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Protein Kinase B Inhibits Endostatin-induced Apoptosis in HUVECs

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Endostatin is a tumor-derived angiogenesis inhibitor, and the endogenous 20 kDa carboxyl-terminal fragment of collagen XVIII. In addition to inhibiting angiogenesis, endostatin inhibits tumor growth and the induction of apoptosis in several endothelial cell types. However, the mechanisms that regulate endostatin-induced apoptotic cell death are unclear. Here, we investigated apoptotic cell death and the underlying regulatory mechanisms elicited of endostatin in human umbilical vein endothelial cells (HUVECs). Endostatin was found to induce typical apoptotic features, such as, chromatin condensation and DNA fragmentation in these cells. Thus, as the phosphoinositide 3-OH kinase (PI3K)/protein kinase B (PKB) signaling pathway has been shown to prevent apoptosis in various cell types, we investigated whether this pathway could protect cells against endostatin induced apoptosis. It was found that the inhibition of PI3K/PKB significantly increased endostatin-induced apoptosis, and that endostatin-induced cell death is physiologically linked to PKB-mediated cell survival through caspase-8.

Keywords: Apoptosis, Caspase-8, Endostatin, HUVEC, Protein kinase B

Introduction

Endostatin is the 20-kDa C-terminal fragment of collagen XVIII, and a potent inhibitor of endothelial cell proliferation (O'Reilly *et al*., 1997), migration (Yamaguchi *et al*., 1999), and angiogenesis (O'Reilly *et al*., 1997). Endostatin can be generated by the proteolytic processing of collagen XVIII by various proteases, including cysteine proteases, matrix metalloproteinases (MMP), and serine protease elastase-like activity (Pepper, 2001). It is also well known that endostatin induces apoptosis in a variety of cells including endothelial cells (Dhanabal *et al*, 1999; Folkman, 2004), and it appears that the antiangiogenic effect of endostatin is mediated by the inhibition of endothelial cell migration. Moreover, a number of combinatorial treatments based on endostatin and chemotherapy, radiotherapy, or immunotherapy, indicate synergism between endostatin and these modalities in clinical trials on tumor-bearing diseases (Cao, 2001).

Angiogenesis is the process of forming new capillaries from preexisting vessels (Choi *et al*., 2003; Folkman, 2004), and thus plays an essential role in many physiological processes, such as, embryonic development (Breier, 2000), the menstrual cycle (Maas *et al*., 2001), and in those of many pathologic conditions (Yoon *et al*., 2003; Krikun *et al*., 2004). Moreover, the initiation of angiogenesis, the so-called 'angiogenic switch', is known to be dependent on a dynamic balance between proangiogenic and antiangiogenic factors in the immediate environment of endothelial cells (Iruela-Arispe and Dvorak, 1997).

bFGF, an important angiogenic factor, inhibits endothelial cell apoptosis induced by radiation or growth factor deprivation (Kondo *et al*., 1996). Like VEGF, bFGF upregulates the expressions of the antiapoptotic proteins, Bcl-2 and survivin (Teh *et al*., 2004), and the overexpression of Bcl-2 in endothelial cells prevents apoptosis induced by serum and growth factor deprivation, but has no effect on bFGF-induced endothelial cell proliferation (Karsan *et al*, 1997). Furthermore, bFGF also activates protein kinase Akt in endothelial cells (Nakashio *et al*., 2002). However, endostatin inhibits the proliferation and migration of bFGF-stimulated endothelial

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; eNOS, endothelial nitroxide synthase; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; PI3K, phosphoinositide 3-OH kinase; PKB, protein kinase B; TUNEL, TdT-mediated dUTP nick end labeling; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor.

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cells (Xu *et al*., 2001; Heljasvaara *et al*., 2005; Reis *et al*., 2005) via an unknown mechanism in HUVECs.

The phosphoinositide 3-OH kinase (PI3K)/protein kinase B (PKB) signaling pathway has been shown to protect various cell types from apoptosis induced by external stimuli (Jeon *et al*., 2002; Jamal *et al*., 2003; Tramontano *et al*., 2003; Shim *et al*., 2004). In addition, PI3K/PKB plays an essential role in mediating cross-talk between survival and cell death pathways (Lawlor and Alessi, 2001; Shim *et al*., 2004), which raises the question as to whether PI3K/PKB signaling is capable of protecting HUVECs from the apoptosis induced by endostatin. Within cells, apoptotic cell death pathways are physiologically linked to survival pathways, and therefore, the identification of points of cross-talk between pro- and anti-apoptotic pathways is of considerable importance in terms of understanding the balance between cell death and survival.

Here, we present evidence that active PI3K and PKB are capable of significantly inhibiting endostatin-induced apoptosis. We also report that endostatin-induced cell death is intrinsically linked to PKB-mediated cell survival via caspase-8 in HUVECs.

Materials and Methods

Cell culture. HUVECs were maintained at 37°C in a 5% CO_2 humid incubator and cultured using EGM-2 BulletKits (BioWhittaker, US & CAN) containing 10% fetal bovine serum (FBS). Prior to experimentation, HUVECs (1×10^6 cells) were starved by incubation in starvation medium supplemented with 1% FBS for 24 h before being treated with the various reagents. Since several distinct differences between early and late passage HUVECs have been reported, e.g., altered expressions of endothelial cell markers and variations in angiogenic potential (Vasile *et al*., 2001), we used HUVECs at passage 7 in this experiments. Moreover, in every case endostatin was treated 30 min before adding bFGF.

Expression and purification of recombinant human endostatin in *E. coli*. *E. coli* BL21 clones transformed with recombinant plasmid were grown in 3 L culture flasks in LB media, and recombinant human endostatin (rhEndostatin) was induced by adding 1 mM IPTG. Cells were harvested by centrifugation for 15 min at 5,000 g. The refolding and purification procedures were as follows. (i) Cells were disrupted and inclusion bodies (IBs) were separated by centrifugation and washed with deoxycholic acid. (ii) IBs were then solubilized in Tris buffer (pH 8.6) containing 7 M guanidine-HCl and 50 mM β-mercaptoethanol, and refolding was performed by rapid dilution in Tris buffer (pH 8.6) containing 2.5 M urea and redox-coupling agents, i.e., reduced and oxidized glutathione. For purification, refolded rhEndostatin fractions were applied to Heparin-Sepharose resin (Pharmacia), which had been pre-equilibrated with 50 mM Tris-Cl, 100 mM NaCl buffer. Refolded rhEndostatin was eluted using an equilibration buffer containing 1 M NaCl, and the purified rhEndostatin was concentrated and was buffer changed using by Amicon Stirred Cells (Millipore).

DAPI staining and fluorescent microscopy. Cell death was studied morphologically using a fluorescent nuclear dye, DAPI (4,6-diamidino-2-phenylindole). Apoptotic cells contain condensed chromatin and show nuclear fragmentation that produce characteristic, irregular nuclear staining. HUVECs treated with various concentrations of endostatin were washed with PBS and cytospin (500 × g, 5 min). Cells were fixed with 4% paraformaldehyde and permeabilized by incubation in 0.1% sodium citrate containing 0.1% Triton X-100 for 2 min at 4°C. They were then stained with DAPI (1 μg/ml) solution for 30 min at 25°C and examined by a fluorescence microscopy (AHPT-514, Olympus) under blue light.

Detection of DNA fragmentation by TUNEL assay. For TdT-mediated dUTP nick end labeling (TUNEL), DNA ends were labeled using an *In Situ* Cell Death Detection Kit (Boehringer Mannheim, Germany) (Lee *et al*., 2003; Qu and Qing, 2004). HUVECs were treated with 0, 0.1, 1, or 5 μg/ml endostatin and then 5 ng/ml bFGF was added. The cells were then washed with PBS and centrifuged at 1,200 rpm for 5 min. Pellets were resuspended in 300 ml of wash buffer and cytospin (500 g, 5 min). Cells were then fixed with 4% paraformaldehyde and permeabilized by incubation with 0.1% Triton X-100 for 2 min at 4°C. The cells were then treated 1 μl of TUNEL reaction mixture and incubated for 0.5-1 h at 37°C in a 5% CO_2 incubator. DNA fragmentation was observed under a confocal fluorescent microscope (MRC-1024, Bio-Rad).

Western blot analysis. HUVECs were cultured **<in what ?>** for 24 h with endostatin (0, 1, or 5 μg/ml), wortmannin (100 nM), or with endostatin plus wortmannin (5 μg/ml and 100 nM, respectively). 5 ng/ml bFGF was then added to each plate. Lysates were prepared in lysis buffer (50 mM Tris (pH 7.5), 0.5% Triton X-100, 3 mM EGTA, 12 mM b-glycerophosphate, 150 mM sodium chloride, 50 mM sodium fluoride, 1 mM sodium vanadate, 2 mM DTT, 1 mM PMSF, 0.1% β-mercaptoethanol, and 0.5 mg/ml aprotinin). Total proteins obtained by centrifugation (13,000 × g, 20 min, 4°C) were size-separated by electrophoresis on 8% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes. Non-specific binding was blocked with TBST (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.05% Tween 20) containing 5% non-fat milk for 1 h at 25°C. Membranes were immunoblotted with primary antibody (1 : 1,000 dilution of rabbit anti-phospho-PKB, anti-PKB, or anti-caspase-8) for 16 h at 4°C and subsequently exposed to a horseradish peroxidase-conjugated secondary antibody (1 : 2,500 dilution in TBST) for 2 h at 25°C. Immunoreactive spots were visualized using an ECL detection system.

Protein kinase B assay. Protein extracts were obtained from pre-treated HUVECs suspended in lysis buffer. Fluorescein-conjugated IRS-1 (^{30}RK*R*S*R*KE**S**YS^{39}) or caspase-8 (^{256}SI*R*D*R*NG**T**HL^{265}) oligopeptides (Peptron Co.) were used as PKB substrates. Briefly, 0.6 μg of fluorescein-conjugated oligopeptide was incubated with 200 μg of protein extract in protein kinase reaction buffer (20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 2 mM DTT, and 1.3 mM $CaCl_2$) containing 1 mM ATP at 30°C for 1 h. Reactions were stopped by heating at 95°C for 10 min, and phosphorylated peptides were separated on a 0.8% agarose gel at 100 V for 15-30 min.

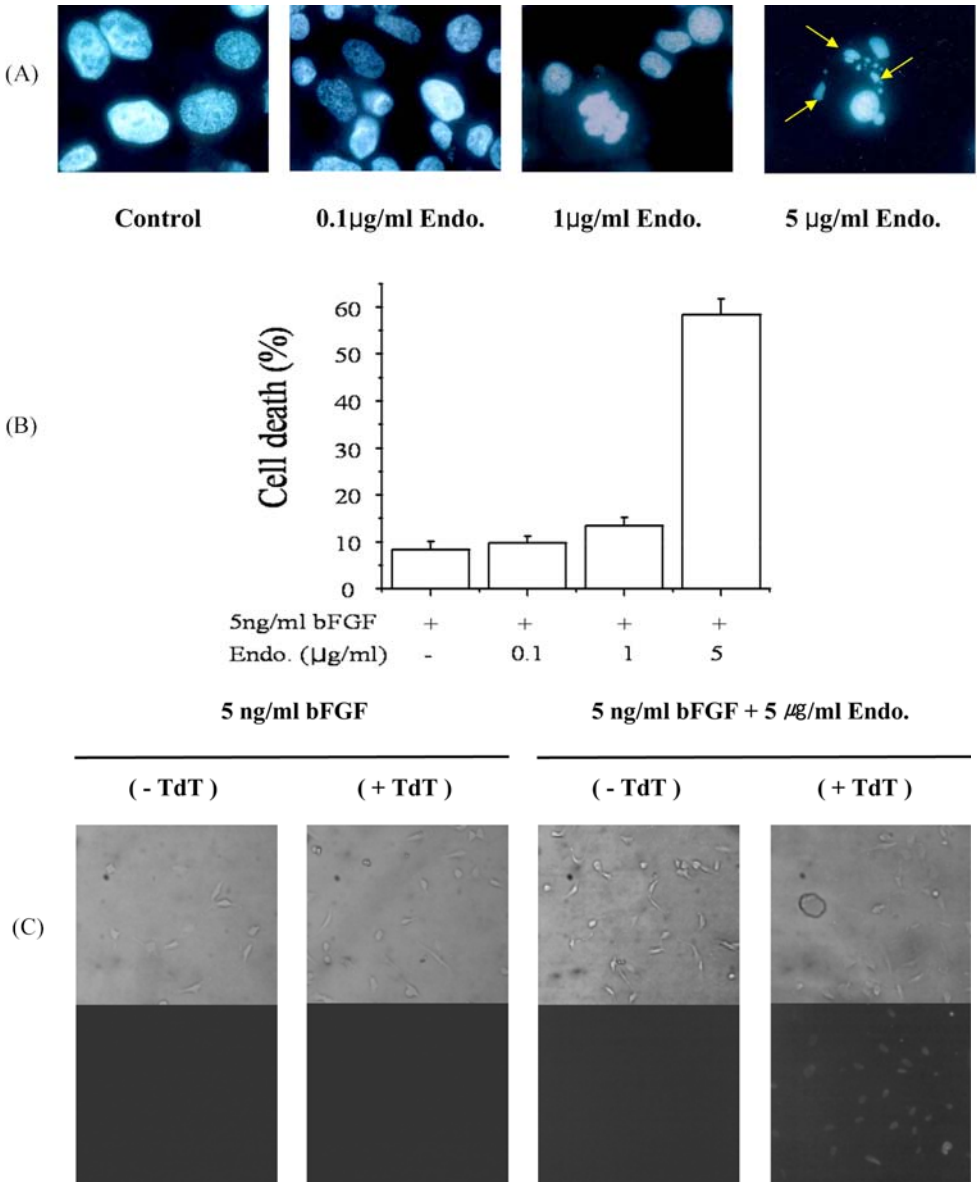

Fig. 1. HUVEC cell death in response to treatment with various concentrations of endostatin. HUVECs were cultured in EGM-2 media containing 5 ng/ml bFGF and treated with 0, 0.1, 1, or 5 ìg/ml endostatin for 24 h. (A) DAPI-stained cells were observed by fluorescence microscopy. Arrows indicate apoptotic cell death. Endo indicates treatment with endostatin. (B) In DAPI-stained cells (1×10^5), we counted apoptotic cells that show irregular nuclear staining. Degrees of apoptotic cell death are expressed as percentages on total cells (approximately 500 cells). Values are averages ± standard deviation of six determinations. (C) DNA fragmentation was assessed in paraformaldehyde-fixed cells by TUNEL staining by laser confocal microscopy. TdT indicates terminal deoxynucleotidyl transferase. Data are representative of three independent experiments.

Results

Endostatin induces apoptotic cell death. The influence of endostatin on HUVEC cell death was determined by analyzing various morphological changes. Initially, we examined cell death in response to various concentrations of endostatin in HUVECs by staining with DAPI (Figs. 1A and 1B). Treatment with endostatin (5 μg/ml) for 24 h caused approximately 58% of the cells to undergo apoptosis. To further establish that endostatin elicits HUVEC apoptosis, we used TUNEL assays, and DNA fragmentation was found to be pronounced in HUVECs treated with endostatin (5 μg/ml) for 24 h (Fig. 1C).

Inhibition of PI3K/PKB signaling enhances the apoptosis evoked by endostatin. The ability of PI3K/PKB to suppress apoptotic cell death has been described in a number of cell types (Jeon *et al*., 2002; Jamal *et al*., 2003; Tramontano *et al*., 2003; Shim *et al*., 2004). Therefore, we examined whether

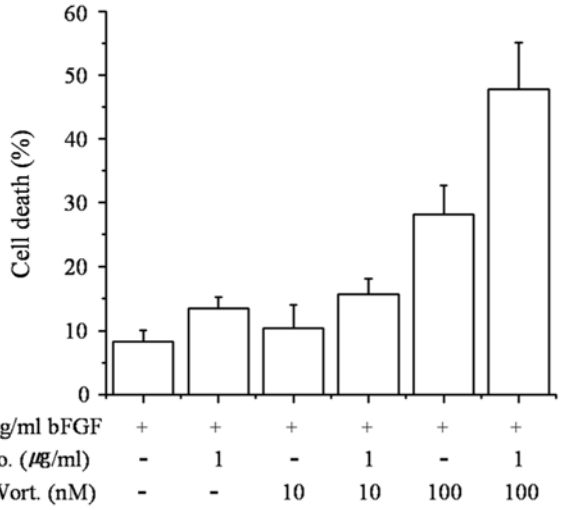

Fig. 2. Inhibition of PI3K enhances the apoptotic cell death elicited by endostatin in HUVECs. Apoptotic cell death was detected by DAPI staining. Proportions of apoptotic cells are expressed as percentages of total cells. Wortmannin (wort) was added 30 min prior to 1 μg/ml endostatin. Values are averages ± standard deviations of six determinations.

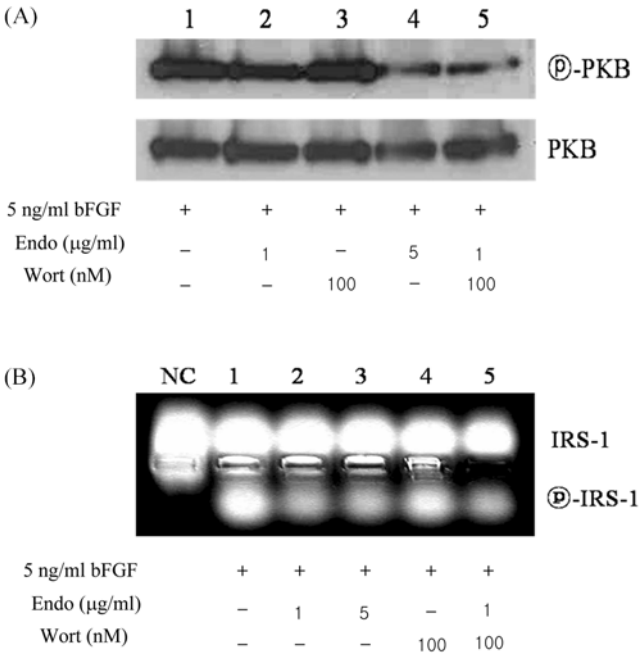

Fig. 3. Endostatin and wortmannin suppress PKB phosphorylation and activity. HUVECs were pretreated with wortmannin for 30 min and then with endostatin or bFGF (in the presence of wortmannin) for 24 h prior and lysed. (A) Western blot analysis of PKB phosphorylation at the serine 473 residue in response to different concentrations of endostatin and wortmannin. 700 μg of protein lysate was loaded per lane. Results are representative of three independent experiments. (B) IRS-1 peptide (0.6 μg), a PKB substrate, was phosphorylated by HUVEC lysates (100 μg protein). Negative control (NC) and control indicate treatments with untreated HUVEC buffer and protein lysate, respectively. Results are representative of five independent experiments. Wort indicates wortmannin treatment.

PI3K/PKB is involved in endostatin-induced cell death. Various concentrations of wortmannin (a PI3K inhibitor) were added to HUVEC cultures 30 min prior to the addition of endostatin (1 μg/ml) with bFGF (5 ng/ml). Endostatin (1 μg/ml) was found to induce apoptotic cell death in approximately 47% of cells pretreated with wortmannin (100 nM) (Fig. 2). Since endostatin alone induced the 13% cell death at the same concentration, it appeared that wortmannin enhances the effect of endostatin. Moreover, LY294002 (also a PI3K inhibitor) had an effect similar (data not shown). These results indicate that the PI3K/PKB signaling pathway is involved in the suppression of apoptotic cell death induced by endostatin in HUVECs.

Endostatin inhibits PKB phosphorylation and activity. The activation of PI3K appears to stimulate PKB activity by phosphorylation (Coffer *et al.*, 1998; Datta *et al.*, 1999; Nicholson and Anderson, 2002). To examine the relationship between the PKB signaling pathway and endostatin-induced apoptosis, we examined the phosphorylation state of PKB and its activity in endostatin-treated HUVECs by using an immunoblot analysis and a nonradioactive protein kinase assay system to measure the phosphorylation and activity of PKB, respectively (Fig. 3). PKB phosphorylation was suppressed by a low dose (1 μg/ml) endostatin in combination with wortmannin (100 nM; Fig. 3A). A fluorescein-conjugated IRS-1 peptide (^{30}RKRSRKESYS^{39}) was employed as a PKB substrate for the nonradioactive protein kinase assay (Jeon *et al.*, 2002). This substrate was basally phosphorylated by HUVEC protein extract and this phosphorylation was found to be inhibited by endostatin and/or wortmannin, indicating that endostatin blocks PKB activity (Fig. 3B). Taken together, these results suggest that endostatin blocks both the phosphorylation and activity of PKB. Moreover, whereas 100 nM wortmannin induced 27% cell death (Fig. 2), it did not inhibit PKB phosphorylation, because wortmannin is a general PI3K inhibitor.

PKB blocks apoptosis via the phosphorylation of caspase-8. Although endostatin is associated with the suppression of PKB activity and phosphorylation, no PKB substrate protein(s) have been identified in HUVECs during endostatin-induced apoptosis. Therefore, we attempted to identify putative PKB substrate proteins in this system using the PKB substrate consensus sequence (*RXRXXS/TXX*; where the underlined residue is a hydrophobic amino acid) (Lawlor and Alessi, 2001; Nicholson and Anderson, 2002). BAD (^{94}*RGR*SRSAP^{101}), caspase-8 (^{258}*RDR*NGTHL^{265}), and caspase-9 (^{191}*RRR*FSSLH^{198}), all apoptosis mediators, contain this conserved sequence and a PKB phosphorylation site (Lawlor and Alessi, 2001). It has be reported that BAD and/or caspase-9 are PKB substrates in

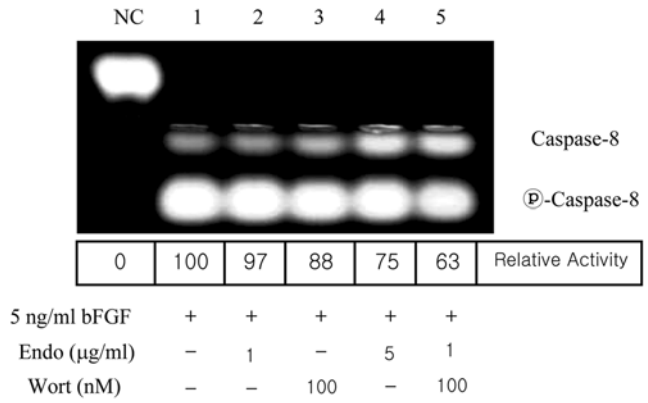

Fig. 4. Endostatin suppresses the phosphorylation of caspase-8 evoked by PKB. Caspase-8 (0.6 μg) was phosphorylated by HUVEC lysate (200 μg protein). HUVECs were pretreated with wortmannin for 30 min and then treated with endostatin for 24 h and lysed. Relative degrees of phosphorylation are shown at the bottom of the figure, and were measured using Image Gauge V2.54. Negative control (NC) and control indicate treatment with untreated HUVEC buffer or protein lysate, respectively. Results are representative of five independent experiments.

Jurkat (Dy *et al*., 2001), melanoma (Li *et al*., 2001), hemopoietic (Scheid and Duronio, 1998) and skin cells (Wan *et al*., 2001). To determine whether caspase-8 and/or caspase-9 are PKB substrates in HUVECs, we employed a nonradioactive protein kinase assay using fluorescein-conjugated caspase-8 (^{256}SI*R*D*R*NGTHL^{265}) and caspase-9 (^{189}KL*RR*F*R*FSSLH^{198}) peptides. The caspase-8 peptide was phosphorylated by HUVEC lysate (Fig. 4), and endostatin and/or wortmannin reduced this phosphorylation, indicating that caspase-8 might mediate the apoptosis evoked by endostatin in HUVECs. Caspase-9 was also phosphorylated by the lysate but its phosphorylation level was not changed by endostatin treatment, indicating that the caspase-9 peptide or protein are PKB substrates, but that they are not related to endostatin-regulate pathways (data not shown).

Discussion

In endothelial cells, endostatin inhibits VEGF induced cell migration by inducing eNOS dephosphorylation on Ser 1177 without affecting Akt activity (Urbich *et al*., 2002). In addition, to this antimigratory effect, endostatin has been demonstrated to induce endothelial cell apoptosis (Dhanabal *et al*., 1999; Dixelius *et al*., 2000). Indeed, endothelial cell stimulation by endostatin leads to a marked reduction in the levels of Bcl-2 and Bcl-XL antiapoptotic proteins without affecting proapoptotic Bax protein levels (Dhanabal *et al*., 1999). Furthermore, Shb adaptor protein has been suggested to be involved in the mediation of the apoptotic signaling induced by endostatin (Dixelius *et al*., 2000). Rehn *et al* (2001) demonstrated that soluble endostatin is capable of binding to α_1- and α_5-integrins, thereby inhibiting integrin functions, such as, endothelial cell migration. It is also conceivable that such an interaction with integrins may affect endothelial cell survival.

The VEGF-induced activation of the MAPK/extracellular signal-regulated kinase (ERK) pathway and its inhibition of the stress-activated protein kinase/c-Jun amino-terminal kinase pathway are also implicated in the antiapoptotic effect mediated by VEGF (Cho and Choi, 2002; Zachary, 2003). Recently, it was reported that several signaling pathways are involved in regulating HUVEC apoptosis (Artwohl *et al*., 2004; Fukushima *et al*., 2005). The PI3K/PKB signaling pathway is one of the most important survival signaling pathways known in various cell types (Hemmings, 1997; Datta *et al*., 1999; Lawlor and Alessi, 2001). Here, we demonstrate that in HUVECs, PI3K/PKB inhibits endostatin-induced apoptosis and that endostatin blocks PKB activity and phosphorylation (Fig. 3).

However, if PKB suppresses the apoptosis induced by endostatin in HUVEC, then where is the point of cross-talk between the survival and apoptotic pathways located, and which proteins serve as substrates for PKB? PKB may maintain cell survival by inhibiting proteins involved in apoptosis, and BAD is one such protein (Blume-Jensen *et al*., 1998). The roles of protein kinase B in the phosphorylation and inactivation of BAD have been well documented in several cell lines (Zha *et al*., 1996; Rusinol *et al*., 2004). Alternatively, PKB could directly phosphorylate caspases, and then block their activities. Indeed, caspases contain a putative phosphorylation site and a consensus PKB substrate sequence. For example, caspase-8 and caspase-9 possess Thr263 (^{258}*RD*R*R*NGTHL^{265}) and Ser196 (^{191}*RR*R*R*FSSLH^{198}), respectively (Lawlor and Alessi, 2001). Moreover, it was previously reported that caspase-9 (but not caspase-8) is phosphorylated and that its protease activity is inhibited by PKB in human cell lines (Cardone *et al*., 1998; Deng *et al*., 2003). In the present study, we concluded that caspase-9 is not a point of cross-talk, since its degree of phosphorylation was unchanged by endostatin treatment, although it was phosphorylated by HUVEC lysate. Interestingly, PKB appeared to phosphorylate caspase-8, and this activity was suppressed by endostatin and/or wortmannin treatment, indicating that caspase-8 is a point of cross-talk between the PI3K/PKB-dependent survival signaling and the endostatin-induced apoptotic pathways (Fig. 4).

Recently, it was reported that endostatin binding to a5b1 integrin leads to the inhibition of cell migration through the ERK1/p38 MAP kinase pathway but not through the PI3K/PKB pathway in HUVECs. In contrast, tumstatin expression found to be mediated by the avb3 integrin inhibition of cell proliferation via the PI3K/PKB pathway (Sudhakar *et al*., 2003). Our results differ, in that we found that apoptosis mediated by endostatin is independent PI3K/PKB. However, when the angiogenic inhibitor, tumstatin inhibits cell proliferation, the PI3K/PKB pathway is mediated. Sudhakar *et al*. (2003) investigated roles of endostatin and tumstatin in

integrin mediate-cell migration and proliferation, but we studied role of endostatin on bFGF activated cell proliferation. It has also been reported that PKB may regulate apoptosis through the modulation of the caspase-8 inhibitor, fas-associated death domain-like interleukin 1β-converting enzyme (FLICE) inhibiting protein (FLIP) (Panka *et al*., 2001; Suhara *et al*., 2001; Skurk *et al*., 2004). Moreover, there is a possibility that caspase-8 activity modulation may be involved in the PI3K/PKB survival pathway in endothelial cells, and it should be recalled that PKB can phosphorylate both procaspase-9 and caspase-9 (Cardone *et al*., 1998; Deng *et al*., 2003). The phosphorylation of procaspase-9 may influence catalytic processing for its activation within Apaf-1/cytochrome C complexes, and phosphorylation of active caspase-9 may inhibit its activity through an allosteric mechanism that affects subunit dimerization or that alters the catalytic machinery of its substrate cleft through conformational change (Cardone *et al*., 1998). A similar situation may occur in the case of caspase-8, namely, that phosphorylation of procaspase-8 may inhibit the catalytic processing required for its activation. Recently, apoptotic pathway was linked to PKB-mediated cell survival through caspase-8 in several cells (Kim *et al*., 2004; Shim *et al*., 2004; Skurk *et al*., 2004; Hu *et al*., 2005).

In conclusion, the present study demonstrates that the PI3K/PKB pathway has a protective effect against endostatin-induced apoptosis. Moreover, caspase-8 was found to be a PKB substrate in HUVECs, which suggests that endostatin-induced apoptotic cell death is physiologically linked to the PI3K/PKB-mediated cell survival pathway through caspase-8. Cross-talk between the PKB and caspase-8 pathways is also a ubiquitous feature in endothelial and cancer cells in terms of the cell survival/death balance. Therefore, an understanding of cross-talk mechanisms contributes to the development of novel therapeutics through the regulation of pro-/anti-apoptotic balance in apoptosis-related diseases such as cancer.

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