phase was calculated using ModFit software.

Real-Time Quantitative Reverse Transcription-PCR. Total RNA was isolated from cell culture, using a Qiagen RNA extraction kit. After RNA isolation, cDNA was prepared from each sample as described previously (13). Quantification of cDNA and an internal reference gene (β-actin) was conducted using a fluorescence-based real-time detection method [ABI PRISM 7700 Sequence Detection system (TaqMan); Perkin-Elmer Applied Biosystems, Foster City, CA], as described previously (14–15). All of the quantitative reverse transcription-PCR experiments were performed as triplicates. The PCR mixture consisted of 600 nM/liter each primer, 200 nM/liter probe (sequences used are given below, in “Plasmid, AS Oligonucleotides, Transfection and Luciferase Assays), 5 units of AmpliTaq Gold polymerase, 200 µM each of nucleotides, and 2x SYBR Green Master Mix (Applied Biosystems). The PCR conditions were as follows: 1 cycle of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec, 60°C for 1 min.
dATP, dCTP, and dGTP, 400 μM dUTP, 5.5 mM MgCl₂, and 1× TaqMan buffer A containing a reference dye, to a final volume of 25 μL. The entire experiment was carried out on a Monolight 3010 Luminometer (PharMingen). Luciferase assays were performed as the protocol from the manufacturer.

After 24 h, cells were irradiated with 3 Gy. Total cellular protein was extracted at the indicated time points after treatment. Shown are the surviving fraction was counted. A control experiment of cells receiving only radiation was also performed.

RESULTS

Irradiation Reduces Survivin Expression in Primary Endothelial Cells but not in Malignant Cells. Survivin plays a critical role in cell viability. To determine the time-dependent expression of survivin in the irradiated endothelial cells and tumor cell lines, we extracted total protein at 5, 15, and 30 min, and at 1, 2, 4, 16, 24 h. Fig. 1 shows the autoradiograph of Western immunoblots. Survivin expression became undetectable at 1 h and remained undetectable at 24 h. However, 3 Gy failed to down-regulate survivin in all of the cancer cell lines that were tested, as shown in Fig. 1B. To determine whether the decrease of survivin is a result of reduction in mitotic cells, we examined cell-cycle distribution at various time points to determine whether there is an increase of nonmitotic cells beyond 16 h postirradiation. HUVECs were irradiated with 3 Gy and were col-
lected at 0, 4, and 24 h. Cells were fixed with 70% ethanol and stained with propidium iodide. Flow cytometry analyses showed no significant variation of G2-M cells at these time points, as shown in Fig. 1C.

**Down-Regulation of Survivin in HUVECs Is Mediated by Transcriptional Suppression.** To examine whether the survivin mRNA level is altered after radiation treatment, we irradiated HUVECs with 3 Gy and collected at 0, 1, 2, 4, 16, and 24 h. Total cellular RNA was isolated. Quantification of survivin mRNA and β-actin (internal reference) mRNA was performed using real-time reverse transcription-PCR. As shown in Fig. 2A, the relative level of survivin mRNA began to decrease at 4 h after irradiation and was further reduced by more than 50% at 24 h. However, this was not observed in colorectal cancer cell lines (SW480 and SW620), as shown in Fig. 2B.

To determine whether the decrease of mRNA is a result of mRNA instability, we treated HUVECs with actinomycin D alone or together with 3 Gy added 1 h later. As shown in Fig. 3, A and B, survivin mRNA was stable after either treatment, whereas Cox-2 mRNA decreased to one-third at 2 h after either treatment. To confirm that the down-regulation of survivin is contributed by reduced transcription, HUVECs were transfected by a luciferase reporter under the control of 1.1 kb survivin promoter fragment. Transfected cells were irradiated and collected at 0, 4, 8, and 16 h after irradiation. As shown in Fig. 3C, luciferase activity was significantly reduced at the various time points after irradiation. These data suggest radiation decreases the promoter activity of survivin gene.

**Role of p53 in Down-Regulating Survivin.** DNA damage from irradiation triggers activation and increase of p53 (18). We therefore studied the p53 status in HUVECs after irradiation. HUVECs were irradiated with 3 Gy and were collected at various time points. Protein extracts were made and analyzed by Western blotting. As shown in Fig. 4A, p53 phosphorylation increased within 15 min of irradiation, whereas the level of total p53 protein did not change. To determine whether overexpression of p53 has an impact on survivin levels in endothelial cells, we used the expression vector Ad.p53. Adenovirus-mediated overexpression of wild-type p53 decreased survivin mRNA level at 24 h after HUVECs were transduced (Fig. 4B). To determine whether p53 mediates the down-regulation of survivin by irradiation, we studied Val138 cell, a lung adenocarcinoma cell line stably transfected with a temperature-sensitive mutant of p53. Cells were cultured at either 39 degrees (predominantly expressing wild-type p53) or 32 degrees (predominantly expressing mutated p53). As shown in Fig. 5A, cleaved caspase 3 was detected in irradiated control (293 cells transfected with a plasmid overexpressing survivin). As shown in Fig. 5B, irradiation failed to inhibit cell proliferation and viability in survivin-overexpressing cells.

To study the biological effects of survivin inhibition in cancer, we

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**Fig. 3. Transcriptional down-regulation of survivin after irradiation.** Human umbilical vein endothelial cells (HUVECs) were treated with actinomycin D and were irradiated 1 h later. Total RNA was extracted at the indicated time points after the irradiation. The RNA samples were reverse-transcribed to cDNA and were quantified by Taqman real-time PCR. Shown are the relative mRNA levels of survivin (A) or Cox-2 (B) in reference to β-actin. C, HUVEC cells were transiently transfected with the SPI plasmid. Transfected cells were irradiated and assayed at the various time points.

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**Fig. 2. Down-regulation of survivin mRNA by 3 Gy in human umbilical vein endothelial cells (HUVECs), but not in colorectal cancer cells.** Cells were irradiated with 3 Gy. Total RNA was extracted at the indicated time points after the irradiation treatment. The RNA samples were reverse-transcribed to cDNA and were quantified by Taqman real-time PCR. Shown are the relative mRNA levels of survivin in reference to β-actin. A, HUVECs; B, SW480 and SW620.
used an AS approach to inhibit survivin expression in H460 lung cancer cells. Either anti-survivin (AS, i.e., antisense) or a missense (mismatch) control oligonucleotide was transiently transfected using Lipofectin. Survivin expression was determined by Western blotting 48 h after transfection. As shown in Fig. 6A, AS oligonucleotide against survivin attenuated survivin expression, compared with the mismatch oligonucleotide. To determine whether inhibition of survivin affects cell survival in irradiated H460 cells, H460 cells transfected with either AS or mismatch were then treated with or without 3 Gy. The 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed at 48 h after irradiation. As shown in Fig. 6B, both 3 Gy alone and AS alone resulted in a 20% reduction of cell viability. However, concurrent survivin inhibition and irradiation caused a 2–3-fold reduction of cell viability. To confirm this result, we performed the clonogenic assay. As shown in Fig. 6C, the inhibition of survivin by AS significantly down-shifted the survival curve.

Fig. 6. Inhibition of survivin enhanced radiation effects in H460 lung cancer cells. A, survivin expression was attenuated 48 h after H460 cells were transfected with anti-survivin antisense oligonucleotide (AS). Mismatch (MS) is the control oligonucleotide. B, H460 cells were transfected with either AS or MS oligonucleotides. 24 h later, the transfected cells were treated with or without 3 Gy. The 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed 48 h after irradiation. Each graph, the mean ± SD from three repeated experiments.

Fig. 5. Survivin affects growth and apoptosis in response to irradiation. A, 293 cell lines stably transfected with or without the survivin-overexpressing plasmid were irradiated with 3 Gy. Cell viability was determined by the 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Each column, the mean ± SD from three repeated experiments.

Fig. 4. Role of p53 in transcriptional down-regulation of survivin by irradiation: human umbilical vein endothelial cells (HUVECs) were irradiated with 3 Gy. Total cellular protein was extracted at the indicated time points after treatment. Shown are the Western immunoblots using antibodies against total p53 or phospho-p53. B, the relative mRNA levels of survivin in reference to β-actin in HUVECs after transduction of Ad.LacZ or Ad.p53. C, the relative mRNA levels of survivin in reference to β-actin in irradiated Val138 cells cultured at either 32 degrees or 39 degrees. D, the promoter activity of survivin in HUVECs cotransfected with the SP1 plasmid plus either a control plasmid or an expression plasmid of mutant p53.

Fig. 3. Survivin affects growth and apoptosis in response to irradiation. A, 293 cell lines stably transfected with or without the survivin-overexpressing plasmid were irradiated with 3 Gy. Total cellular protein was extracted at the indicated time points after treatment. Shown are the Western immunoblots using antibodies against total p53 or phospho-p53.
of H460 after 2 Gy and 5 Gy. These data suggest that the inhibition of survivin leads to radiation sensitization in H460 lung cancer cells.

**DISCUSSION**

In this study, we have shown that survivin is transcriptionally down-regulated by radiation in normal human endothelial cells. However, this mechanism is defective in malignant cell lines. p53 is shown to affect survivin levels in general, but it does not mediate radiation-inducible suppression of survivin. We have shown that overexpression of survivin leads to radiation resistance by inhibiting apoptosis and promoting cell survival. Inhibition of survivin in H460 lung cancer greatly enhanced the cytotoxic effects of radiotherapy.

Both cell-cycle-dependent and cell-cycle-independent regulation of survivin have been described (4). It is believed that the cell-cycle-dependent regulation is mediated transcriptionally by a cell-cycle-dependent element/cell-cycle gene homology region in the survivin promoter (18). Posttranslationally, proteasome-dependent degradation may lead to the decrease of survivin during interphase (19). However, survivin expression was increased by interleukin 11 and angiotensin in endothelial cells, independent of cell-cycle progression, via phosphorylation of signal transducer and activator of transcription 3 (STAT3) or phosphatidylinositol 3-kinase (20). In the present study, transcriptional down-regulation of survivin in HUVECs was induced by irradiation and was independent of cell cycle. However, a similar decrease of survivin was not detected in various cancer cell lines. We found that the protein level of survivin decreased significantly at 16 h postirradiation in HUVECs. But its promoter activity was reduced at 4 h after irradiation. However, at the same time point, the mRNA level of survivin had only a slight drop, presumably because of the stability of its mRNA.

We explored the mechanism by which survivin is down-regulated in HUVECs. We found that radiation induced a down-regulation of survivin mRNA. Radiation had decreased the activity of survivin promoter without altering its mRNA stability. This suggests that survivin is transcriptionally down-regulated by irradiation. Because p53 may bind to the survivin promoter and suppress its transcription (7), we examined the role of p53 in the down-regulation of survivin. We have shown that p53 can be activated in irradiated HUVECs. Overexpression of wild-type p53 in HUVECs resulted in a lower level of survivin mRNA. However, we found that restoration of p53 function failed to down-regulate survivin mRNA in Val138 lung cancer cells. In addition, cotransfection of mutant p53 failed to abolish the down-regulation of survivin promoter in HUVECs. However, increased survivin expression was found in the presence of mutant p53 in both HUVECs and Val138 cells. This suggests that p53 affects survivin level in general but does not mediate transcriptional suppression by irradiation. Another possible mechanism of down-regulating survivin protein by irradiation is through its phosphorylation. It has been shown that Thr34 of survivin is phosphorylated by p34CDC2, a process that is critical for survivin function (21). Inhibition of this phosphorylation event by cyclin-dependent kinase inhibitors results in rapid clearance of survivin protein (22). Because irradiation has been shown to down-regulate CDC2 (23), it is possible that phosphorylation of Thr34 may contribute to posttranslational degradation of survivin in response to irradiation.

Survivin not only protects cells from apoptosis, it is also required for cell division. Deletion of survivin results in a catastrophic defect of microtubule assembly, with absence of mitotic spindles, disorganized tubulin aggregates, and multinucleation (24–25). Colocalization of survivin with Aurora-B and the inner centromere protein (INCENP) suggests that these proteins interact throughout mitosis and are essential for chromosome condensation and segregation as well as for completion of cytokinesis (26). Similar centrosome overduplication was present in several irradiated tumor cell lines and was believed to lead to the radiation-induced cell death (27).

The persistent expression of survivin may be a mechanism of radiation resistance. Radioreistant colorectal cell lines have a higher level of survivin expression (28). The inhibition of survivin using ribozyme or its dominant-negative molecule has been shown to sensitize melanoma and pancreatic cancer cells to radiation (29–30). We found that overexpression of survivin inhibits caspase 3 cleavage, presumably by binding caspase 9. Irradiation has less growth-suppressive effects on the survivin-overexpressed cells. Because survivin is selectively expressed in tumor and its neovasculature and is important to maintain their viability, it is an attractive target for cancer therapy. Strategies of targeting survivin include AS oligonucleotides, ribozymes, dominant-negative mutants, and cancer vaccine (29–33). One of the dominant-negative mutants has Thr34 changed to Ala, the consequence of which is the defective phosphorylation of survivin by cyclin-dependent kinase p34CDC2, dissociation of survivin-caspase 9 complex, and induction of apoptosis (21). Inhibition of p34CDC2 by cyclin-dependent kinase inhibitors such as Purvalanol (Purv.A) and flavopiridol significantly enhance the apoptosis and antitumor activity of Taxol and Adriamycin, respectively, in a breast cancer xenograft model (22, 34). Adenoviral-mediated overexpression of T34A mutant results in tumor reduction in xenograft models of breast cancer and melanoma (21, 34, 35). We found that anti-survivin oligonucleotides significantly enhanced cytotoxic effects of radiation in lung cancer cells.

In summary, our results suggest that survivin is down-regulated by radiation in endothelial cell but not in various cancer cell lines. The combination of survivin inhibition and irradiation resulted in significantly decreased cell survival of cancer cells. Additional studies are ongoing to determine the transcription factors that mediate radiation-induced suppression of survivin as well as to validate survivin as a target for radiation sensitization in animal models of lung cancer.

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