Protein Kinase B/Akt-Dependent Phosphorylation of Glycogen Synthase Kinase-3β in Irradiated Vascular Endothelium

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Abstract

The vascular endothelium plays a critical role in the response of cancer to ionizing radiation. Activation of the phosphoinositide-3-kinase/Akt pathway is one initial signaling event in irradiated endothelial cells. Specifically, a low dose of ionizing radiation (3 Gy) induces phosphorylation of Akt at Ser^{473} in the vascular endothelium within minutes of irradiation. However, signaling events that are downstream of Akt have not been well defined. Here, we show that phosphorylation of the Akt downstream target glycogen synthase kinase-3β (GSK-3β) at Ser^{9} also occurred within minutes of exposure to ionizing radiation. In addition, ionizing radiation caused the dissociation of GSK-3β from the cell membrane, consistent with the inactivation of GSK-3β enzyme activity. Overexpression of the dominant negative mutant Akt attenuated GSK-3β phosphorylation at Ser^{9} and enhanced radiation-induced apoptosis. X-irradiated endothelial cells formed capillaries in both *in vitro* and *in vivo* models, whereas overexpression of the dominant negative mutant Akt inhibited capillary tubule formation. Studies using GSK-3β antagonists showed that GSK-3β activity was required for apoptosis in endothelial cells treated simultaneously with Akt antagonists and radiation. In mouse vascular models, radiation-induced microvascular destruction in response to Akt antagonists also required GSK-3β function. These data indicate that on exposure of vascular endothelium to ionizing radiation, activation of Akt signaling contributes to GSK-3β inhibition, which in turn promotes endothelial cell survival and capillary formation. Thus, pharmacologic regulation of Akt/GSK-3β signaling may present a new approach to the radiation response in the tumor microvasculature. (Cancer Res 2006; 66(4): 2320-7)

Introduction

The vascular endothelium plays a critical role in the response to ionizing radiation (1, 2). The physiologic response of the microvasculature to ionizing radiation is in part dependent on signaling through phosphoinositide-3-kinase (PI3K)/Akt (3–5). Overexpression of the mutant p85 component of PI3K enhances radiation-induced apoptosis and minimizes capillary tubule formation (5). The PI3K/Akt signal transduction pathway has been implicated in survival signaling in various cell types (6–10). In endothelial cells, PI3K/Akt can be activated through a family of receptor tyrosine kinases that are activated by growth factors (11, 12). PI3K, the downstream effector of receptor tyrosine kinases, has been shown to be a molecular target for enhancement of the radiation response in tumor endothelium (3, 5, 13). For example, Akt signaling participates in angiogenesis following vascular endothelial growth factor (VEGF) stimulation of endothelial cells and regulates capillary-like tubule formation (14). Akt can be activated independently of growth factor or PI3K signaling (15). Recently, it has been shown that radiation induces VEGF-independent phosphorylation of Akt within endothelial cells (5). These studies suggest that Akt may be a molecular target for the development of drugs that enhance the efficacy of cancer therapy.

The results demonstrating the role of Akt in endothelial cell viability led to questions about how this signal transduction pathway contributes to the vascular response to ionizing radiation. Although high-dose irradiation exceeds the threshold for radiation-induced apoptosis in the endothelium (1, 2), lower doses do not induce programmed cell death (4, 16). We speculated that low-dose irradiation activates the Akt-mediated cell viability pathway, which also involves inhibitory phosphorylation of the downstream kinase glycogen synthase kinase-3β (GSK-3β; refs. 17, 18). PI3K-induced activation of Akt results in the phosphorylation of GSK-3β at Ser^{9}, which in turn inactivates GSK-3β enzymatic activity (19–21). We therefore studied the role of Akt and GSK-3β in endothelial cell survival and function in response to low-dose irradiation.

GSK-3β is a ubiquitously expressed protein serine/threonine kinase which was initially identified as an enzyme that regulates glycogen synthesis in response to insulin (19–23). Stimulation of cells with insulin causes inactivation of GSK-3β through a PI3K/Akt-dependent mechanism (24). GSK-3β is subject to multiple regulatory mechanisms. The activity of GSK-3β can be reduced by phosphorylation at Ser^{9}. Several kinases could mediate this modification, including p70S6 (25), p90RSK (26), PKC (27), and Akt (28). In contrast to the inhibitory phosphorylation of GSK-3β at Ser^{9}, phosphorylation of GSK-3β at Tyr^{216} increases its enzyme activity (29). In addition to phosphorylation, GSK-3β activity is also regulated by protein complex formation (30) and intracellular localization, in which the membrane-bound form generally represents protein with higher kinase activity (31). GSK-3β participates in multiple cellular processes including cell growth, differentiation, cell survival, and cytokinesia (32–34). Interestingly, GSK-3β has emerged as a regulator of neuronal, endothelial, hepatocyte, fibroblast, and astrocyte death (33, 34). Direct overexpression of wild-type GSK-3β is known to induce apoptosis in various cell types in culture, and specific inhibitors of GSK-3β are able to ameliorate this apoptotic process (20, 33–41).

Akt is a key effector of the PI3K survival pathway and enhances cell survival by minimizing the induction of apoptosis (12, 20, 34). The purpose of the studies described herein was to more directly test the role of Akt and its downstream target GSK-3β in the response of the endothelium to ionizing radiation. Phosphorylation of both Akt at Ser^{473} and GSK-3β at Ser^{9} occurred within minutes

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of irradiation of endothelial cells with 1 to 3 Gy. Radiation-induced phosphorylation of GSK-3β was also associated with decreased binding of GSK-3β to the membrane. To determine whether Akt signaling contributes to the response of the microvasculature to ionizing irradiation, endothelial cells were transduced with adenovirus vector containing double mutant Akt (T308A, S473A). This dominant negative Akt (dn-Akt) could not be activated by phosphorylation (42, 43). Inhibition of radiation-induced Akt activity by the introduction of dn-Akt attenuated GSK-3β phosphorylation at Ser^9, confirming that this phosphorylation is Akt-dependent and that GSK-3β is a part of the radiation-triggered PI3K/Akt signaling pathway. Furthermore, transduction of endothelial cells with dn-Akt enhanced radiation-induced apoptosis and prevented capillary tubule formation. Because overexpression of dominant negative GSK-3β (dn-GSK-3β) can protect cells from programmed cell death (34), we introduced dn-GSK-3β to endothelial cells that were treated with Akt inhibitors. This combination eliminated the radiation sensitizing effect of Akt antagonists on irradiated endothelial cells. Taken together, these findings show for the first time that ionizing radiation induces inhibitory phosphorylation of GSK-3β within the vascular endothelium. These findings also show that radiation-induced GSK-3β phosphorylation is dependent upon Akt activity. Moreover, programmed cell death induced by Akt inhibition and radiation is dependent on GSK-3β activity. In conclusion, these results indicate that activity of the radiation-induced PI3K/Akt/GSK-3β signal transduction pathway regulates endothelial cell resistance to low-dose irradiation.

Materials and Methods

Adenovirus vectors and other reagents. The adenovirus vector encoding a dominant-negative double mutant of Akt (T308A, S473A) was obtained from Dr. K. Walsh (Department of Pharmacology, Yale University School of Medicine, New Haven, CT) (42). The dominant-negative mutant GSK-3β was a gift from Dr. M.J. Birnbaum (Department of Pharmacology, Yale University School of Medicine, New Haven, CT) (24). The adenovirus vector encoding green fluorescent protein (GFP) was purchased from Clontech (Palo Alto, CA). Lithium chloride (LiCl) was obtained from Sigma (St. Louis, MO). GSK-3β inhibitor III and capase-3 cellular activity assay kit were purchased from Calbiochem, San Diego, CA. Akt tyrosine kinase inhibitor, ALX-349, was purchased from Alexis Biochemical (San Diego, CA). Viruses were propagated in HEK-293 cells, purified by column chromatography, quantitated, and dosed by particle yield.

Cell cultures and treatment. Primary culture of human umbilical vein endothelial cells (HUVEC) pooled from multiple donors were obtained from Cambrex (East Rutherford, NJ) and were maintained in EBM-2 medium (Cambrex). Cells from passages four to six were used in this study. HEK-293 cells were cultured in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum. In experiments with GSK-3β inhibitors, cells were treated with LiCl at 10 mmol/L for 2 hours prior to irradiation or with GSK3β inhibitor III at 10 μmol/L for 1 hour prior to irradiation. For adenovirus infections, HUVEC (8×10^5 cells) were cultured in 100 cm plates and were transduced with adenovirus at 10 to 100 plaque-forming units/cell. Transduced cells were subjected to further treatment 24 hours later. An Eldorado 8 Teletherapy ^{60}Co Unit (Atomic Energy of Canada Limited, Ontario, Canada) was used to irradiate endothelial cell cultures at a dose rate of 0.84 Gy/min. The delivered dose was verified by using thermoluminescence detectors.

Apoptosis quantitation. Morphologic analysis of primary culture endothelial cell apoptosis was done under a microscope using propidium iodide staining. Apoptotic cells were identified according to their nuclear condensation and fragmentation. Briefly, HUVEC were transduced with GFP, dn-Akt, and/or dn-GSK-3β adenoviruses. When chemical inhibition of GSK-3β was used, cells were treated with LiCl or GSK-3β inhibitor III and irradiated with 3 Gy 24 hours later. Cells were washed with PBS several times at 24 hours after treatment, and then permeabilized with 30% methanol and stained with propidium iodide in PBS. Apoptotic and nonapoptotic cells were counted in multiple randomly selected fields, and data were presented as a percentage of the cells that were apoptotic. Student's test was used to analyze statistical difference.

Caspase-3 cellular activity. Caspase-3 activity was assessed according to the Calbiochem protocol. Cells were transduced with GFP or dominant negative double mutant Akt adenoviruses. After 24 hours, cells were treated with LiCl or GSK3β inhibitor III and irradiated with 3 Gy. Cells were then washed twice with PBS and collected by trypsinization followed by centrifugation. The cells were lysed in the lysis buffer and incubated on ice for 10 minutes. Lysates were centrifuged for 10 minutes at 12,000 rpm, and the supernatants were added to microcentrifuge tubes containing caspase activity assay buffer. Caspase substrate was added to each sample and incubated for 2 hours at room temperature. Caspase enzyme activity was detected at 405 nm. Student's test was used to analyze statistical difference.

Immunoblot analysis. HUVEC were starved in MCDB 131 medium (Invitrogen) with 0.2% bovine serum albumin for 6 hours prior to irradiation. Then HUVEC were irradiated with the indicated dose and harvested at the indicated times after irradiation as previously described (3, 16). Total protein extraction was done using M-PER kit (Pierce, Rockford, IL). Membrane and cellular protein fractions were isolated using M-PEK kit (Calbiochem). Protein concentration was quantified using bicinchoninic acid reagent (Pierce). Protein extracts (40 μg) were subjected to Western immunoblot analysis using antibodies for the detection of phospho-Akt (Ser^{473}), Akt, phospho-GSK3β (Ser^9), GSK3β, cleaved caspase-3 and caspase-3 (Cell Signaling Technology, Danvers, MA), Flk-1 (A-3, Santa Cruz Biotechnology, Santa Cruz, CA), actin (Sigma). The secondary antibodies conjugated with horseradish peroxidase were purchased from Sigma. Protein bands were visualized with Western Lightning Chemiluminescence Reagent (Perkin-Elmer, Boston, MA).

Cell migration assay. HUVEC were transduced with GFP or dn-Akt adenoviruses, irradiated with 3 Gy 24 hours later and then placed at 1×10^5 cell amount into fibronectin-coated Boyden chambers at 37°C for 6 hours. The filter was carefully removed and cells which attached to the upper side were wiped off by cotton tips. The HUVEC migrating through the filter and appearing on the lower side were fixed by careful immersion of the filter into 70% ethanol for 15 minutes and staining. Cells were washed with PBS and detached from the filter with dissociation buffer. The amount of migrated cells were detected by measuring the absorbance of cell dissociation buffer at 550 nm. Each experiment was done in duplicate, and three separate experiments were done for each group. Student's test was used to analyze statistical difference.

Capillary-like network formation in Matrigel. A mixture of Matrigel (200 μL of a 10 mg/mL mixture; BD Bioscience, Bedford, MA) was placed in each well of an ice-cold 24-well plate. The plate was allowed to sit at room temperature for 15 minutes and then incubated at 37°C for 30 minutes to allow the Matrigel to polymerize. HUVEC were transduced with GFP or dn-Akt adenoviruses, irradiated with 3 Gy 24 hours later, and then seeded at 5×10^5 into each Matrigel-coated well. The cells were incubated for 24 hours to allow capillary-like structure formation. For ease of handling and optimal visualization of the capillary-like network, the medium was removed carefully after incubation, and agarose was gently added to the cells. After solidification of agarose, immobilized tubes were fixed and stained with Diff-Quik solution. The tubules were counted under microscopy and presented as a percentage of the control (GFP). Student's test was used to analyze statistical difference.

***In vivo* assay of microvascular response using Matrigel injection.** Capillary formation in the irradiated mouse used 10 mL Matrigel with a 5 mL solution of heparin (1 μg/mL) and VEGF (1 μg/mL). Adenovirus vectors containing GFP, dn-Akt or dn-GSK-3β were added to Matrigel at 2×10^8 plaque-forming units/500 μL. Virus-laden Matrigel (500 μL) was then injected s.c. into the flank of C57BL6 mice. The flank was then treated with radiation thrice at 3 Gy of superficial X-rays each day over 72 hours. After treatment, FITC-dextran was injected by tail vein and mice were euthanized 30 minutes later. After this, Matrigel plugs were excised and the FITC dextran–containing endothelium within the Matrigel plug was imaged

by fluorescence microscopy. Capillaries were counted from three mice and presented as a percentage of the control (GFP). Student's test was used to analyze statistical difference.

***In vivo* assay of microvascular response using skin fold window.** The dorsal skin fold window is a 3 g plastic frame applied to the skin of the C57BL6 mouse before tumor implantation and remains attached for the duration of the study as we have previously described (3, 4). The chamber was screwed together, whereas the epidermis was incised and remained open with a plastic covering. The midline was found along the back, and a clip was placed to hold the skin in position. The epidermis of the four flaps was then removed using a scalpel with an effort to follow the hypodermis superior to the fascia. The bottom portion of the chamber was put in place, and the top was carefully positioned on the cut side so that the window and the circular incision were fitted. Animals were placed under a heating lamp for several days. Microvasculature within the window was monitored by microscopy. Blood vessels developed in the dorsal skin fold window within 1 week of implantation of Lewis lung carcinoma (LLC) cells. Microvascular windows were treated with 3 Gy of superficial X-rays using 80 kVp (Pantak X-ray Generator, East Haven, CT). Five mice were studied in each of the treatment groups. ALX-349 (25 mg/kg) was injected i.p. 30 minutes before irradiation. The window frame was marked with coordinates, which were used to photograph the same microscopic field each day. Vascular windows were photographed using a 4× objective to obtain a 40× total magnification. Color photographs were used to catalogue the appearance of blood vessels on days 0 to 7. Photographs were scanned into Photoshop software, and vascular centerlines were positioned by ImagePro Software and verified by an observer blinded to the treatment groups. Tumor blood vessels were quantified by the use of ImagePro software, which quantifies the vascular length density of blood vessels within the microscopic field. Student's test was used to analyze statistical difference.

Statistical analysis. The mean and SE of each treatment group were calculated, and variance was analyzed by Student's test. $P = 0.05$ was considered statistically significant.

Results

Akt-dependent phosphorylation of GSK-3β in irradiated endothelial cells. We have previously reported that Akt phosphorylation at Ser^{473} was increased within minutes of exposure to a low dose of ionizing radiation (5). Because GSK-3β is one of the key effectors of Akt signaling, radiation-induced Akt activation may also lead to phosphorylation of GSK-3β, specifically phosphorylation at Ser^9, which is known to be Akt-dependent (25). To study radiation-induced GSK-3β phosphorylation, endothelial cells were irradiated with 3 Gy and total protein was extracted at the indicated times after treatment (Fig. 1*A*). Western immunoblots were probed with antibodies specific for phosphorylated GSK-3β at Ser^9 and total GSK-3β. Minimal phosphorylation of GSK-3β at Ser^9 was found in untreated control cells. Increased phosphorylation was first noted at 5 minutes and maximal phosphorylation occurred at 15 minutes. To further characterize radiation-induced phosphorylation of GSK-3β at Ser^9, endothelial cells were treated with varying radiation doses. Radiation-induced phosphorylation of GSK-3β at Ser^9 was dose-dependent, requiring as little as 1 Gy. Maximal phosphorylation was observed in response to 3 Gy (Fig. 1*B*). Radiation-induced phosphorylation of GSK-3β was also associated with intracellular localization of GSK-3β. The active form of GSK-3β is membrane-bound. A substantial decrease in membrane-associated GSK-3β was observed in irradiated endothelial cells at 30 minutes after treatment with 3 Gy (Fig. 1*C*).

To determine whether Akt participates in the phosphorylation of GSK-3β at Ser^9 following irradiation, the dominant negative double mutant Akt was overexpressed in HUVEC. Total protein was extracted from cells at the indicated time points after irradiation. Overexpression of dn-Akt abrogated radiation-induced phosphorylation of GSK-3β at Ser^9 (Fig. 1*D*). In comparison, cells transduced with control adenovirus GFP showed no interruption of radiation-induced GSK-3β phosphorylation (Fig. 1*D*).

Inhibition of Akt induces apoptosis in irradiated endothelium. We studied Akt contribution to the response of the microvasculature to ionizing irradiation by inhibiting Akt activity in endothelial cells using either transduction with dn-Akt adenovirus vector or chemical Akt inhibitor (Figs. 2 and 3). Figure 2 shows that 2% to 5% of HUVEC undergo apoptosis following treatment with dn-Akt as compared with 2% to 3% in the control cells or 4% to 6% in irradiated cells. However, when cells transduced with dn-Akt were irradiated, they show markedly increased amounts of apoptotic cells of 11% to 25%, which corresponds to 3- to 5-fold increase as compared with treatment

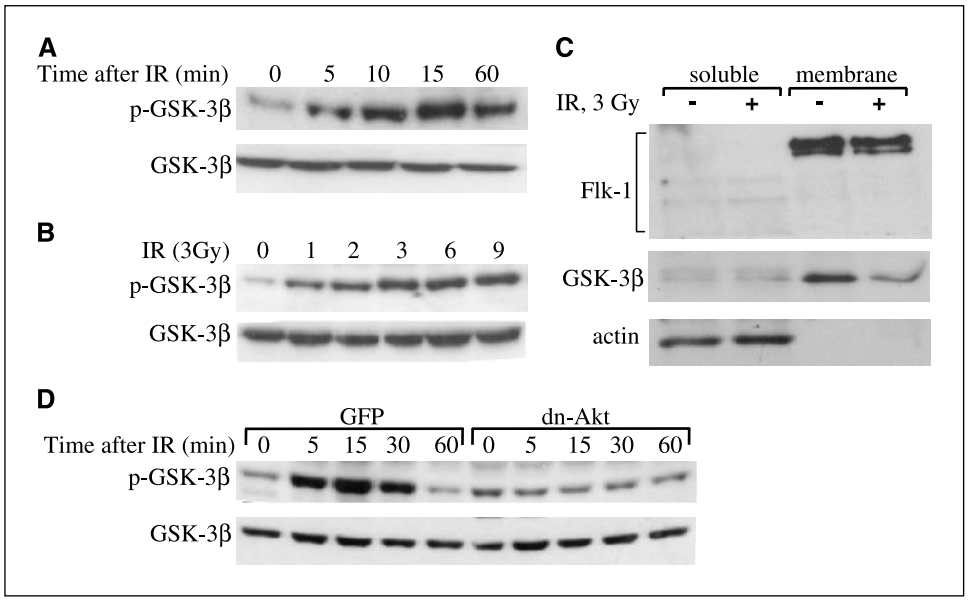

Figure 1. Phosphorylation of GSK-3β in irradiated endothelial cells is Akt-dependent. HUVEC were treated as indicated and total cell lysate (*A, B,* and *D*) or subcellular fractions (*C*) were extracted. Samples (20 μg of protein) were subjected to Western blot analysis with antibodies specific for phosphorylated GSK-3β at Ser^9 and total GSK3β. *A,* time course of GSK-3β phosphorylation at Ser^9 in response to irradiation with 3 Gy. *B,* dose response of GSK-3β phosphorylation at Ser^9 in irradiated HUVEC harvested 30 minutes after treatment. *C,* dissociation of GSK-3β from membrane in irradiated HUVEC harvested 30 minutes after treatment with 3 Gy. Flk-1 was used as a positive control for membrane fraction, actin was used as a positive control for soluble fraction. *D,* HUVEC were transduced with adenovirus vector expressing GFP or dn-Akt. Transduced cells were irradiated with 3 Gy 24 hours later. Akt-dependent time course of GSK-3β phosphorylation at Ser^9 in response to irradiation is shown.

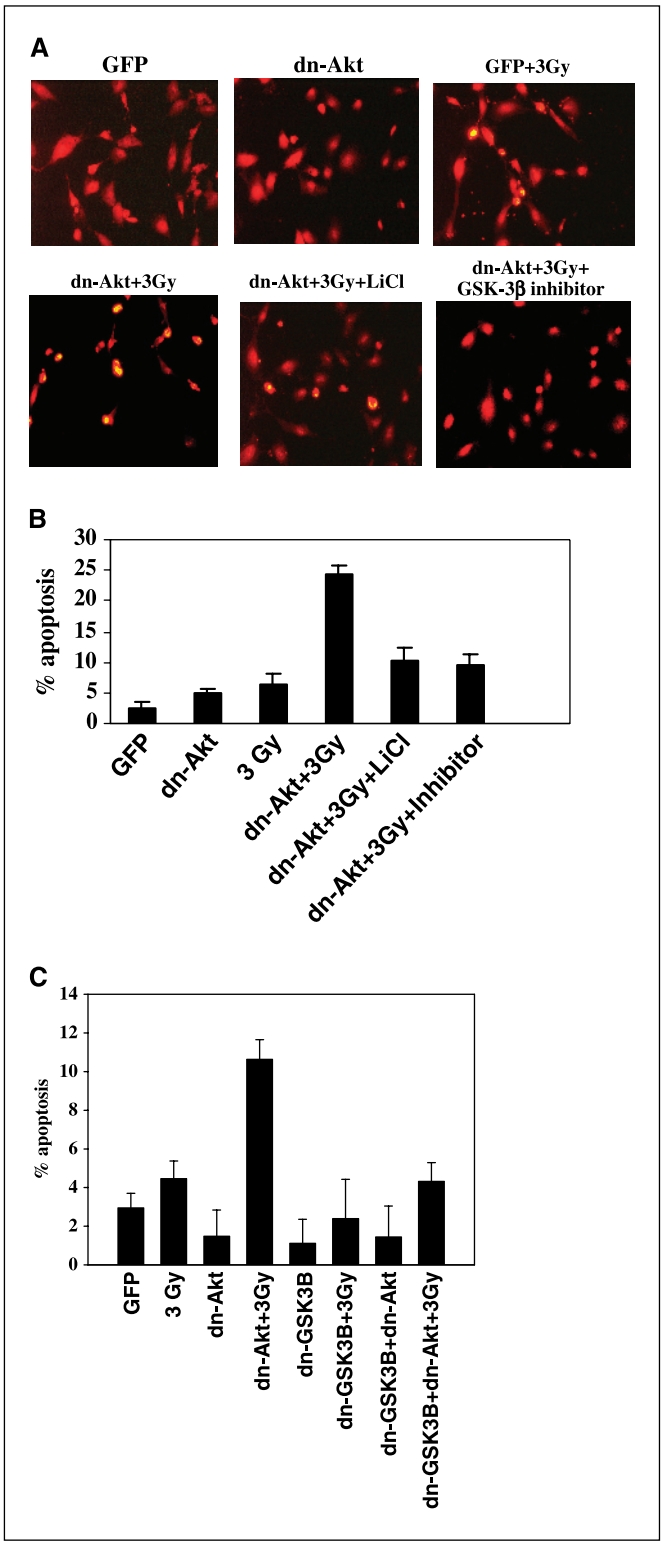

Figure 2. GSK-3β inhibition protects endothelial cells from apoptosis induced by Akt inhibition and irradiation. Akt was inhibited in HUVEC by transduction with adenovirus vector containing dn-Akt for 24 hours (*A* and *B*); GSK-3β was inhibited by using chemical GSK-3β inhibitors, 10 mmol/L LiCl for 2 hours or 10 μmol/L GSK-3β inhibitor III for 1 hour (*A* and *B*) or by transduction with adenovirus vector containing dn-GSK-3β for 24 hours (*C*). Cells were then sham-irradiated or irradiated with 3 Gy as indicated, and stained with propidium iodide. Microscopic photographs of PI-stained cells (*A*) and bar graphs of the corresponding average percentage of apoptotic cells with SE from three experiments (*B* and *C*).

with dn-Akt or radiation alone ($P < 0.001$; Fig. 2*B* and *C*). To confirm these findings, Western blot analysis of caspase-3 cleavage was used. Apoptosis-dependent activation of caspase-3 requires lower molecular weight protein, cleaved caspase-3. Figure 3*A* and *C*

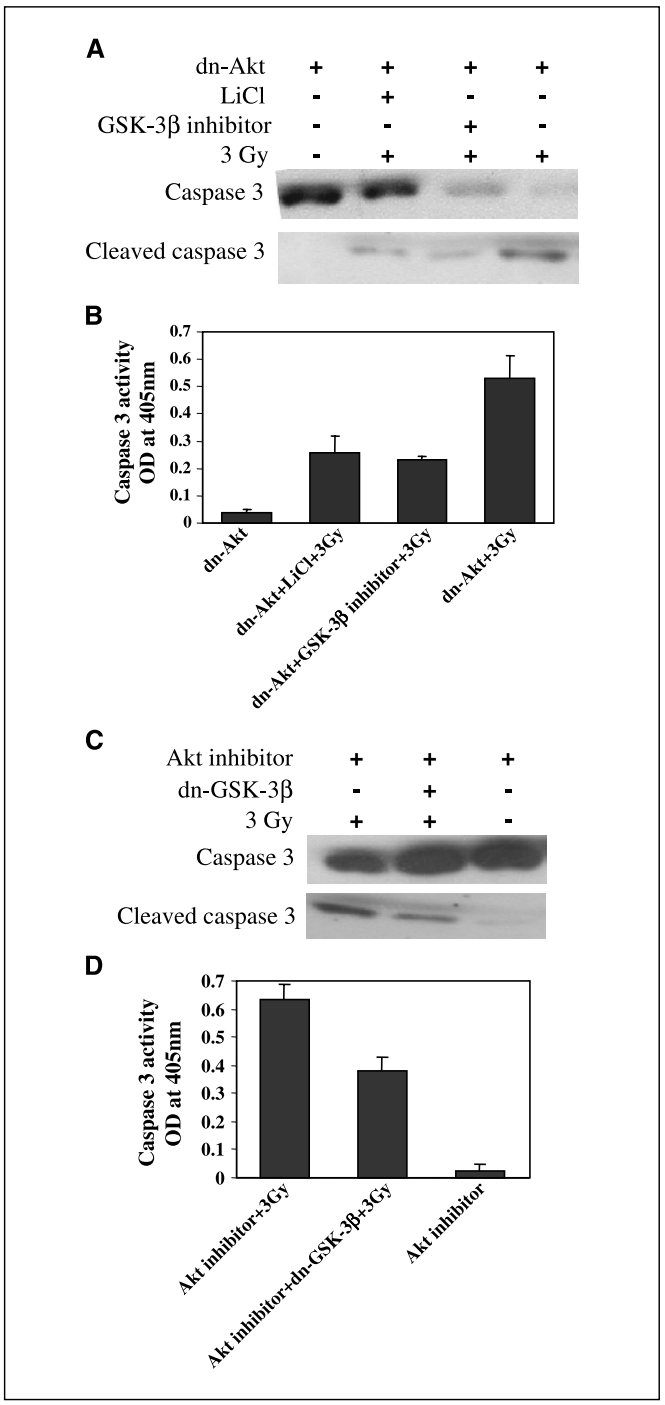

Figure 3. GSK-3β inhibition prevents caspase cleavage induced by Akt inhibition and irradiation. HUVEC were transduced with adenoviral vectors containing GFP, dn-Akt, or dn-GSK-3β for 24 hours and/or Akt inhibitor for 1 hour and GSK-3β inhibitors (10 mmol/L LiCl for 2 hours or 10 μmol/L GSK-3β inhibitor III for 1 hour). Cells were then sham-irradiated or irradiated with 3 Gy as indicated and assayed for caspase-3 activity. Western blot analysis of caspase-3 cleavage in irradiated endothelial cells using antibodies to intact and cleaved caspase-3 (*A* and *C*) and caspase-3 enzyme activity measured by absorbance of caspase-specific substrate at 405 nm with SE from three experiments (*B* and *D*).

show that caspase-3 was cleaved in HUVEC following Akt inhibition prior to irradiation. In comparison, dn-Akt or chemical inhibition of Akt alone resulted in minimal caspase-3 cleavage in endothelial cells. Radiation-induced caspase-3 activation was verified by measuring caspase-3-specific substrate absorbance (Fig. 3*B* and *D*), which confirmed results from Western blot analysis. Again, caspase enzyme activity was markedly increased in irradiated cells with inhibited Akt, whereas Akt inhibition alone resulted in minimal activation of caspase-3 enzyme activity.

GSK-3β antagonists prevent apoptosis in endothelial cells. To determine whether inhibition GSK-3β could protect endothelial cells from programmed cell death induced by inhibition of Akt and radiation, we examined caspase-3 activity and propidium staining of the cells. Figures 2 and 3 show that inhibition of GSK-3β using either transduction with dn-GSK-3β adenovirus vector or chemical GSK-3β inhibitors (GSK-3β inhibitor III or LiCl) prevented apoptosis, which was induced by inhibition of Akt and radiation. In a separate experiment, HUVEC were cotransduced with dominant negatives to both Akt and GSK-3β (Fig. 2*C*). In this experiment, inhibition of Akt with dn-Akt increased the percentage of HUVEC in apoptosis to 12% as compared with 3% in control cells. Transduction of dn-GSK-3β in addition to dn-Akt significantly attenuated the induction of apoptosis in irradiated HUVEC to 4% as compared with 12% in irradiated cells transduced with dn-Akt only (Fig. 2*C*).

Akt regulates capillary tubule formation and cell migration in irradiated endothelium. We investigated the role of Akt in capillary tubule formation using two approaches: a mouse model with the dorsal skin fold window and capillary-like network formation in Matrigel. For the first approach, the Akt inhibitor ALX-349 was given to mice bearing tumors within the tumor vascular window. Dorsal skin fold windows were implanted with LLC tumors and tumor blood vessels developed within the windows after 7 days (Fig. 4). Animals were randomly assigned into each of four treatment groups including untreated control, Akt inhibitor ALX-349 alone, 3 Gy alone, or ALX-349 prior to 3 Gy. Figure 4*B* shows that radiation alone or ALX-349 alone had little effect on established blood vessels (92% and 81% of control, respectively). However, mice treated with ALX-349 prior to irradiation had significant vascular regression to 26% of control as compared with ALX-349 alone or radiation alone ($P = 0.04$).

In a separate experiment, we used dn-Akt- or GFP-transduced HUVEC plated onto Matrigel (Fig. 5*A* and *B*). HUVEC transduced with GFP attached to Matrigel when plated, and formed capillary-like structures within 24 hours following irradiation. HUVEC containing dn-Akt showed a reduction in the number of capillary-like tubules up to 70% as compared with that of GFP-transduced cells (Fig. 5*B*). However, irradiated HUVEC overexpressing dn-Akt showed a more pronounced decrease of 10-fold (10% of control) in a number of capillary-like tubules, whereas irradiated cells transduced with GFP did not show any significant reduction in tubule formation.

We also investigated the role of Akt in endothelial cell migration by use of the Boyden chamber cell migration assay (Fig. 5*C*). Irradiated HUVEC showed a slight increase in cell migration (~ 15% of control), whereas inhibition of Akt using dn-Akt adenovirus resulted in a significant decrease in endothelial cell migration of up to 40% of the cells transduced with GFP adenovirus. However, inhibition of Akt followed by radiation maximally abolished HUVEC migration leaving only 10% of the cells capable of moving (Fig. 5*C*).

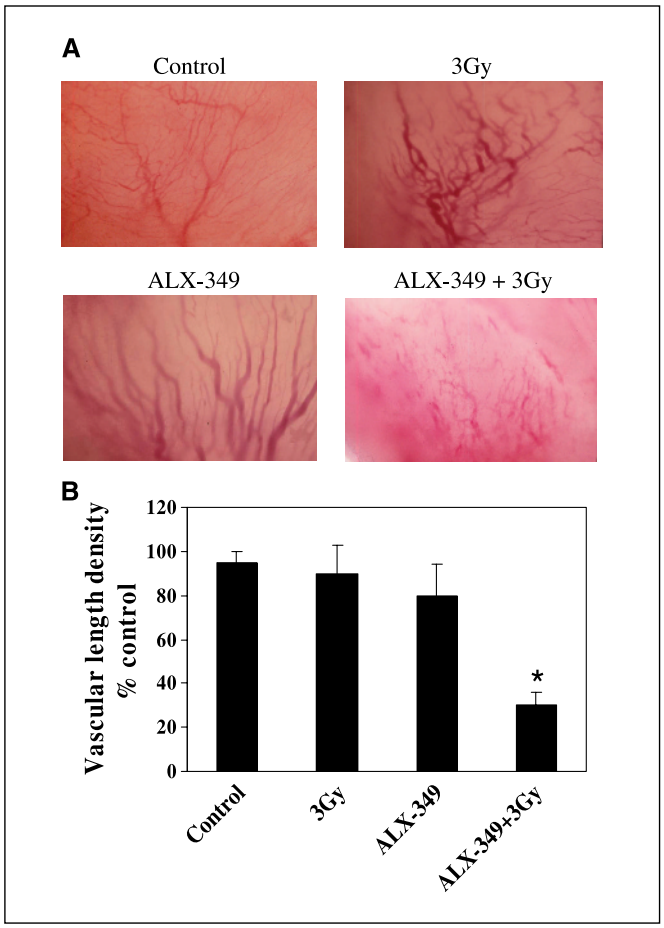

Figure 4. Akt inhibition decreases *in vivo* tubule formation. Tumor angiogenesis was monitored by microscopy using mouse dorsal skin fold window implanted with LLC tumors. Tumor blood vessels developed within the model over the course of 1 week after tumor implantation. Mice were randomly assigned to four treatment groups that included untreated control, Akt inhibitor ALX-349 alone, 3 Gy alone, and ALX-349 plus 3 Gy. The tumor vascular window was treated with 3 Gy of superficial X-rays. Vascular length density of tumor blood vessels was measured by use of Image Pro software analysis of digital images. Microscopic photographs of microvasculature obtained at 48 hours after the indicated therapies (*A*). *Columns*, mean of corresponding percentage change in vascular length density from three mice entered into each of the treatment groups; *bars*, SE; *, $P < 0.05$ (*B*).

GSK-3β inhibition improves endothelial function. To verify the role of GSK-3β signaling during *in vivo* vascular response, we studied VEGF-induced capillary formation in the irradiated mouse within Matrigel implanted s.c. into the mouse flank. FITC-dextran was injected into the tail vein to visualize capillaries within the Matrigel plug using fluorescent microscopy (Fig. 6). VEGF-induced capillary formation could be observed within Matrigel in the mouse flank (Fig. 6*A*). In the irradiated mouse, microvasculature formation was not affected. Inhibition of Akt activity by using dn-Akt alone decreased capillary formation by 50%, whereas combined with irradiation, it entirely eliminated capillary-like tubule formation (Fig. 6*B*). But additional inhibition of GSK-3β using dn-GSK-3β reversed the effect of Akt inhibition on capillary formation in irradiated mouse (Fig. 6*B*).

Discussion

PI3K/Akt signaling participates in angiogenesis following VEGF stimulation of endothelial cells and regulates capillary-like tubule

formation (14). Akt can also be activated by radiation in the absence of growth factor (3, 44). This observation led to the present investigation to determine whether the biological effects of radiation are mediated through Akt signaling and its downstream target, GSK-3β. To study the involvement of Akt, we inhibited Akt activity either with chemical inhibitors or with overexpression of the dominant negative mutant. Inhibition of Akt alone did not significantly affect the survival of vascular endothelial cells, whereas combined irradiation and inhibition of Akt sensitized these cells to radiation-induced apoptosis. In addition, overexpression of the dn-Akt prevented capillary tubule formation and endothelial cell migration. Similarly, Akt antagonists enhanced radiation-induced destruction of the microvasculature within the dorsal skin fold window model. Taken together, these findings show that Akt signaling contributes to the regulation of endothelial cell functions and survival in response to X-irradiation.

GSK-3β is one of the established downstream targets of Akt (29). In the present study, we found that GSK-3β is phosphorylated at Ser^9 in response to irradiation, which has been shown to inactivate GSK-3β. Overexpression of dn-Akt showed that radiation-induced GSK-3β phosphorylation is Akt-dependent. In addition to phosphorylation at Ser^9, irradiation of endothelial cells led to a substantial reduction of membrane-bound GSK-3β, which is associated with the inactivation of enzyme activity. Taken together, these findings show for the first time that ionizing radiation induces inhibitory phosphorylation of GSK-3β within the vascular endothelium. These findings also show that radiation-induced GSK-3β phosphorylation is dependent on Akt activity. These data are consistent with the premise that the PI3K/Akt signaling pathway is a mediator of GSK-3β phosphorylation in response to irradiation and suggests a biological role for GSK-3β in irradiated endothelial cells.

GSK-3β is involved in diverse cellular processes, including glycogen synthesis, proliferation, apoptosis, and development (32). Overexpression of dn-GSK-3β can protect cells from death induced by deprivation of growth factors or serum starvation of neurons (20). GSK-3β inhibitors such as OTDZT and lithium could rescue neurons from trophic deprivation (38). Furthermore, overexpression of constitutively active GSK-3β can induce neuronal cell death (34). To further investigate GSK-3β in regulating the viability of irradiated endothelial cells, we used the dn-GSK-3β or GSK-3β chemical inhibitors, GSK-3β inhibitor III or LiCl, to block GSK-3β-mediated signal transduction. Although LiCl has nonspecific effects (17, 45), overexpression of dn-GSK3β is a specific means to inhibit this signaling pathway (24). The present study shows that GSK-3β inhibition attenuates endothelial cell death that is induced by combined Akt antagonists and irradiation. This role of GSK-3β in regulating cell death in endothelial cells is consistent with the reports about other cell types (20, 38).

GSK-3β signal transduction was shown to inhibit the migration of endothelial cells and block angiogenesis in the Matrigel plug assay (44). Furthermore, inhibition of GSK-3β signaling enhanced capillary formation (29). Radiation stimulates endothelial cell colonization of Matrigel implanted into mice, where they formed capillary-like structures (40). Previously, we and other investigators have shown increased blood flow following low-dose irradiation (3–5). This increase in blood flow is primarily due to vasodilation within the tumor microvasculature, as shown in Fig. 4. The present study analyzes vascular length density in the tumor vascular window. Low-dose radiation has a minimal effect on microvascular

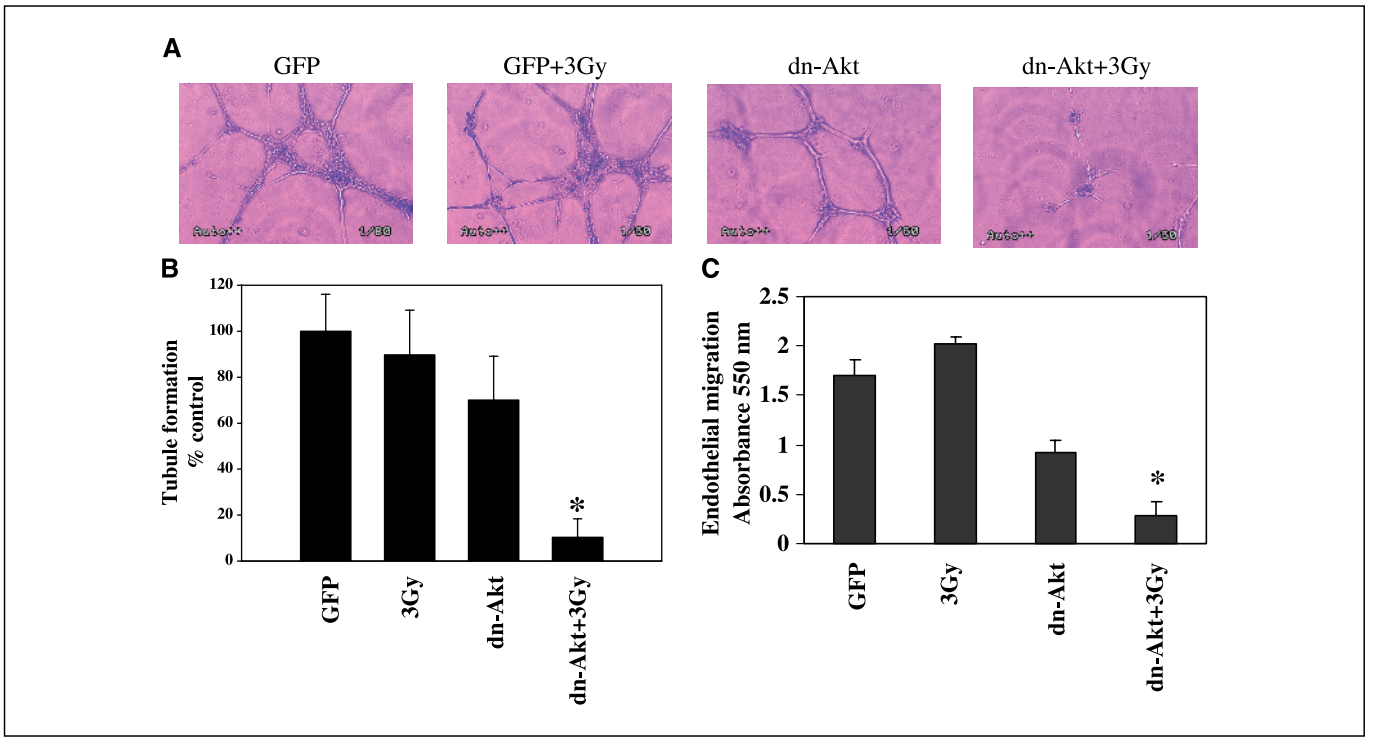

Figure 5. Akt inhibition decreases capillary tubule formation and migration in irradiated endothelial cells. HUVEC were transduced with GFP or dn-Akt adenoviruses and treated with 3 or 0 Gy 24 hours later. *A* and *B,* for evaluation of capillary tubule formation, cells were immediately cultured onto Matrigel, and tubules were counted under microscopy 24 hours later. Capillary tubule formation (*A*) and amount of formed tubules presented as a percentage of controls in the bar graph with SE of three experiments; *, $P < 0.01$ (*B*). *C,* endothelial cell migration was studied in fibronectin-coated Boyden chambers at 37°C for 6 hours. The amount of migrated cells was detected by measuring the absorbance of cell dissociation buffer at 550 nm. *Columns*, mean from three experiments; *bars*, SE.

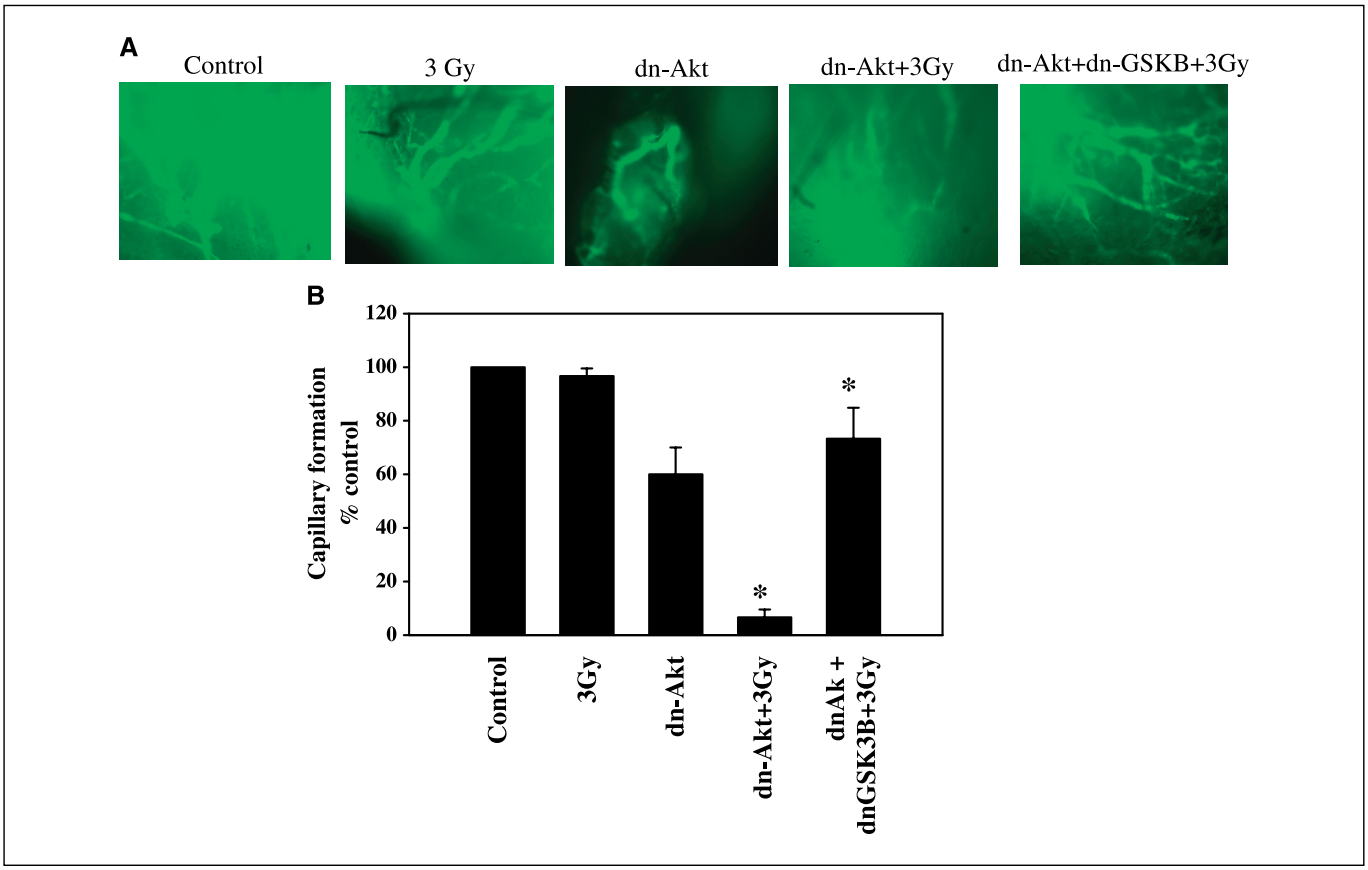

Figure 6. GSK-3β inhibition improves endothelial function. Matrigel containing VEGF, heparin, and adenoviral vectors (dn-AKT and dn-GSK-3β) were implanted s.c. in the flanks of mice. Flanks were then treated with radiation thrice at 3 Gy each day >72 hours. After FITC-labeled dextran injection by tail vein, Matrigel plugs were excised and imaged by fluorescence microscopy. Shown are fluorescence microscopy images of capillary formation following different treatments: untreated control, 3 Gy alone, dn-Akt alone, dn-Akt + 3 Gy, and dn-Akt + dn-GSK-3β + 3 Gy (*A*). *Columns*, percentage of change in capillary formation from three experiments; *bars*, ±SE; **P* < 0.01 (*B*).

length in the tumor vascular window (Fig. 4). GSK-3β signal transduction was shown to inhibit the migration of endothelial cells and block angiogenesis in the Matrigel plug assay (44). Furthermore, inhibition of GSK-3β signaling enhanced capillary formation (29). In the present study, the contribution of GSK-3β activity in the radiation response in endothelium was verified by use of the dn-GSK-3β within Matrigel placed s.c. into the flank of mouse.

The PI3K/Akt signaling pathway plays a central role in the survival of growth factor–dependent endothelial cells during angiogenesis (9). Because inhibition of PI3K/Akt activity enhances radiation-induced tumor vascular destruction, PI3K/Akt inhibitors have recently gained significant attention (3–5, 46). For example, overexpression of dominant negative Akt leads to selective induction of apoptosis in tumor cells. On other hand, dominant negative Akt has a minimal effect on normal cells (43). These results are supported by other studies using RNAi technique to inactivate Akt (47). These results indicate that GSK-3β plays a key role in controlling endothelial cell survival and angiogenesis, similar to the roles previously defined for PI3K and Akt, and suggest that the response of the microvasculature to radiation can be modified by regulating the activity of PI3K/Akt/GSK-3β signaling pathway. Considering that the microvascular response to ionizing radiation plays a predominant role in the response of normal tissues and tumors to radiotherapy, signal transduction through Akt/GSK-3β provides molecular targets for the augmentation of cancer therapy.

The clinical implications of the findings are that specific inhibitors of Akt enhance the therapeutic effects of low-dose radiation and that the radiosensitization requires GSK-3β signaling. Furthermore, the results implicate GSK-3β inhibitors as radioprotectors. Previous studies have shown that high-dose irradiation is required for the induction of apoptosis in tumor endothelium (1, 2). The present study shows that specific inhibitors of Akt, which lead to activation of GSK-3β, reduce the threshold dose that is required for the induction of apoptosis in the vascular endothelium. We found that doses as low as 2 or 3 Gy induced apoptosis in the presence of Akt antagonists. Akt is a molecular target for drug development. Conversely, inhibitors of GSK-3β could serve as radioprotectors of normal tissues. Lithium, in particular, is being studied as a radioprotector in phase I clinical trials.

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