

Growth Factor-Independent Activation of Protein Kinase B Contributes to the Inherent Resistance of Vascular Endothelium to Radiation-Induced Apoptotic Response

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ABSTRACT

The phosphatidylinositol 3'-kinase (PI3K)/protein kinase B (Akt) signal transduction pathway plays a critical role in mediating endothelial cell survival during oxidative stress. The role of the PI3K/Akt pathway in promoting cell viability was studied in vascular endothelial cells treated with ionizing radiation. Western blot analysis showed that Akt was phosphorylated rapidly in response to radiation in primary culture human umbilical vein endothelial cells in the absence of serum or growth factors. Akt phosphorylation occurred after doses as low as 1 Gy. PI3K consists of p85 and p110 subunits, which play a central role in Akt activation in response to exogenous stimuli. A mutation within the Src homology region 2 domain of mutant p85 (Δ p85) prevented radiation-induced Akt phosphorylation, when overexpressed in endothelial cells. Vascular endothelial cells transduced with control vector were resistant to radiation-induced apoptosis, whereas endothelial cell transduction with adenovirus encoding the mutated p85 (Ad. Δ p85) reversed this resistance to apoptosis after treatment with intermediate radiation doses (2–6 Gy). Δ p85 overexpression alone had no effect on the viability or apoptosis of endothelial cells. However, irradiated endothelial cells overexpressing Δ p85 released cytochrome *c* into the cytosol fraction and activated proteolytic cleavage of caspases 3 and 9, thereby inducing the apoptotic response. Inhibition of caspase 3 blocked endothelial apoptosis induced by overexpression of Δ p85 and radiation. These findings suggest that growth factor-independent activation of Akt contributes, in part, to the inherent resistance of irradiated vascular endothelium to the activation of apoptotic response.

INTRODUCTION

Ionizing radiation is used to ablate aberrant or pathological vasculature in the setting of arteriovenous malformation, carotid artery restenosis, and neoplastic diseases. The vascular endothelium, however, does not demonstrate an apoptotic response to low-dose irradiation (1, 2), thereby necessitating high-dose irradiation for ablative therapy. Cytotoxic effects of radiotherapy can be enhanced by inhibition of cell viability signaling pathways such as receptor tyrosine kinases (1). We studied downstream signaling from receptor tyrosine kinases to determine the mechanisms of radioresistance in vascular endothelium.

Growth factors reduce cellular apoptosis through PI3K⁴/Akt signal transduction (3). Apoptotic signaling pathways in endothelial cells are attenuated through a variety of mechanisms. Growth factors attenuate programmed cell death in the vascular endothelium through PI3K/Akt-dependent inhibition of mitochondrial release of factors such as

cytochrome *c* (4, 5). Cytochrome *c* is sequestered in mitochondria through the Bcl gene family of proteins. Akt phosphorylation substrates include Bid and Bad members of the Bcl family of proteins (5). These proteins, in turn, close mitochondrial channels to sequester proteins that participate in the apoptotic cascade.

PI3K is composed of a heterodimer of a M_r 85,000 (p85) adaptor subunit and a M_r 100,000 (p110) catalytic subunit (6, 7). Mutant p85 (Δ p85) contains two SH2 domains that bind to tyrosine-phosphorylated receptors after stimulation of cells with growth factors and, in this manner, recruit p110 into the complex at the cell membrane (8). Mutation of the SH2 domain of the Δ p85 of PI3K prevents recruitment of the p110 component and thereby prevents PI3K-dependent signal transduction. Amino acid sequence analysis of p85 showed that the protein contains two SH2 domains, separated by the iSH2, and one SH3 domain. It has been established that iSH2 domains are involved in interactions with the p110 subunit (9). The dominant negative mutant of the M_r 85,000 subunit of PI3K (p85) lacks the binding site (iSH2) for the catalytic M_r 110,000 subunit (10). Δ p85 eliminates the link between p110 and receptor tyrosine kinases, thereby negating PI3K function. Downstream signaling of PI3K, through Akt, is attenuated in endothelial cells transduced with the Ad. Δ p85 (an adenovirus encoding the mutated p85) genetic construct. In the present study, we confirmed our previous conclusion that Akt activation by radiation is dependent on PI3K by transfecting endothelial cells with an adenoviral vector containing the dominant negative Δ p85.

Akt-mediated phosphorylation of caspases inactivates these enzymes that are required for apoptosis. Two caspase activation pathways have been well elucidated; one of these centers on tumor necrosis factor family of receptors, which activate caspase 8 to initiate apoptosis. The other caspase-dependent pathway involves the participation of mitochondria, which in turn release cytochrome *c* into the cytosol, thereby triggering caspase 9 activation (5). In the present study, cytochrome *c* release and caspase activation were studied in irradiated vascular endothelial cells. Inactivation of this apoptotic cascade after exposure to ionizing radiation was PI3K dependent.

MATERIALS AND METHODS

Cell Culture and Adenoviral Infection. HUVECs, obtained from Clonetics (San Diego, CA), were maintained in endothelial basal medium 2 supplemented with EGM-2 MV Singlequots (BioWhittaker). Only fourth or fifth passage cells were used. For adenoviral infection, 5×10^5 cells in 60-cm plates were treated with adenovirus vector at 10–100 plaque-forming units/cell as described by Kotani *et al.* (11). More than 90% of the HUVECs were infected at a similar multiplicity of infection with Ad.GFP (adenovirus encoding the GFP). Infected cells were subjected to further treatment 24 h later. An Eldorado 8 Teletherapy ⁶⁰Co Unit (Atomic Energy of Canada Ltd.) was used to irradiate endothelial cell cultures at a dose rate of 0.84 Gy/min. Radiation dose was verified by use of thermoluminescence detectors.

Antibodies and Other Reagents. A mutant regulatory subunit of p85 (Ad. Δ p85) was kindly provided by W. Ogawa (Kobe University School of Medicine, Kobe, Japan; Ref. 11). Antibodies to phospho-Ser-473 Akt, total Akt, caspases 3 and 9, and caspase 9-cleaved fragment were obtained from New England Biolabs (Beverly, MA). Antibodies to cytochrome *c* and PARP were obtained from Oncogene Research Products (Boston, MA). Antibodies to

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⁴ The abbreviations used are: PI3K, phosphatidylinositol 3'-kinase; SH, Src homology domain; iSH2, inter-SH2; HUVEC, human umbilical vein endothelial cell; GFP, green fluorescent protein; PARP, poly(ADP-ribose) polymerase.

cytochrome *c* oxidase subunit IV (CytoC4) were obtained from CLONTECH (Palo Alto, CA), and antibodies to actin and tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Apoptosis Assays. Cells were then assayed for apoptosis, caspase 3, and PARP cleavage by Western immunoblot. Apoptosis assays used propidium iodide staining of nuclei. One hundred cells were counted by an observer who was not aware of the experimental conditions for each of the cultures. Apoptotic nuclei were counted, and the percentage of cells undergoing apoptosis was quantified in three separate experiments. The mean and SE were calculated. Caspase 3 inhibitor IV was obtained from Oncogene Research Products. Caspase inhibitor was added to the cells at a concentration of 100 μ M for 60 min before irradiation. Statistical difference of $P < 0.05$, as measured by Student's *t* test, was considered statistically significant.

Cell Lysis and Immunoblot Analysis. HUVECs were treated at the indicated times and washed twice with PBS, and lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM Na PP₁, 1 mM phenylmethylsulfonyl fluoride, and 1 μ g/ml leupeptin) was added. Protein concentration was quantified by the Bio-Rad method. Twenty μ g of total protein were loaded into each well and separated by 8% or 12% SDS-PAGE gel, depending on the size of the target protein being investigated. The proteins were transferred onto nitrocellulose membranes (Hybond enhanced chemiluminescence; Amersham, Arlington Heights, IL) and probed with antibodies for phosphorylated Akt, total Akt, caspase 3, caspase 9, cytochrome *c*, or PARP.

Mitochondria-Enriched Assays. Cells were lysed in hypotonic fractionation buffer mix from CLONTECH, incubated on ice for 10 min, and homogenized in an ice-cold Dounce tissue grinder from Fisher Scientific. Cell homogenates were transferred to a 1.5-ml microcentrifuge tube and centrifuged at 700 $\times g$ for 10 min. Proteins within the supernatant are cytosolic fractions, and proteins within the pellet are mitochondrial fractions. Five to 10 μ g of each cytosolic and mitochondrial fraction were separated on a 12% SDS-PAGE. PAGE gels were transferred to Western blots and probed with cytochrome *c* antibody at 1:100 dilution in blocking buffer.

RESULTS

Vascular endothelial cell viability is enhanced by growth factor stimulation and subsequent Akt activation (12). To determine whether the vascular endothelial growth factor receptor contributes to Akt phosphorylation in irradiated endothelial cells, the Flk1-specific inhibitor, SU5416, was added to the HUVECs 30 min before irradiation. Fig. 1 shows that SU5416 does not eliminate Akt phosphorylation in response to 3 Gy. Furthermore, serum deprivation eliminated baseline Akt phosphorylation but did not prevent radiation-induced Akt phosphorylation. To characterize the time-dependent phosphorylation of Akt in irradiated HUVECs, total protein was extracted at 5, 15, 30, 60, and 120 min. Fig. 1 shows the autoradiograph of Western immuno-

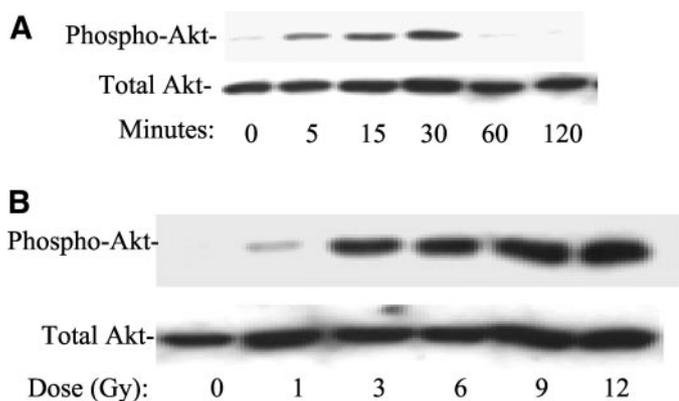


Fig. 1. Dose- and time-dependent Akt phosphorylation in irradiated HUVECs. *A*, cells were treated with 3 Gy, and total protein was extracted at the indicated time points after irradiation. *B*, cells were treated with the indicated doses, and protein was extracted at 30 min after irradiation. Shown are autoradiographs of Western immunoblots using antibodies to total Akt and phosphorylated Akt.

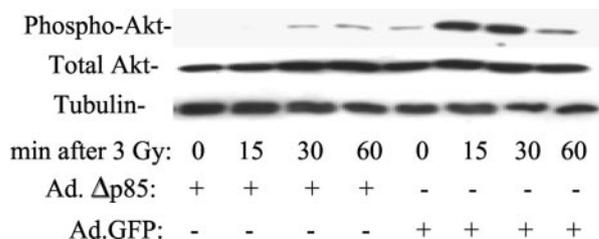


Fig. 2. HUVECs transduced with Ad. Δ p85 attenuate radiation-induced Akt phosphorylation. HUVECs were transduced with Ad.GFP or Ad. Δ p85 at 10 plaque-forming units/cell. Greater than 90% of cells were transduced with GFP at this multiplicity of infection. HUVECs were irradiated at 24 h after transduction. Transduced HUVECs were irradiated with 3 Gy, and total protein was extracted at 30 min after irradiation. Shown are autoradiographs of Western immunoblots using antibodies to total Akt and phosphorylated Akt.

blots using phospho-specific Akt antibody as compared with an antibody that binds both the phosphorylated and unphosphorylated Akt (total Akt). Akt phosphorylation was first noted at 5 min and increased progressively at 15 and 30 min. In comparison, there was no change in total Akt protein. Akt phosphorylation was transient and resolved at 120 min (Fig. 1A).

Dose-dependent Akt phosphorylation in irradiated HUVECs was studied by extraction of total protein at 30 min after irradiation with doses ranging from 1 to 12 Gy. Autoradiographs of Western immunoblots show a dose-dependent Akt phosphorylation with a small increase in response to 1 Gy and a plateau after doses of 3 Gy or more (Fig. 1B).

Growth factor-induced Akt phosphorylation requires the p85 component of PI3K, which binds directly to receptor tyrosine kinases through the SH2 domain (11). Δ p85 was overexpressed as a PI3K dominant negative genetic construct to determine whether radiation-induced Akt phosphorylation is PI3K dependent. Δ p85 was transduced into HUVECs by use of the adenovirus vector Ad. Δ p85. HUVECs transduced with Ad.GFP gene were used to control for the effects of adenovirus transduction. HUVECs were transduced with Ad. Δ p85 or Ad.GFP at a ratio of 10 plaque-forming units/cell, which showed a transduction efficiency of approximately 90%. Transduced cells were irradiated, and total cellular protein was extracted at 0, 15, 30, and 60 min after irradiation. Autoradiographs of Western immunoblots using antibodies specific for total Akt or phosphorylated Akt are shown in Fig. 2. Cells transduced with Ad.GFP showed no attenuation of radiation-induced Akt phosphorylation as compared with radiation alone. In comparison, HUVECs transduced with Ad. Δ p85 showed attenuated Akt phosphorylation after irradiation.

The PI3K/Akt pathway enhances cell viability by inhibiting apoptotic machinery. To determine whether mutation within the SH2 domain of the p85 component of PI3K alters cell viability after irradiation, HUVECs were transduced with Ad. Δ p85 at 24 h before irradiation. Fig. 3 shows photographs of fluorescence microscopy of propidium iodide-stained cells at 6 h after irradiation. HUVECs transduced with Δ p85 showed chromatin condensation and nuclear changes consistent with apoptosis. Apoptosis was quantified by counting the percentage of cells with apoptotic nuclei. Control HUVECs transduced with Ad.GFP showed 2% of cells with apoptotic nuclei (Fig. 3B). Either radiation or Ad. Δ p85 alone produced 9% and 8% of cells with apoptotic nuclei, respectively. Irradiated HUVECs transduced with Ad. Δ p85 showed 22% of cells with apoptotic nuclei, a value that was increased significantly as compared with either radiation or Ad. Δ p85 alone ($P < 0.001$).

Apoptosis is preceded by cytochrome *c* release from mitochondria (13). To determine whether cytochrome *c* is released from the mitochondria after irradiation, the subcellular localization of cytochrome *c*

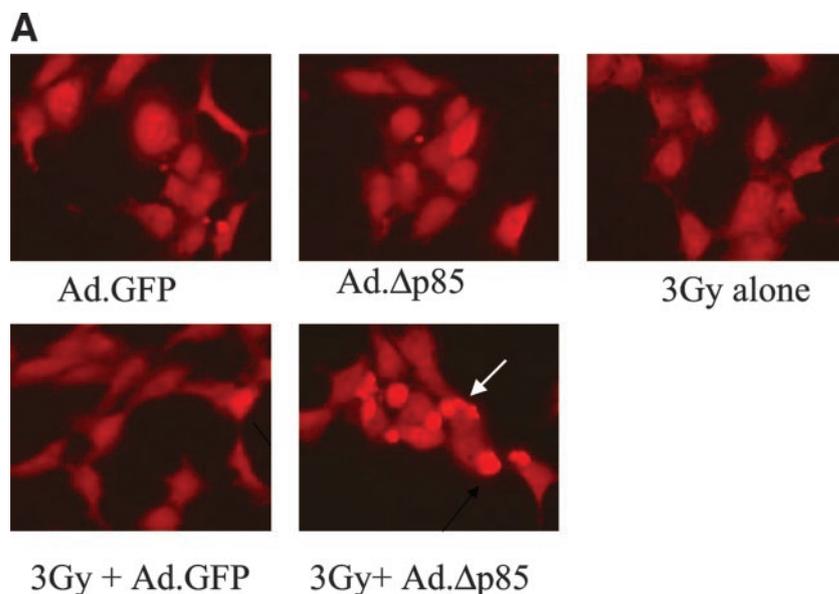
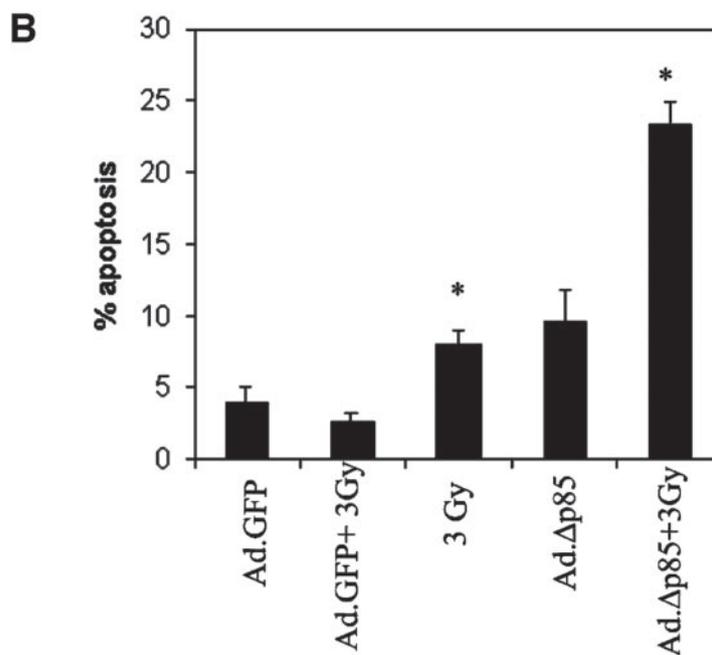


Fig. 3. Overexpression of $\Delta p85$ enhances radiation-induced apoptosis in HUVECs. HUVECs were transduced with adenovirus vectors Ad.GFP or Ad.p85. Cells were irradiated with 0 or 3 Gy at 24 h after transduction. Cells were fixed and stained with propidium iodide 6 h later. A, shown are photographs of fluorescence microscopy to image nuclei of apoptotic cells. *Arrow* indicates apoptotic nucleus. B, bar graph showing the mean and SE of the percentage of cells with apoptotic nuclei for each experimental group (*, $P < 0.001$).



was assessed. Cells were lysed in hypotonic buffer, and protein within the cytosolic and mitochondrial fractions was separated by centrifugation. Fig. 4 shows autoradiographs of Western immunoblots using antibody specific for cytochrome *c*. Immunoblots using antibodies specific for cytochrome *c* oxidase 4 and actin were used as controls. Western blot analysis revealed that mitochondrial cytochrome *c* decreased, whereas cytochrome *c* in the cytosolic fraction increased in HUVECs treated with radiation and Ad.Δp85. Cytochrome *c* remained within the mitochondria after treatment with either Ad.Δp85 alone or radiation alone (Fig. 3).

Cytochrome *c* activates the apoptotic cascade, resulting in cleavage of caspase 9 (13, 14). To characterize the mechanisms by which Ad.Δp85 regulates apoptosis in irradiated endothelial cells, we studied caspases 3 and 9. HUVECs were transduced with Ad.GFP or Ad.Δp85 for 24 h, followed by irradiation. Total protein was extracted at 6 h after irradiation. Fig. 3 shows autoradiographs of Western immunoblots using antibodies specific for caspases 3 and 9. After serum

starvation, each of the caspases was cleaved to form proteolytic products. Caspases 3 and 9 were not cleaved after treatment with either Ad.Δp85 alone or radiation alone. HUVECs transduced with Ad.Δp85 showed cleavage of caspases 3 and 9 at 6 h after irradiation (Fig. 4).

On activation, caspase 3 cleaves numerous proteins involved in cell structure, signaling, and repair and is essential for DNA fragmentation. PARP is one of the downstream substrates for caspase 3 (14). To determine whether caspase 3 activation is essential for apoptosis induced by radiation and mutant Ad.Δp85, HUVECs were treated with caspase 3 inhibitor IV. PARP cleavage and subsequent apoptosis were studied in Western immunoblots using anti-PARP antibodies (Fig. 5). After treatment with radiation and Ad.Δp85, PARP was cleaved within endothelial cells, whereas caspase 3 inhibitor IV prevented PARP cleavage. Caspase 3 inhibitor IV also prevented apoptosis induced by radiation in Ad.Δp85-transduced endothelial cells (Fig. 5B). Untreated control and Ad.GFP-transduced cells each had

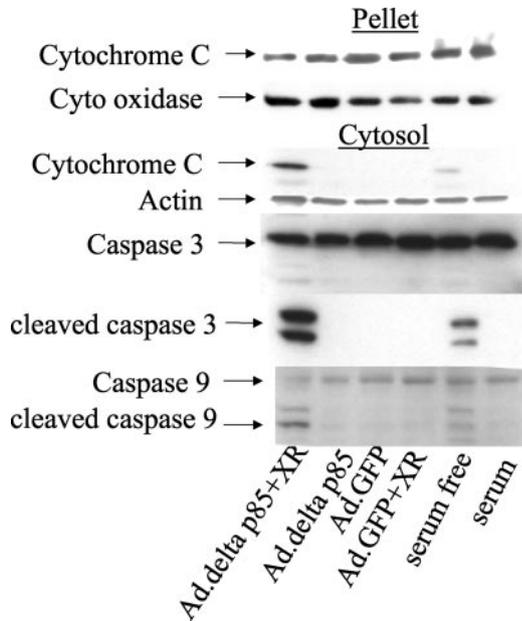


Fig. 4. Cytochrome *c* released from mitochondria induced by mutant p85 and radiation. HUVECs were transduced with Ad.Δp83, and the Ad.GFP gene insert was used as a control. Transduced cells were irradiated with 0 or 3 Gy. Cells were lysed in hypotonic buffer, and cytochrome *c* in the cytosolic and mitochondrial fraction was measured by Western immunoblot using cytochrome *c*-specific antibody. Shown are autoradiographs of Western immunoblots using antibody to cytochrome *c* oxidase 4 and actin as controls. Cleavage of caspases 3 and 9 in response to Ad.Δp85 and radiation was examined. HUVECs were transduced with Ad.Δp85 and irradiated. Total cellular protein was extracted 6 h after irradiation. Protein lysates (20 μg) from each treatment condition were separated by PAGE and transferred for Western immunoblot. Shown are autoradiographs of Western blots using antibodies specific for caspases 3 and 9. Arrows indicate proteolytic cleavage products for caspases 3 and 9.

3% apoptotic cells. Cells treated with 3 Gy alone or Ad.GFP + 3 Gy had 6% and 7% apoptotic cells, respectively. Cells treated with Ad.Δp85 + 3 Gy showed 21% apoptotic cells. Caspase 3 inhibitor IV added to cells treated with Ad.Δp85 + 3 Gy reduced the percentage of apoptotic cells to 3% ($P = 0.043$). Caspase inhibitor did not significantly attenuate apoptosis in other treatment groups.

DISCUSSION

The resilience of vascular endothelium to cytotoxic effects of ionizing radiation can be overcome by overexpression of the Δp85 component of PI3K. This dominant negative construct inhibited radiation-induced Akt phosphorylation and subsequently enhanced radiation-induced apoptosis within vascular endothelial cells. We found a radiation dose- and time-dependent activation of Akt phosphorylation in endothelial cells. PI3K inhibition resulted in enhancement of endothelial apoptosis and diminished viability. Taken together, these data indicate that the PI3K participates in radiation-induced phosphorylation of Akt and subsequent enhancement of cell viability.

Signal transduction through PI3K/Akt has been demonstrated to enhance cancer cell viability after X-irradiation (15–18). In particular, the PI3K inhibitor LY294002 has been shown to radiosensitize human bladder cancer, breast cancer, and head and neck cancer cell lines (15–17). In the present study, we explored the role of PI3K and radiation-induced Akt phosphorylation. The PI3K/Akt signal transduction pathway has been shown to enhance endothelial cell viability (2). We found that dominant negative constructs of PI3K attenuate radiation-induced activation of Akt and in turn allow apoptosis to proceed in response to X-irradiation.

Akt-mediated attenuation of programmed cell death occurs through several independent mechanisms. One such mechanism involves the

Bcl gene family members, which are phosphorylated directly by Akt (19). Bcl-2 and its homologue, Bcl-XL, encode membrane-associated proteins that protect cells from DNA damage-induced apoptosis (20). Studies of Akt phosphorylation are associated with phosphorylation of Bad protein, which can enhance viability of small cell lung cancer cells (18). In contrast, bax expression is induced by radiation and promotes cell death (21). Akt-enhanced cell survival occurs through maintenance of mitochondrial membrane potential. Induction of apoptosis after inactivation of Akt correlated with the disruption of mitochondrial membrane integrity and cytochrome *c* release. Activation of Akt alone was sufficient to promote cell survival in the hematopoietic stem cells (22). We found that the number of apoptotic bodies within HUVECs increased dramatically when treated with the PI3K dominant negative genetic construct and radiation together as compared with treatment with radiation or the PI3K dominant negative alone.

In a cell-free system, radiation induces the loss of mitochondrial membrane potential, opening of the permeability transition pore, and release of cytochrome *c* (23). The cell-free system lacks PI3K/Akt signaling, which is required for cell viability after X-irradiation. The present study found that a PI3K dominant negative construct promotes

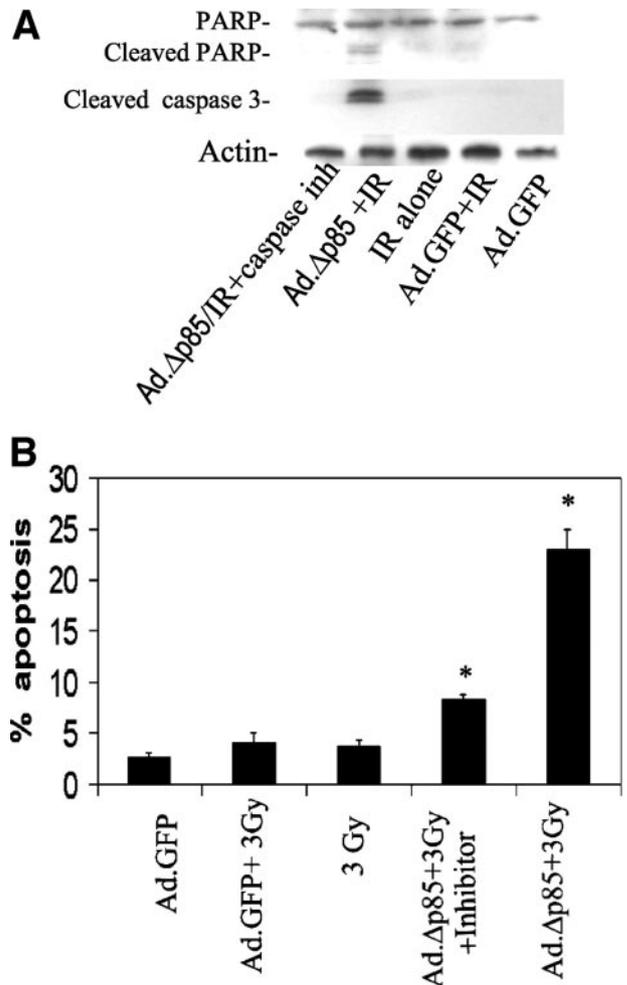


Fig. 5. Attenuation of apoptosis in HUVECs using caspase 3 inhibitor. Caspase inhibitor IV was added to HUVEC cultures before irradiation. HUVECs were lysed 6 h after irradiation, and protein extracts were separated by SDS-PAGE. A, shown are autoradiographs of Western immunoblots using antibodies to caspase 3 and PARP. B, apoptosis in HUVECs. HUVECs were transduced with adenovirus vectors Ad.GFP or Ad.Δp85. Cells were irradiated with 0 or 3 Gy at 24 h after transduction. Caspase inhibitor IV was added to cells. Cells were fixed and stained with propidium iodide 6 h later. B, bar graph shows the percentage and SE of cells with apoptotic nuclei (*, $P = 0.043$).

apoptosis within irradiated HUVECs. Cytochrome *c* was released into the cytosol only after irradiation of cells overexpressing $\Delta p85$. Cytochrome *c* release into cytosol interacts directly with Apaf-1 within the cytoplasm, leading to the ATP-dependent formation of the apoptosome. This complex recruits and activates the protease caspase 9 (24). Caspase 3 has been shown to be indispensable for apoptosis induced by numerous cytotoxic stimuli (25). For that reason, we used caspase 3 inhibitor IV to determine that caspase 3 is essential for apoptosis induced within HUVECs treated with Ad. $\Delta p85$ and radiation. Taken together, these findings indicate that interruption of signal transduction through PI3K/Akt allows apoptosis to proceed in irradiated endothelial cells.

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