

# Bcl-2 Protects Endothelial Cells against $\gamma$ -Radiation via a Raf-MEK-ERK-Survivin Signaling Pathway That Is Independent of Cytochrome *c* Release

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## Abstract

The Bcl-2 oncoprotein is a potent inhibitor of apoptosis and is overexpressed in a wide variety of malignancies. Until recently, it was generally accepted that Bcl-2 primarily mediates its antiapoptotic function by regulating cytochrome *c* release from mitochondria. However, more recent studies have shown that Bcl-2 is present on several intracellular membranes and mitochondria may not be the only site where Bcl-2 exercises its survival function. In this study, we investigated if Bcl-2 can protect endothelial cells against  $\gamma$ -radiation by a cytochrome *c*-independent signaling pathway. Human dermal microvascular endothelial cells (HDMEC), when exposed to  $\gamma$ -radiation, exhibited a time-dependent activation of caspase-3 that was associated with increased cytochrome *c* release from mitochondria. Bcl-2 expression in endothelial cells (HDMEC-Bcl-2) significantly inhibited irradiation-induced caspase-3 activation. However, Bcl-2-mediated inhibition of caspase-3 was significantly reversed by inhibition of the Raf-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)-ERK pathway. Interestingly, caspase-3 activation in HDMEC-Bcl-2 cells was not associated with cytochrome *c* release. We also observed that endothelial cell Bcl-2 expression significantly increased the expression of survivin and murine double minute-2 (Mdm2) via the Raf-MEK-ERK pathway. Endothelial cells expressing Bcl-2 also inhibited  $\gamma$ -radiation-induced activation of p38 MAPK and p53 accumulation. Inhibition of p53 accumulation in HDMEC-Bcl-2 could be due to the enhanced expression of Mdm2 in these cells. Taken together, these results show three mechanisms by which Bcl-2 may mediate endothelial cell cytoprotection independently of cytochrome *c* release: (a) increased survivin expression, (b) inhibition of p53 accumulation, and (c) inhibition of p38 MAPK. [Cancer Res 2007;67(3):1193–202]

## Introduction

The Bcl-2 family of proteins are key regulators of apoptosis (1). The Bcl-2 family is subdivided into two classes, antiapoptotic members, such as Bcl-2 and Bcl-x<sub>L</sub>, and proapoptotic members,

Bax and Bak (2). Previous studies have focused mainly on the antiapoptotic functions of Bcl-2 at the mitochondrial level because it was assumed that Bcl-2 and Bcl-x<sub>L</sub> function mainly within mitochondria (3). However, it is now known that only Bcl-x<sub>L</sub> has a signal sequence that specifically targets it to the mitochondrial outer membrane whereas Bcl-2 is distributed on several intracellular membranes including plasma membrane, nuclear membrane, and endoplasmic reticulum (4). Artificial targeting of Bcl-2 to the endoplasmic reticulum membrane in Madin-Darby canine kidney cells and Rat-1/myc cells by exchanging the Bcl-2 COOH-terminal insertion sequence for an equivalent sequence from cytochrome *b<sub>5</sub>*, an endoplasmic reticulum protein, still protects the cells from apoptosis (5). In contrast, specific targeting of Bcl-2 to the mitochondrial membrane by exchanging the Bcl-2 COOH-terminal insertion sequence for an equivalent sequence from monoamine oxidase B, a mitochondrial membrane protein, converts it into a proapoptotic molecule (6). These studies suggest that mitochondria may not be the preferred site for executing the survival function of Bcl-2, and the inhibition of cytochrome *c* release might not be the predominant mechanism of its antiapoptotic function.

Although Bcl-2 has been found to inhibit cell death induced by a wide variety of apoptotic signals in many cell types, the mechanism underlying its protective effects still remains unclear. Bcl-2 has been shown to bind to a multifunctional chaperone protein BAG-1 (7). In turn, BAG-1 binds to and activates the protein kinase Raf-1 (8). Raf-1 kinase plays a central role in the conserved Ras-Raf-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)-ERK pathway, acting to relay signals from activated Ras proteins via MEK1/2 to the ERK1/2 (9). ERK can phosphorylate a number of downstream targets including Rsk, Elk1, cFos, and transcription factors (10), leading to the enhanced expression of a number of proteins including survivin (11). Survivin, a member of the inhibitor of apoptosis family, has been shown to inhibit activation of downstream effectors of apoptosis, caspase-3 and caspase-7, in cells exposed to apoptotic stimuli (12). Elevated levels of survivin have been observed in a number of human cancers (13, 14) and enhanced expression of survivin is associated with more aggressive forms of cancer with poor survival rates (15).

Activation of the Raf-MEK-ERK survival pathway has also been shown to regulate the tumor suppressor protein p53 by regulating the transcription of both murine double minute-2 (Mdm2) and its inhibitor p19ARF (16). In normal unstressed cells, p53 is an unstable protein with a half-life of <20 min and is kept at very low cellular levels because of its continuous degradation by Mdm2 (17). These studies suggest that a Raf-MEK-ERK survival signaling cascade can indirectly regulate p53 accumulation by targeting it for Mdm2-mediated degradation.

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In this study, we sought to determine if Bcl-2 can also mediate its antiapoptotic function by activating a prosurvival signaling cascade independent of cytochrome *c* release. Here, we report that Bcl-2, by activating the prosurvival Raf-MEK-ERK-survivin signaling cascade, protects endothelial cells against  $\gamma$ -radiation-induced apoptosis that is independent of Bcl-2-mediated inhibition of cytochrome *c* release. In addition, endothelial cells expressing Bcl-2 also inhibited  $\gamma$ -radiation-induced p53 accumulation, due in part to the enhanced expression of Mdm2.

## Materials and Methods

**Cells and reagents.** Human dermal microvascular endothelial cells (HDMEC), endothelial cell growth medium-2, Mito Tracker Red, and 4',6-diamidino-2-phenylindole (DAPI) nucleic acid stain were purchased from Cambrex (Walkersville, MD). A squamous cell carcinoma line (UM-SCC-74B), generously provided by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI), was cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Raf-1 kinase inhibitor (BAY 43-9006), MAPK inhibitor (U0126), and p38 MAPK inhibitor (SB202190) were obtained from Calbiochem (San Diego, CA). Rabbit polyclonal anti-phospho-c-Raf-1, rabbit polyclonal anti-c-Raf-1, rabbit polyclonal anti-phospho-MEK1/2, rabbit monoclonal anti-MEK1/2, rabbit polyclonal anti-phospho-ERK1/2, rabbit polyclonal anti-ERK1/2, rabbit polyclonal anti-phospho-p38 MAPK, rabbit polyclonal anti-p38 MAPK, mouse monoclonal anti-caspase-3, rabbit monoclonal anti-cleaved caspase-3, and monoclonal anti-cytochrome *c* antibodies were purchased from Cell Signaling (Beverly, MA). Mouse monoclonal anti-Bcl-2 antibodies and mouse monoclonal antisurvivin antibodies were purchased from Biosource International (Camarillo, CA). Mouse monoclonal anti-cytochrome *c* (clone 7H8.2C12), mouse monoclonal anti-cytochrome *c* (clone 6H2.B4), and anti-cleaved caspase-3 phycoerythrin were purchased from BD PharMingen (San Diego, CA). Mouse monoclonal anti-p53 and rabbit polyclonal anti-Mdm2 antibodies were obtained from Lab Vision (Fremont, CA). Transfection reagents and Western blotting reagents were purchased from Invitrogen.

**Retroviral vector construction and HDMEC transduction.** The generation of HDMEC-Bcl-2 and HDMEC-LXSN was done as previously described (18). The Bcl-2 construct or the vector alone was introduced into PA317 amphotropic packing cells with Lipofectin. Viral supernatants were collected after 24 h, centrifuged, filtered, and stored at  $-70^{\circ}\text{C}$ . HDMECs were transduced with either Bcl-2 (HDMEC-Bcl-2) or control vector (HDMEC-VC) by overnight incubation with 1:10 dilution of the viral supernatant in the presence of 4  $\mu\text{g}/\text{mL}$  polybrene. Endothelial cell growth medium supplemented with 400  $\mu\text{g}/\text{mL}$  G418 was used to select the resistant clones. Bcl-2 expression was confirmed by Northern and Western blot analyses.

**Transient transfection of HDMEC with small interfering RNA.** HDMECs were transfected with small interfering RNA (siRNA) for Raf-1, ERK1, survivin, and p38 MAPK using Signal Silence siRNA kits from Cell Signaling according to the manufacturer's instructions. In brief, HDMECs ( $2 \times 10^5$ ) were cultured in 60-mm dishes and transfected with 50 nmol/L siRNA for Raf-1, ERK1, survivin, p38 MAPK, or control siRNA (fluorescein conjugated). After 18 h of incubation, HDMECs were rinsed with HBSS and further incubated in fresh medium. Forty-eight hours posttransfection, cells were treated with  $\gamma$ -radiation and processed for cytochrome *c* and caspase-3 analysis or terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis. Transfection efficiency was calculated by counting the HDMECs transfected with fluorescein-conjugated control siRNA and was found to be  $>80\%$ .

**Immunohistochemistry.** Human head and neck squamous cell carcinoma tissue microarrays (TMA) were stained with Bcl-2 and factor VIII to analyze the expression of Bcl-2 in tumor microvasculature. At least three tissue cores (0.6-mm diameter) from the tumor tissues from each case or adjacent normal control tissues were sampled. TMAs were deparaffinized and antigen retrieval was done by incubating the slides at  $92^{\circ}\text{C}$  for

20 min in target retrieval solution (DAKO, Carpinteria, CA). Nonspecific sites in the TMAs were blocked by incubating in nonserum protein block solution (DAKO) for 10 min at room temperature and then the TMAs were incubated with mouse anti-human Bcl-2 antibody (ready to use, DAKO) and rabbit anti-human factor VIII antibody (1:60; DAKO) for 1 h at room temperature. The sections were washed with PBS and incubated further with antimouse FITC (1:50; Sigma, St. Louis, MO) and antirabbit rhodamine (1:50; Sigma) for 30 min at room temperature. Slides were washed and mounted in aqua mount (Polyscience, Inc., Warrington, PA). Separate pictures of each tissue samples for factor VIII and Bcl-2 were taken and then superimposed to quantify the double positive vessels.

**Western blot analysis.** HDMECs ( $2 \times 10^5$ ) were plated in 60-mm dishes containing a thin layer of type I collagen gel and cultured overnight in endothelial cell growth medium-2 (19). HDMECs were further incubated in serum-free medium for additional 2 or 24 h for signal transduction or survivin expression studies, respectively. At the end of each time period, supernatants were aspirated and whole-cell lysates were prepared in cell lysis buffer (100 mmol/L Tris, pH 7.4) containing 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 0.5% deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 100 mmol/L sodium orthovanadate, and protein inhibitor cocktails (Sigma). Thirty micrograms of each sample were separated by 4% to 12% NuPAGE Bis-Tris gel (Invitrogen) and transferred onto nitrocellulose membranes using NuPAGE transfer buffer (Invitrogen). To block nonspecific binding, membranes were incubated with 5% nonfat milk in TBS containing 0.1% Tween 20 (TBST) for 1 h at room temperature. Afterwards, the blots were incubated in the respective primary antibody in TBST + 5% nonfat milk at  $4^{\circ}\text{C}$  overnight. After washing with TBST, the blots were incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (IgG; 1:10,000) or with goat anti-rabbit IgG (1:10,000) for 1 h at room temperature. An enhanced chemiluminescence-plus detection system (Amersham Life Sciences, Piscataway, NJ) was used to detect specific protein bands. Protein loading in all the experiments was normalized by stripping the blots and then reprobing with antitubulin or respective pan antibody.

**Analysis of cytochrome *c* release and caspase-3 activation by flow cytometry.** Bcl-2 expression in HDMECs was induced either by treatment with vascular endothelial growth factor (VEGF; 50 ng/mL) for 96 h or by retroviral transduction. Cells were treated with siRNA (Raf-1, ERK1, and p38 MAPK) for 48 h or pretreated with different inhibitors for 1 h and then exposed to  $\gamma$ -radiation (15 Gy). Twenty-four hours after irradiation, cells were harvested, fixed in 4% paraformaldehyde for 15 min at  $4^{\circ}\text{C}$ , and stored overnight in 70% ethanol at  $-20^{\circ}\text{C}$ . We have used flow cytometry technique to analyze cytochrome *c* release and caspase-3 activation as previously reported by Stahnke et al. (20). Cells were washed with PBS supplemented with 1% bovine serum albumin (BSA) and 0.1 sodium azide and treated with 1  $\mu\text{g}/\text{mL}$  digitonin for 2 min on ice. Cells were then incubated with 5  $\mu\text{g}/\text{mL}$  mouse IgG to block nonspecific binding. Anti-cytochrome *c* (clone 7H8.2C12, BD PharMingen) and anti-cleaved caspase-3 phycoerythrin (BD PharMingen) antibodies were added for 20 min at room temperature. For cytochrome *c* staining, the cells were treated with goat anti-mouse IgG-FITC for 20 min at room temperature. After fixation with 4% paraformaldehyde, the cells were analyzed on a flow cytometer (BD Biosciences, San Jose, CA).

**Analysis of cytochrome *c* release by immunohistochemistry.** HDMECs were cultured on Lab-Tech chambers and pretreated with different inhibitors for 1 h and then exposed to  $\gamma$ -radiation (15 Gy). Twenty-four hours after irradiation, cells were incubated with Mito Tracker Red stain (100 nmol/L) for 30 min, washed, fixed in 4% paraformaldehyde for 15 min at  $4^{\circ}\text{C}$ , and stored overnight in 70% ethanol at  $-20^{\circ}\text{C}$ . Cells were washed with PBS supplemented with 1% BSA and 0.1 sodium azide and treated with 1  $\mu\text{g}/\text{mL}$  digitonin for 2 min on ice. Cells were then incubated with 5  $\mu\text{g}/\text{mL}$  mouse IgG to block nonspecific binding. Cells were stained with anti-cytochrome *c* antibody (clone 6H2.B4, BD PharMingen; ref. 21) for 20 min at room temperature and further incubated with goat anti-mouse IgG-FITC for 20 min at room temperature. After fixation with 4% paraformaldehyde, nuclei were stained with DAPI and analyzed by fluorescence microscopy.

**Analysis of TUNEL-positive cells by flow cytometry.** HDMECs treated with different inhibitors or transfected with siRNAs were exposed to  $\gamma$ -radiation (15 Gy). After 72 h, cells were washed, fixed in 4% paraformaldehyde for 15 min at 4°C, and then stored overnight in 70% ethanol at -20°C. The percentage of apoptotic cells was evaluated by the APO-BrdU TUNEL assay according to the manufacturer's instructions (Sigma). Apoptotic cells were quantified by flow cytometry using an argon laser excited at 488 nm (BD Biosciences).

**Statistical analysis.** Data from all the experiments are expressed as mean  $\pm$  SE. Statistical differences were determined by two-way ANOVA and Student's *t* test.  $P < 0.05$  was considered significant.

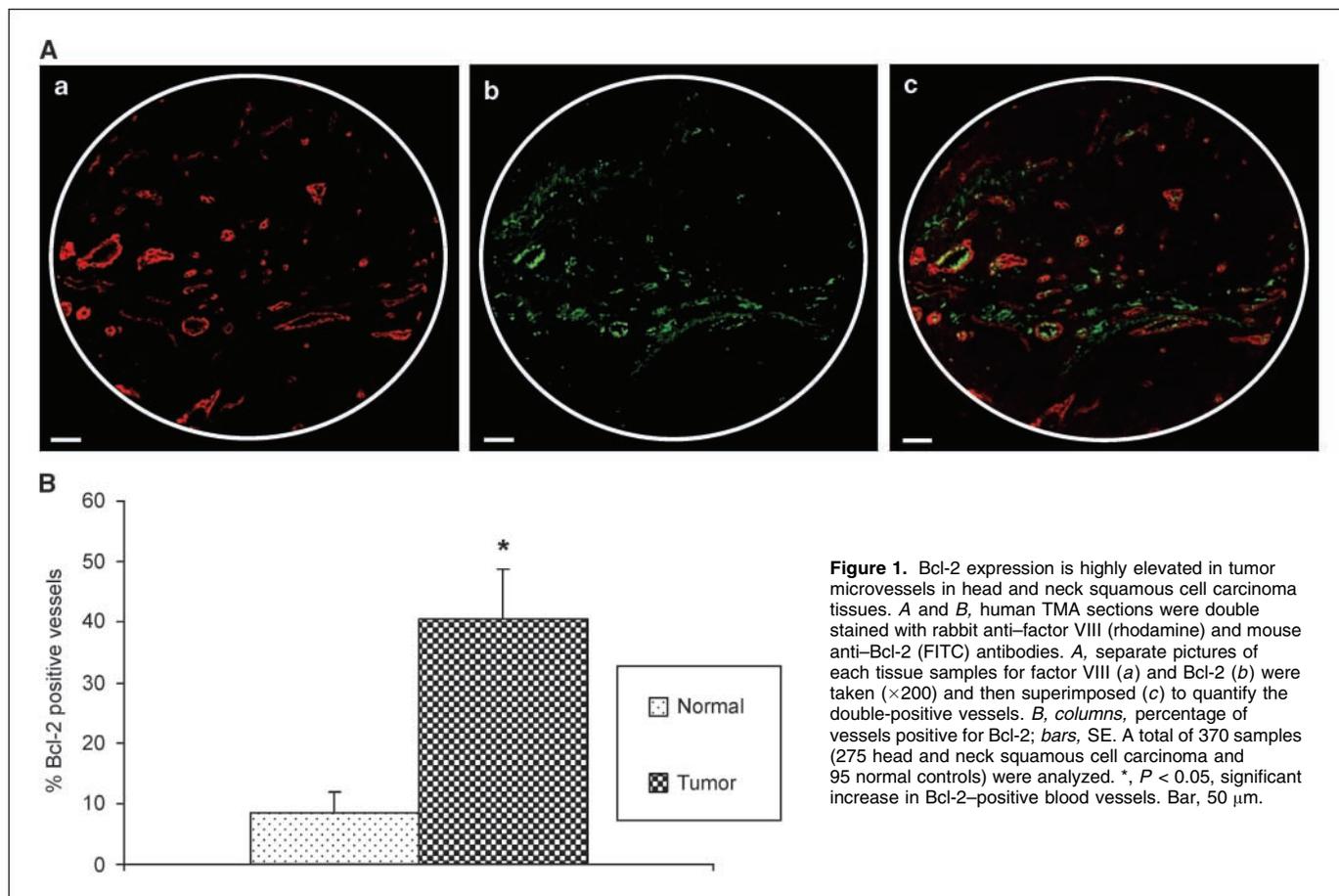
## Results

**Tumor samples from head and neck cancer patients show significantly higher Bcl-2-positive vessels.** It is well known that Bcl-2 overexpression occurs in many cancer types and is associated with enhanced chemoresistance and radioresistance (22, 23). We have previously shown that up-regulation of Bcl-2 in endothelial cells is sufficient to enhance tumor progression *in vivo* (18). However, little is known about the Bcl-2 expression profile in tumor blood vessels, particularly in head and neck squamous cell carcinoma. To examine the expression profile of Bcl-2 in tumor blood vessels, we double stained head and neck squamous cell carcinoma TMAs with factor VIII and Bcl-2. Our results suggest that Bcl-2 expression is significantly higher in tumor blood vessels as compared with normal tissue (Fig. 1). Interestingly, this Bcl-2 expression was markedly higher in tumor blood vessels as

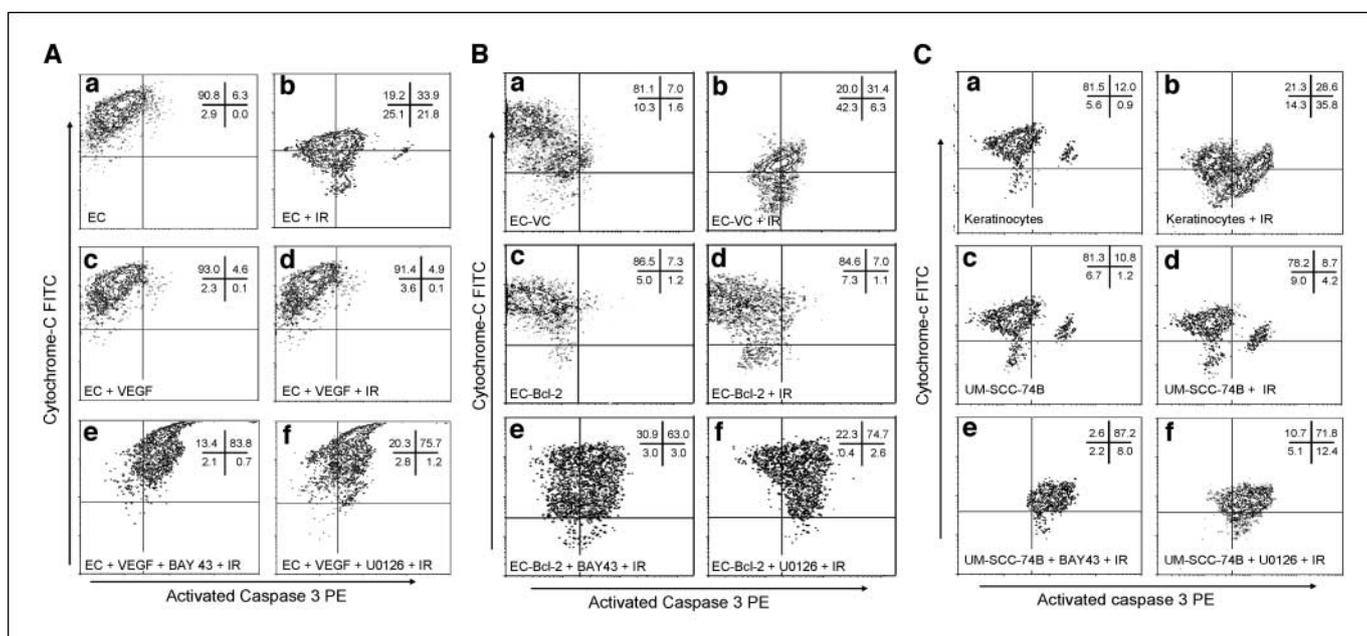
compared with tumor cells. In contrast, Bcl-xL expression was higher in tumor cells as compared with tumor vessels (data not shown).

### Bcl-2-mediated endothelial cell protection against $\gamma$ -radiation is independent of its inhibition of cytochrome *c* release.

The role of Bcl-2 in cell survival has extensively been studied and it has been assumed that Bcl-2 is a mitochondrial protein that mediates its antiapoptotic function principally by inhibiting mitochondrial cytochrome *c* release. However, recent studies have shown that Bcl-2 is distributed on several intracellular membranes including plasma membrane, nuclear membrane, and endoplasmic reticulum (4). In addition, directional targeting studies of Bcl-2 have indicated that mitochondria may not be the only site where Bcl-2 mediates protection (5). In this study, we investigated if Bcl-2 might also mediate its survival function through an alternative pathway that is independent of its role in mitochondria. To address this hypothesis, we induced the expression of Bcl-2 in endothelial cells by treating with VEGF, a key mediator of angiogenesis, for 72 h. We have previously shown that VEGF mediates endothelial cell survival by inducing the expression of Bcl-2 (19). We used a flow cytometry technique to quantitatively analyze the release of cytochrome *c* and activation of caspase-3 in the same cells as reported by Stahnke et al. (20).  $\gamma$ -Radiation treatment of HDMEC showed a significantly higher number of cells with activated caspase-3 and reduced cytochrome *c* signal as compared with nontreated cells (Fig. 2A, *a* and *b*). In contrast,  $\gamma$ -radiation treatment of HDMEC stimulated with VEGF did not induce



**Figure 1.** Bcl-2 expression is highly elevated in tumor microvessels in head and neck squamous cell carcinoma tissues. *A* and *B*, human TMA sections were double stained with rabbit anti-factor VIII (rhodamine) and mouse anti-Bcl-2 (FITC) antibodies. *A*, separate pictures of each tissue samples for factor VIII (*a*) and Bcl-2 (*b*) were taken ( $\times 200$ ) and then superimposed (*c*) to quantify the double-positive vessels. *B*, columns, percentage of vessels positive for Bcl-2; bars, SE. A total of 370 samples (275 head and neck squamous cell carcinoma and 95 normal controls) were analyzed. \*,  $P < 0.05$ , significant increase in Bcl-2-positive blood vessels. Bar, 50  $\mu$ m.



**Figure 2.** Bcl-2 mediates endothelial cells protection via a signaling cascade that is independent of mitochondrial cytochrome *c* release. **A**, HDMECs were cultured in 100-mm dishes containing a thin layer of type I collagen gel and were treated with either a Raf kinase inhibitor (BAY 43-9006) or a MAPK inhibitor (U0126) for 1 h and then exposed to  $\gamma$ -radiation. After 24 h, endothelial cells (EC) were stained with anti-cytochrome *c* (FITC) and anti-active caspase-3 [phycoerythrin (PE)] antibodies. A representative contour plot from each group from three independent experiments. **a** to **f**, untreated HDMECs and HDMECs treated with  $\gamma$ -irradiation, VEGF-treated HDMECs, VEGF-treated HDMECs + irradiation, VEGF-treated HDMECs + BAY 43-9006 + irradiation, and VEGF-treated HDMECs + U0126 + irradiation, respectively. **B**, HDMECs transduced with Bcl-2 or vector alone were cultured in 100-mm dishes containing a thin layer of type I collagen gel and were treated with either BAY 43-9006 or U0126 for 1 h and then exposed to  $\gamma$ -radiation. After 24 h, cells were stained with anti-cytochrome *c* (FITC) and anti-active caspase-3 (phycoerythrin) antibodies. A representative contour plot from each group from three independent experiments. **a** to **f**, untreated HDMEC-VC, HDMEC-VC treated with irradiation, untreated HDMEC-Bcl-2 cells, HDMEC-Bcl-2 treated with irradiation, HDMEC-Bcl-2 cells treated with BAY 43-9006 + irradiation, and HDMEC-Bcl-2 treated with U0126 + irradiation, respectively. **C**, UM-SCC-74B cells or human dermal keratinocytes (control cells) were cultured in 100-mm dishes and were treated with either BAY 43-9006 or U0126 for 1 h and then exposed to  $\gamma$ -radiation. After 24 h, cells were stained with anti-cytochrome *c* (FITC) and anti-active caspase-3 (phycoerythrin) antibodies. A representative contour plot from each group from three independent experiments. **a** to **f**, untreated keratinocytes, keratinocytes treated with irradiation, untreated UM-SCC-74B cells, UM-SCC-74B cells treated with irradiation, UM-SCC-74B cells treated with BAY 43-9006 + irradiation, and UM-SCC-74B treated with U0126 + irradiation, respectively.

caspase-3 activation or cytochrome *c* release (Fig. 2A, c and d). These VEGF-treated endothelial cells were significantly protected against irradiation-induced apoptosis and also exhibited high levels of Bcl-2 expression and ERK1/2 activation as we have previously reported (19). In addition, treatment of VEGF-stimulated HDMECs with either Raf-1 inhibitor or MAPK inhibitor showed a marked increase in caspase-3 activation when exposed to  $\gamma$ -radiation. Interestingly, this increase in caspase-3 activation in Raf-1 inhibitor- or MAPK inhibitor-treated cells was not associated with cytochrome *c* release (Fig. 2A, e and f).

Because VEGF can induce the expression of a number of proteins in addition to Bcl-2, we next used a genetic approach to study the role of Bcl-2 in endothelial cell survival by transducing Bcl-2 gene in endothelial cells using a retroviral vector. HDMEC-VC cells were used as control. Similar to VEGF treatment, endothelial cells expressing Bcl-2 blocked cytochrome *c* release from the mitochondria and inhibited caspase-3 activation (Fig. 2B, c and d). In contrast, treatment of HDMEC-Bcl-2 with either Raf-1 inhibitor or MAPK inhibitor showed a marked increase in caspase-3 activation when exposed to  $\gamma$ -radiation, and this increase in caspase-3 activation in Raf-1 inhibitor- or MAPK inhibitor-treated cells was not associated with cytochrome *c* release (Fig. 2B, e and f). We next examined if cells that naturally express Bcl-2 were also resistant to  $\gamma$ -irradiation-mediated apoptosis via a similar survival mechanism. For these experiments, we used a human squamous carcinoma cell line, UM-SCC-74B. This cell line naturally expresses high levels of Bcl-2 (24).

UM-SCC-74B cells also showed a marked resistance to  $\gamma$ -irradiation treatment that was mediated by the Raf-MEK pathway (data not shown). In addition,  $\gamma$ -irradiation treatment of UM-SCC-74B cells did not induce caspase-3 activation or cytochrome *c* release (Fig. 2C, d). In contrast, control keratinocytes showed a marked increase in caspase-3 activation and this caspase-3 activation was associated with cytochrome *c* release (Fig. 2C, c). Similar to endothelial cells expressing Bcl-2, when UM-SCC-74B cells were treated with BAY 43-9006 (or U0126) and irradiated, they showed significantly higher caspase-3 activation that was not associated with cytochrome *c* release (Fig. 2C, e and f).

We next used an immunohistochemistry technique (21) to validate the cytochrome *c* release data from our flow analysis experiments. Endothelial cells expressing Bcl-2 or vector controls were cultured on Lab-Tech chambers and were treated as described in Materials and Methods. Radiation treatment of HDMEC-VC induced a change in cytochrome *c* staining from a punctuate mitochondrial profile that nicely colocalized with Mito Tracker staining in untreated cells to a cytosolic distribution (Fig. 3A, a and b). In contrast, Bcl-2 expression in endothelial cells nearly completely blocked radiation-induced cytochrome *c* release from mitochondria to cytosol (Fig. 3A, c and d). Similarly, inhibition of Raf and MAPK in HDMEC-Bcl-2 cells did not alter the cytochrome *c* distribution in these cells (Fig. 3A, e and f).

As pharmacologic inhibitors of Raf-1 (BAY 43-9006) and MAPK (U0126) are known to partially inhibit other kinases, we next used

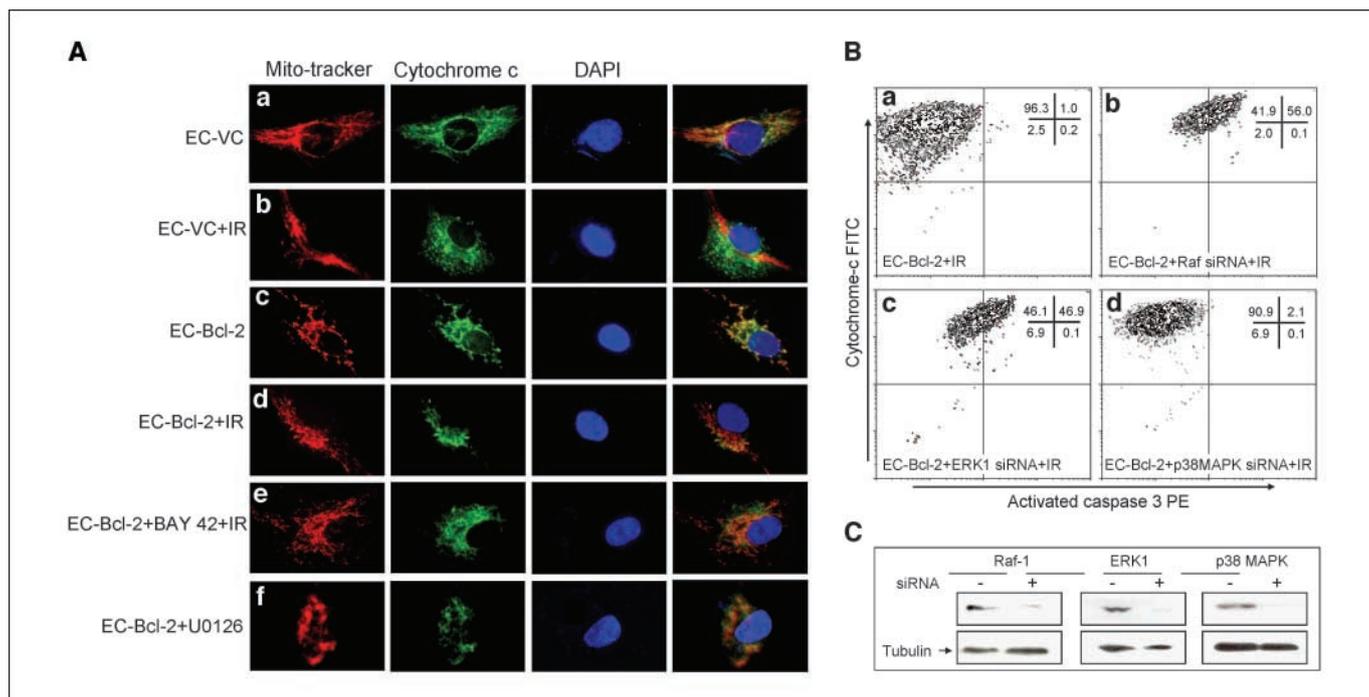
siRNA technology to knock down Raf, ERK, and p38 MAPK. In these studies, we observed >80% transfection efficiency with siRNAs and the effectiveness of these siRNAs was assessed by Western blotting at 48 h posttransfection. We observed a marked decrease in Raf-1, ERK1, and p38 MAPK protein levels with siRNA for Raf-1, ERK1, and p38 MAPK, respectively (Fig. 3C). As observed earlier with pharmacologic inhibitors, inhibition of Raf and ERK kinases with siRNAs in HDMEC-Bcl-2 showed a marked increase in caspase-3 activation independently of mitochondrial cytochrome *c* release (Fig. 3B, *b* and *c*). In contrast, inhibition of p38 MAPK by siRNA did not induce caspase-3 activation or cytochrome *c* release (Fig. 3B, *d*). These results suggest that Bcl-2, in addition to its function in mitochondria, might also be mediating its antiapoptotic function via an alternative signaling pathway that is independent of mitochondrial cytochrome *c* release.

**Bcl-2 mediates survivin up-regulation via activation of Raf-1, MEK1/2, and ERK1/2.** We next examined if Bcl-2 may be mediating endothelial cell survival by activating a signaling cascade. HDMEC-Bcl-2 and HDMEC-VC were cultured in 60-mm dishes on a thin layer of collagen and incubated in serum-free endothelial cell growth medium-2. After 2 or 16 h, cell lysates were prepared and analyzed by Western blotting. HDMECs expressing Bcl-2 showed markedly enhanced phosphorylation of Raf-1, MEK1/2, and ERK1/2 proteins as compared with vector control cells (Fig. 4A, *b* and *d*). Bcl-2 expression in endothelial

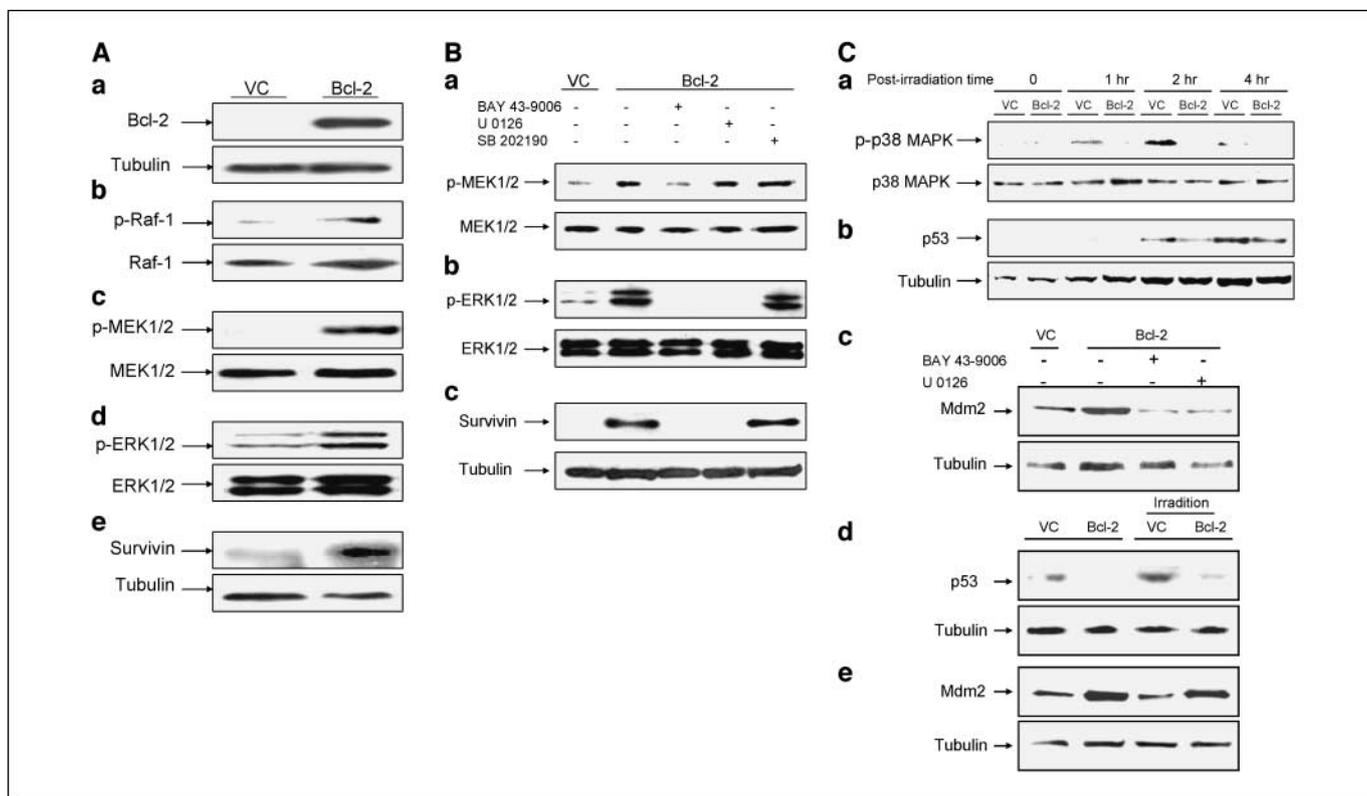
cells also led to a significant up-regulation of survivin, a key antiapoptotic protein (Fig. 4A, *e*). Similarly, VEGF-induced Bcl-2 expression in endothelial cells also led to increased phosphorylation of ERK1/2 and up-regulation of survivin levels (data not shown).

We next investigated if the Bcl-2 induced up-regulation of survivin is mediated via activation of the Raf-MEK-ERK cascade. HDMEC-Bcl-2 cells were treated with Raf-1 inhibitor (BAY 43-9006), MAPK inhibitor (U0126), or p38 MAPK inhibitor (SB203580) and cell lysates were prepared. HDMEC-Bcl-2 cells treated with Raf-1 inhibitor showed marked inhibition of MEK1/2 and ERK1/2 phosphorylation as well as survivin expression (Fig. 4B, *a-c*). Similarly, MAPK inhibitor treatment significantly inhibited ERK1/2 phosphorylation and survivin expression. In contrast, p38 MAPK inhibitor had no effect on MEK1/2 and ERK1/2 phosphorylation as well as survivin up-regulation (Fig. 4B, *b* and *c*). These results suggest that Bcl-2 mediates a signaling cascade via the Raf-MEK-ERK pathway to up-regulate the expression of survivin.

**$\gamma$ -Radiation-induced p38 MAPK activation and p53 expression are inhibited by Bcl-2.** We have previously shown that  $\gamma$ -radiation induces endothelial cell apoptosis predominantly via activation of p38 MAPK (19). In this study, we examined if Bcl-2 could inhibit  $\gamma$ -radiation-mediated p38 MAPK activation. HDMEC-VC showed a time-dependent activation of p38 MAPK



**Figure 3.** Inhibition of Raf-MAPK pathway in endothelial cells expressing Bcl-2 leads to activating of caspase-3 that is independent of cytochrome *c* release. **A**, HDMECs transduced with Bcl-2 or vector alone were cultured in Lab-Tech chambers until they were ~70% to 80% confluent. Cells were then pretreated with BAY 43-9006 or U0126 for 1 h and then exposed to irradiation. After 24 h, cells were incubated with Mito Tracker Red dye for 15 min, fixed with paraformaldehyde, and stained with anti-cytochrome *c* (FITC). Nuclei were stained with DAPI. Separate pictures of each sample for mitochondria (*red*), cytochrome *c* (*green*), and nucleus (*blue*) were taken ( $\times 1,000$ ) and then superimposed. *a* to *f*, untreated HDMEC-VC, HDMEC-VC treated with irradiation, untreated HDMEC-Bcl-2, HDMEC-Bcl-2 treated with irradiation, HDMEC-Bcl-2 treated with BAY 43-9006 + irradiation, and HDMEC-Bcl-2 treated with U0126 + irradiation, respectively. **B**, cells were transfected with siRNA for Raf-1, ERK1, and p38 MAPK using Signal Silence siRNA kits from Cell Signaling according to the manufacturer's instructions. Forty-eight hours posttransfection, cells were treated with  $\gamma$ -irradiation. After 24 h, cells were stained with anti-cytochrome *c* (FITC) and anti-active caspase-3 (phycoerythrin) antibodies. A representative contour plot from each group from three independent experiments. *a* to *d*, HDMEC-Bcl-2 treated with irradiation, HDMEC-Bcl-2 cells treated with Raf-1 siRNA + irradiation, HDMEC-Bcl-2 treated with ERK1 siRNA, and HDMEC-Bcl-2 treated with p38 MAPK siRNA + irradiation, respectively. **C**, cells were transfected with siRNA for Raf-1, ERK1, and p38 MAPK as described above and, 48 h posttransfection, cell lysates were prepared and analyzed for the expression of Raf-1, ERK1, or p38 MAPK. Immunoblots were then stripped of the primary antibody and reprobbed with antitubulin antibody.



**Figure 4.** Bcl-2 mediates survivin and Mdm2 up-regulation via activation of the Raf-MEK-ERK pathway. **A**, HDMEC-VC and HDMEC-Bcl-2 were cultured in 60-mm dishes containing a thin layer of type I collagen gel until they were ~90% confluent. HDMEC-VC and HDMEC-Bcl-2 were further cultured in serum-free endothelial cell growth medium-2 for 2 h and cell lysates were prepared. Thirty micrograms of each sample were Western blotted and probed with anti-Bcl-2, anti-phospho-Raf-1, anti-phospho-MEK1/2, anti-phospho-ERK1/2, or antisurvivin antibodies. Immunoblots were then stripped of the primary antibody and re probed with respective pan antibody or antitubulin antibody. **B**, HDMEC-VC and HDMEC-Bcl-2 were treated with specific Raf-1 inhibitor (BAY 43-9006), MAPK inhibitor (U0126), or p38 MAPK inhibitor (SB203580) for 2 h (Raf-1, MEK1/2, and ERK1/2) or 16 h (survivin) and cell lysates were prepared. Thirty micrograms of each sample were Western blotted and probed with anti-phospho-MEK1/2, anti-phospho-ERK1/2, or antisurvivin antibodies. Immunoblots were then stripped of the primary antibody and re probed with antitubulin or respective pan antibody. **C**, **a** and **b**, HDMEC-VC and HDMEC-Bcl-2 cells were exposed to  $\gamma$ -radiation (15 Gy) and at different time points postirradiation (0, 1, 2, and 4 h) and cell lysates were prepared. Thirty micrograms of each sample were Western blotted and probed with anti-phospho-p38 or anti-p53 antibodies. Immunoblots were then stripped of the primary antibody and re probed with anti-pan-p38 MAPK or antitubulin antibody. **c**, HDMEC-VC and HDMEC-Bcl-2 were pretreated with specific Raf-1 inhibitor (BAY 43-9006) or MAPK inhibitor (U0126) and incubated for 4 h. Thirty micrograms of each sample were Western blotted and probed with anti-Mdm2 antibody. Immunoblots were then stripped of the primary antibody and re probed with antitubulin antibody. **d** and **e**, HDMEC-VC and HDMEC-Bcl-2 cells were treated with a single dose of  $\gamma$ -radiation (15 Gy). At 4 h postirradiation, cell lysates were prepared. Thirty micrograms of each sample were Western blotted and probed with anti-Mdm2 or anti-p53 antibodies. Immunoblots were then stripped of the primary antibody and re probed with antitubulin antibody. A minimum of three independent experiments were done for each experimental group.

in response to  $\gamma$ -radiation (Fig. 4C, a). In contrast, Bcl-2 expression in endothelial cells inhibited  $\gamma$ -radiation-induced activation of p38 MAPK. A number of studies have shown that ionizing radiation can induce the accumulation of p53 (25, 26). We next examined if Bcl-2 expression inhibited  $\gamma$ -radiation-mediated p53 accumulation. Figure 4C(b) shows the time course of p53 induction in HDMEC-VC and HDMEC-Bcl-2. In HDMEC-VC, p53 protein was markedly induced after  $\gamma$ -radiation. Increased p53 levels were detectable as early as 2 h postirradiation and reached a maximum at 4 h postirradiation. Even at 8 h postirradiation, high levels of p53 protein was observed in HDMEC-VC (data not shown). In contrast, irradiation of HDMEC-Bcl-2 showed markedly lower p53 protein levels as compared with HDMEC-VC.

**Bcl-2 enhances Mdm2 expression via activation of the Raf-MEK-ERK1/2 pathway and attenuates p53 accumulation in response to  $\gamma$ -radiation.** Because the Raf-MEK-ERK pathway has been shown to positively control *Mdm2* gene expression (16) and Mdm2 can regulate the p53 expression by targeting it for degradation (27), we next examined if Bcl-2 can also regulate p53 expression. HDMECs expressing Bcl-2 showed enhanced

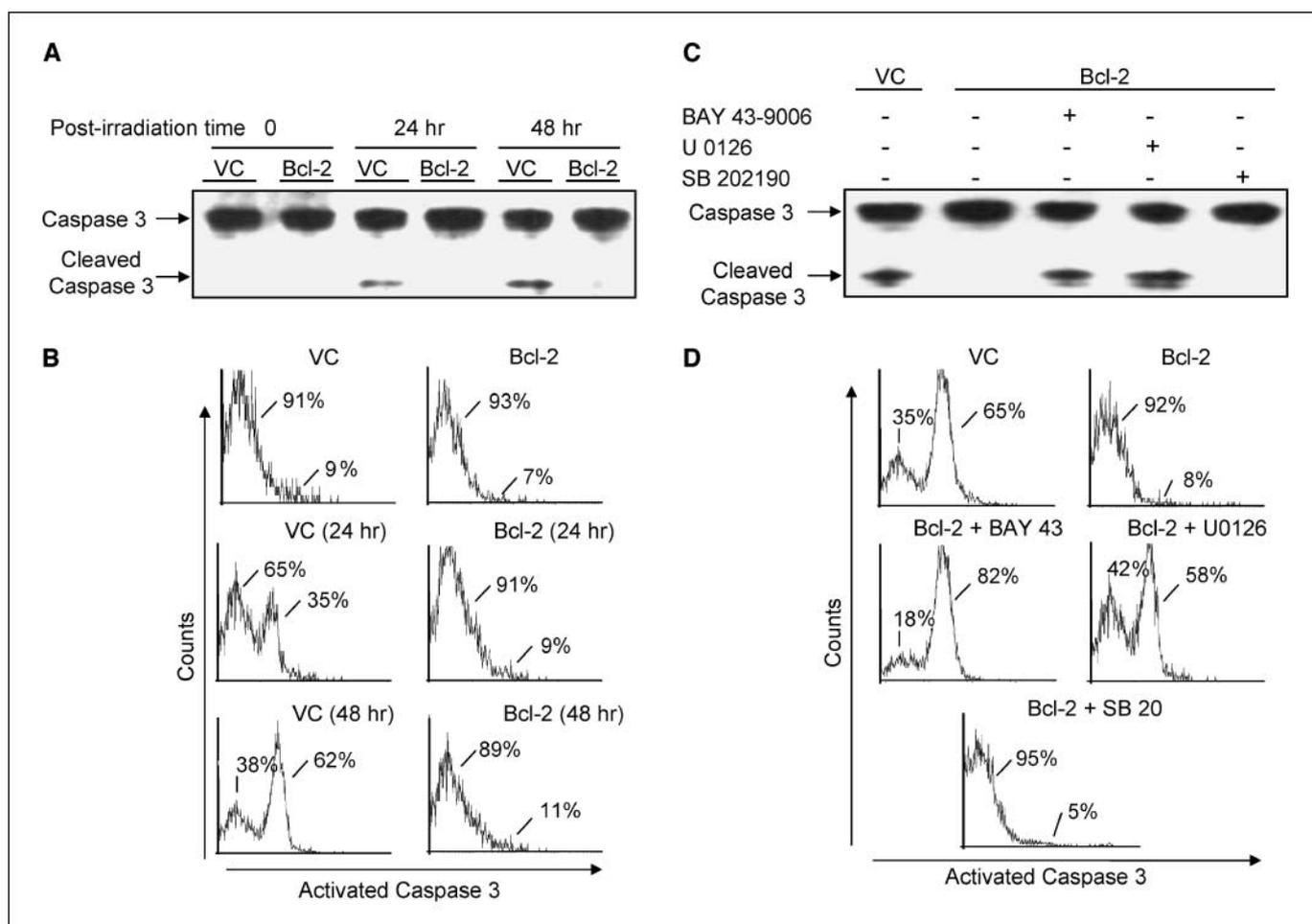
expression of Mdm2 via activation of the Raf-MEK-ERK pathway (Fig. 4C, c). In addition, a high level of Mdm2 expression was maintained in HDMEC-Bcl-2 cells even after  $\gamma$ -radiation (Fig. 4C, e). These results suggest that Bcl-2 can inhibit p53 accumulation via the Mdm2 pathway. Additionally, these results suggest that Bcl-2-mediated Mdm2 expression is independent of p53 transcriptional activity as high levels of Mdm2 were observed in HDMEC-Bcl-2 that showed markedly reduced levels of p53.

**$\gamma$ -Radiation induces time-dependent activation of caspase-3 and Bcl-2 inhibits caspase-3 activation via the Raf-MEK-ERK pathway.** We have previously shown that  $\gamma$ -radiation can induce endothelial cell apoptosis predominantly via activation of the p38 MAPK pathway (19). In this study, we show that HDMECs expressing Bcl-2 significantly inhibit p38 MAPK phosphorylation as well as p53 accumulation (Fig. 4C, a and b). We further examined if HDMECs expressing Bcl-2 are resistant to  $\gamma$ -radiation-induced caspase-3 activation.  $\gamma$ -Radiation treatment of HDMEC-VC induced a time-dependent activation of caspase-3 and showed a substantial increase in cleaved caspase-3 fraction by 24 h, with peak caspase-3 cleavage observed at 48 h postirradiation

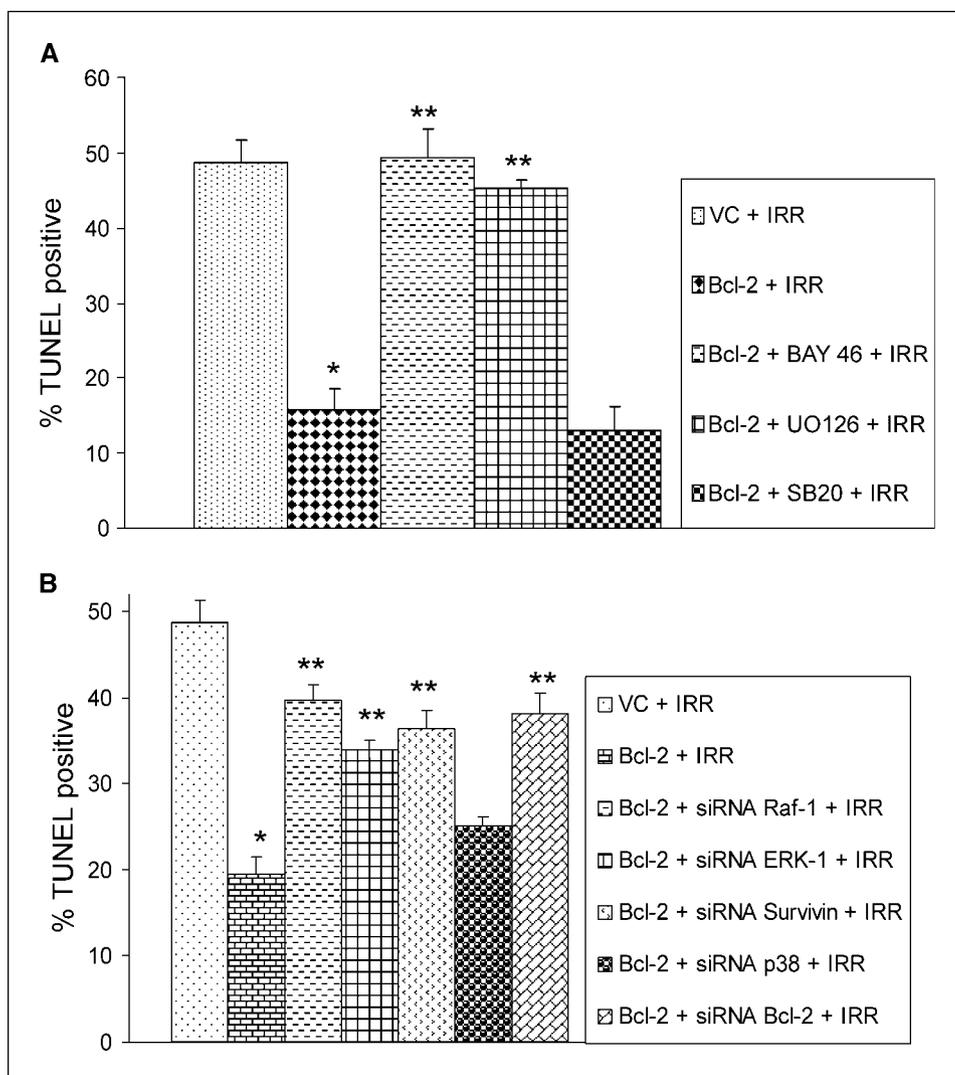
(Fig. 5A and B). In contrast, HDMECs expressing Bcl-2 cells were resistant to  $\gamma$ -radiation-mediated caspase-3 activation. This inhibition of caspase-3 activation by Bcl-2 was predominantly mediated via the Raf-MEK pathway as inhibition of both Raf-1 (BAY 43-9006) and MAPK (U0126) significantly reversed the ability of Bcl-2 to inhibit  $\gamma$ -radiation-induced caspase-3 activation (Fig. 5C and D).

**Bcl-2 protects endothelial cells against  $\gamma$ -radiation-induced apoptosis via activation of the Raf-MEK-ERK-survivin pathway.**  $\gamma$ -Radiation-induced apoptosis of HDMECs was evaluated by TUNEL assay. We did a dose-response study to identify the appropriate dose for these experiments. We used 5, 10, 15, and 20 Gy irradiation doses and observed 9%, 32%, 50%, and 87% HDMEC apoptosis (TUNEL positive), respectively. Untreated HDMEC showed <3% apoptosis. We selected the 15-Gy dose for our studies because this dose allowed us to observe potential additive effect when used in combination with other inhibitors. As we observed before (19), HDMECs expressing Bcl-2 were quite

resistant to  $\gamma$ -radiation-mediated apoptosis and showed significantly lower TUNEL-positive cells as compared with HDMEC-VC cells (Fig. 6A and B). We next examined the role of the Raf-MEK-ERK-survivin pathway in Bcl-2-mediated protection of HDMECs against  $\gamma$ -radiation. We used both pharmacologic inhibitors to block Raf-1, MAPK, and p38 MAPK, as well as siRNA, to inhibit Raf-1, ERK1, p38 MAPK, and survivin expression. Inhibition of Raf-1 and ERK by either pharmacologic inhibitors or siRNA significantly reversed the Bcl-2-mediated HDMEC protection against irradiation-mediated apoptosis (Fig. 6A and B). Similarly, inhibition of survivin by siRNA led to the reversal of Bcl-2-mediated HDMEC protection from irradiation-induced apoptosis. In contrast, p38 MAPK inhibition by either pharmacologic inhibitor or siRNA did not interfere with Bcl-2-mediated HDMEC protection against  $\gamma$ -radiation (Fig. 6A and B). These results suggest that Bcl-2 protects endothelial cells against irradiation-mediated apoptosis via activation of the Raf-MEK-ERK-survivin pathway.



**Figure 5.**  $\gamma$ -Radiation induces time-dependent activation of caspase-3 and Bcl-2 inhibits caspase-3 activation via the Raf-MEK-ERK pathway. *A* and *B*, HDMEC-VC and HDMEC-Bcl-2 were cultured in 100-mm dishes containing a thin layer of type I collagen gel until they were ~90% confluent. Cells were treated with  $\gamma$ -radiation (15 Gy). At different time points postirradiation (0, 24, and 48 h), cells were harvested for activated caspase-3 analysis by flow cytometry or cell lysates were prepared for Western blotting. *A*, 50  $\mu$ g of each sample were Western blotted and immunoblots were cut into two parts at 25 kDa and higher immunoblot was probed with anti-caspase-3 antibody and the 25 kDa and lower immunoblot was probed with anti-cleaved caspase-3 antibodies. *B*, cells were stained with activated caspase-3 antibody (phycoerythrin labeled) and analyzed by flow cytometry. A representative histogram of each group from three independent experiments. *C* and *D*, HDMEC-VC and HDMEC-Bcl-2 were pretreated with specific Raf-1 inhibitor (BAY 43-9006) or MAPK inhibitor (U0126) for 1 h and then were exposed to  $\gamma$ -radiation (15 Gy). After 48 h, cells were harvested for activated caspase-3 analysis by flow cytometry or cell lysates were prepared and Western blotted for caspase-3 as described above.



**Figure 6.** Bcl-2 protects endothelial cells against  $\gamma$ -radiation-induced apoptosis via activation of the Raf-MEK-ERK-survivin pathway. HDMEC-VC and HDMEC-Bcl-2 were cultured in 100-mm dishes containing a thin layer of type I collagen gel until they were ~90% confluent. **A**, cells were cultured in serum-free endothelial cell growth medium-2 in the presence or absence of specific Raf-1 inhibitor (BAY 43-9006) or MAPK inhibitor (U0126) for 1 h and then were exposed to  $\gamma$ -radiation (15 Gy). **B**, cells were transfected with siRNA for Raf-1, ERK1, survivin, and p38 MAPK using Signal Silence siRNA kits from Cell Signaling according to the manufacturer's instructions. Forty-eight hours posttransfection, cells were treated with  $\gamma$ -radiation. Seventy-two hours postirradiation, cells were harvested and the percentage of apoptotic cells was evaluated by the TUNEL assay according to the manufacturer's instructions (Sigma). \* and \*\*,  $P < 0.05$ , a significant decrease or increase, respectively, between the test and the respective control group from three independent experiments.

## Discussion

Ionizing radiation is widely used in the treatment of a variety of solid tumors and it is thought to act directly by targeting clonogens (28). It is now well recognized that successful clonogenic cell dysfunction requires tumor endothelial cell apoptosis (29). Endothelial cells lining the tumor blood vessels are normally quite resistant to radiation therapy and require quite high doses of ionizing radiation to induce apoptosis (30). One of the reasons for endothelial cell radioresistance in tumors could be due to the presence of numerous growth factors, particularly VEGF in the tumor microenvironment, where it functions to enhance endothelial cell survival (19). We have previously shown that VEGF protects endothelial cells against ionizing radiation by up-regulating the expression of Bcl-2 (19). However, the precise mechanism by which Bcl-2 protects endothelial cells is not well defined. Previous studies have mainly focused on the antiapoptotic activity of Bcl-2 at the mitochondrial level because it was assumed that Bcl-2 is mainly functional on mitochondria (3). It is now known that only Bcl-x<sub>L</sub> is specially targeted to the mitochondrial outer membrane whereas Bcl-2 is distributed on several intracellular membranes including plasma membrane, nuclear membrane, and endoplasmic reticulum (4). In addition, recent studies have shown that organelle-specific

expression of Bcl-2 at the endoplasmic reticulum protects cells against stress-induced apoptosis (31). These studies suggest that the mitochondria may not be the only site for the survival function of Bcl-2, and Bcl-2 might mediate its antiapoptotic effect via alternative overlapping pathways.

In this study, we show that Bcl-2 can protect endothelial cells against  $\gamma$ -radiation by up-regulating a key antiapoptotic protein, survivin, and down-regulating an important tumor suppressor protein, p53, via activation of the Raf-MEK-ERK signaling cascade. Furthermore, this Bcl-2-mediated protection in endothelial cells against ionizing radiation was significantly reversed by inhibition of the Raf-MAPK pathway without affecting mitochondrial cytochrome *c* distribution. To the best of our knowledge, this is the first study that has shown direct evidence that Bcl-2, in addition to its function in mitochondria, might also protect endothelial cells via an alternative pathway that is independent of mitochondrial cytochrome *c* release. These results could help us better understand how Bcl-2, which is present on nonmitochondrial membranes, particularly endoplasmic reticulum, protects endothelial cells. In addition, we show that Bcl-2 exerts its antiapoptotic effect by Mdm2-mediated inhibition of p53 accumulation. Recently, Chen et al. (32) have shown that inhibition of

Src kinase enhanced paclitaxel-mediated ovarian cancer cell apoptosis, which was independent of cytochrome *c* release and caspase-9 activation.

Our findings from this study suggest that Bcl-2 may use multiple pathways to inhibit death signals in endothelial cells. One of these mechanisms involves the up-regulation of survivin. Survivin, a member of the inhibitor of apoptosis family, has been shown to inhibit cell death initiated via both the extrinsic and intrinsic apoptotic pathways (13, 15). Survivin mediates its antiapoptotic function predominantly by inhibiting the activation of downstream effectors of apoptosis, caspase-3 and caspase-7, in cells exposed to apoptotic stimuli (12). Survivin is a relatively short-lived protein (half-life of 30 min; ref. 33), and its enhanced expression has been shown to be mediated by phosphatidylinositol 3-kinase-Akt and signal transducer and activator of transcription 3 activation (34, 35). In this study, we show that Bcl-2-mediated Raf-MEK-ERK pathway can also up-regulate the expression of survivin. Bcl-2 expression in endothelial cells can also regulate survivin protein levels via down-regulation of p53 accumulation, as it is well established that survivin is one of the genes transcriptionally repressed by wild-type p53 (36) and loss of p53 in Bcl-2 expressing endothelial cells may further enhance survivin expression at the transcription level.

Another mechanism used by Bcl-2 to protect endothelial cells is by regulating the accumulation of p53 protein by Mdm2. Mdm2 is a ubiquitin E3 ligase that promotes ubiquitination of p53 and itself, leading to rapid degradation by the 26S proteasomes (37). Mdm2 is a transcriptional target of p53 as p53-responsive elements have been identified in the intronic promoter of the *Mdm2* gene (38, 39). Induction of Mdm2 transcription by p53 establishes a negative feedback loop in which p53 itself initiates its own destruction (40). Mdm2 expression has also been shown to be modulated by mitogenic activation with a number of growth factors including basic fibroblast growth factor (41). Our results suggest that Bcl-2 modulates the expression of Mdm2 via activation of the Raf-MEK-ERK pathway, and this enhanced expression of Mdm2 is independent of p53 protein. Similarly, Ries

et al. (16) have shown that activation of Ras-induced Raf-MEK-ERK pathway inhibits p53 accumulation by up-regulating Mdm2 expression (16).

A third mechanism by which Bcl-2 protects endothelial cells is by down-regulating the activation of p38 MAPK and caspase-3, also via the Raf-MEK-ERK pathway. p38 MAPK is a key kinase that is activated by a number of DNA-damaging agents, including ionizing radiation (19, 42). Activation of p38 MAPK can lead to the phosphorylation of a number of downstream targets (43–45). p38 MAPK has been shown to phosphorylate p53 directly and, thus, is of particular significance for cancer therapy (46, 47). p53 phosphorylation, especially at the Ser<sup>15</sup> residue, can increase its accumulation by inhibiting its degradation (48). Grethe et al. (49) have shown that the activation of p38 MAPK leads to caspase-3 activation in endothelial cells. We and others have shown that the Raf-MEK-ERK and phosphatidylinositol 3-kinase-Akt pathways can cross-regulate p38 MAPK activation (19, 50). In this study, we show that Bcl-2 can inhibit  $\gamma$ -radiation-induced activation of p38 MAPK and caspase-3 via activation of the Raf-MEK-ERK pathway.

In summary, our results suggest that Bcl-2 protects endothelial cells against  $\gamma$ -radiation-induced apoptosis by mechanisms that include up-regulation of survivin and Mdm2 expression, inhibition of p38 MAPK and caspase-3, and inhibition of p53 accumulation. These novel findings may have important implications in the development of antiangiogenic as well as antitumor therapies.

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## Bcl-2 Protects Endothelial Cells against $\gamma$ -Radiation via a Raf-MEK-ERK-Survivin Signaling Pathway That Is Independent of Cytochrome c Release

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