Phosphatidylinositol 3-Kinase/Akt Signaling Controls Endothelial Cell Sensitivity to Fas-Mediated Apoptosis via Regulation of FLICE-Inhibitory Protein (FLIP)

Toshimitsu Suhara, Toshiaki Mano, Beatriz Enes Oliveira, Kenneth Walsh

Abstract—Fas is constitutively expressed on endothelial cells, but in contrast to smooth muscle and other cell types, endothelial cells are highly resistant to Fas-mediated apoptosis. In this study, we examined the role of the serine/threonine kinase Akt/PKB in controlling the sensitivity of endothelial cells to Fas-mediated apoptosis. Serum deprivation inhibited expression of the caspase-8 inhibitor FLICE-inhibitory protein (FLIP), which functions downstream from Fas. FLIP expression levels were restored when serum-depleted cells were treated with vascular endothelial growth factor. Treatment with the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitors wortmannin or LY294002 or infection of the adenoviral construct expressing dominant-negative Akt (Adeno-dnAkt) also inhibited the expression of FLIP in endothelial cells, whereas the MEK inhibitor PD98059 had no effect. Conversely, adenovirus-mediated transfection of a constitutively-active Akt gene abolished the wortmannin- and LY294002-mediated downregulation of FLIP. Suppression of PI 3-kinase signaling sensitized endothelial cells to Fas-mediated apoptosis. Under conditions of suppressed PI 3-kinase signaling, restoration of FLIP expression reversed the induced sensitivity of endothelial cells to Fas-mediated apoptosis. These data suggest that inhibition of Fas-mediated apoptosis, via promotion of FLIP expression, is a mechanism through which Akt signaling can promote endothelial cell survival. (Circ Res. 2001;89:13-19.)

Key Words: apoptosis n cell signaling n signal transduction n growth factors n cytokines

Fas (also called APO-1 or CD95) is a type I membrane protein belonging to the TNF receptor family that functions to transmit a death signal to the cell. Activation of this pathway requires receptor cross-linking with Fas ligand (FasL) or anti-Fas antibodies. Fas-mediated apoptosis is an essential mechanism for the maintenance of homeostasis in multicellular organisms, and disruption of the Fas/FasL system can lead to lymphoproliferative disorders and accelerate autoimmune diseases. Conversely, its overactivation can cause pathological tissue destruction. With regard to the vasculature, expression of Fas has been detected in both the normal and diseased vessel wall. Furthermore, it has been proposed that Fas-mediated apoptosis of vascular cells is a feature of atherogenesis, atherosclerotic plaque vulnerability, and allograft arteriopathy.

Fas ligation induces the recruitment of the proapoptotic proteins Fas-associated death domain (FADD) and procaspase-8, to form a complex in which the proteolytic activation of caspase-8 leads to the generation of an apoptotic cascade. The antiapoptotic FLICE-inhibitory protein (FLIP) is a cytoplasmic protein that is homologous to caspase-8 (also referred to as FLICE). FLIP acts in a dominant-negative manner to inhibit caspase-8 because it lacks a cysteine residue at the active site that is essential for proteolysis. FLIP expression is elevated during early stages of T-cell activation and during macrophage differentiation, and this is believed to make these cells resistant to Fas-mediated apoptosis. Multiple isoforms of FLIP (two of which are designated FLIP-L [long isoform] and FLIP-S [short isoform]) result from alternative splicing. Endothelial cells express abundant FLIP-L, but the level of FLIP-S protein is very low or undetectable. Endothelial cells are naturally resistant to Fas-mediated apoptosis, and specific stimuli are thought to sensitize endothelial cells to Fas ligation via downregulation of FLIP or upregulation of Fas expression.

The serine/threonine protein kinase Akt/PKB promotes viability in various cell types, including endothelial cells. Activation of Akt involves the binding of phosphatidylinositol 3-kinase (PI 3-kinase)—generated inositol lipids to Akt via its pleckstrin homology domain. PI 3-kinase–dependent activation of Akt also involves PDK1-mediated phosphorylation of threonine 308, leading to the autophosphorylation of serine 473. Akt-mediated viability is dependent, at least in
part, on the ability of Akt to phosphorylate and inactivate proapoptotic proteins.20

In the present study, we examined the role of Akt/PKB signaling in controlling sensitivity to Fas-mediated apoptosis in endothelial cells. We found that like serum deprivation, suppression of either PI 3-kinase or Akt signaling induced downregulation of FLIP expression and sensitized endothelial cells to Fas-mediated apoptosis. Under these conditions, adenovirus-mediated FLIP gene transfer protected endothelial cells from apoptosis. These data suggest that Akt signaling controls the sensitivity of cells to Fas-mediated apoptosis via the regulation of FLIP expression.

Materials and Methods

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVECs) were isolated as described.23 Human vascular smooth muscle cells (VSMCs) were isolated from an internal mammary artery obtained during coronary bypass surgery. The human T-cell leukemia cell line, Jurkat clone E6-1, was obtained from the American Type Culture Collection. HUVECs were grown to confluence on gelatin-coated 10-cm dishes, 6-well plates, or slide chambers with medium containing 2% FBS. HUVECs were grown to confluence on gelatin-coated 10-cm dishes, 6-well plates, or slide chambers with medium containing 2% FBS. Medium was replaced with fresh medium, with or without serum, typically at the time reagents were added. Mouse monoclonal antibody against human FLIP (NF6) was a gift from Dr Marcu E. Peter (German Cancer Research Center, Heidelberg, Germany). Caspase-8 activity was determined with a colorimetric assay from R&D Systems.

Adenoviral Constructs

AdTet-FLIP was constructed by inserting a DNA cassette containing seven consecutive tetracycline-responsive elements (TRE), a CMV minimal promoter, and SV40 polyaA into the multicloning site of adenovirus shuttle vector pAE1sp1A (Microbix Biosystems) to create pΔ1ATRE. A DNA fragment containing the FLAG-tagged protein-coding sequence of human FLIP-L was then inserted downstream from the TRE repeats and CMV minimal promoter of pΔ1ATRE. Recombinant E1-E3–deleted adenovirus constructs were generated by homologous recombination with adenovirus genome plasmid pM17 in human embryonic kidney 293 cells. The adenoviral vector AdTet-LacZ encoding β-galactosidase (β-gal) was generated by using pΔ1ATRE, as described previously.25 The adenoviral vector AdCMV-rtTA encodes a chimeric transcription activator under control of the CMV promoter/enhancer. Replication-defective adenovirus vectors expressing dominant-negative and constitutively active forms of murine Akt from the CMV promoter have been described previously.24 The dominant-negative Akt mutant (Adeno-dnAkt) has alanine residues substituted for threonine at position 308 and serine at position 473. This protein functions as a dominant negative for endogenous Akt.25 The constitutively active Akt (Adeno-myrAkt) has an in-frame fusion of the c-src myristoylation sequence to the N-terminus of the wild-type Akt coding sequence, thereby targeting the fusion protein to the membrane. All recombinant Akt vectors are fused in frame to the hemagglutinin (HA) epitope. Ad-β-gal expresses the LacZ gene from the CMV promoter.26 All viral constructs were purified by CsCl gradient ultracentrifugation. HUVECs were typically infected with adenoviral constructs overnight, followed by replacement with fresh medium with or without serum or test agent (wortmannin, LY294002, or PD98059). After 6 hours in fresh medium, cells were harvested for caspase-8 cleavage and activity. After 24 hours, cells were harvested for RT-PCR and Western blot analyses (except for caspase-8), and apoptosis was assessed at 48 hours.

Western Immunoblot Analysis

Cells were washed with PBS twice and harvested by scraping. Cell lysates were prepared in cell lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 20 mmol/L EDTA, 1% SDS, and 100 mmol/L NaCl). Protein concentration was determined by using a protein assay kit (Bio-Rad). Ten to 20 μg of protein extract was fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The membrane was blocked with T-PBS (1× PBS and 0.3% Tween 20) containing 3% dry milk and incubated with primary antibody overnight at 4°C. After three washes with T-PBS, the membrane was incubated with secondary antibody (anti-mouse IgG HRP conjugate) for 1 hour and then washed with 0.05% Tween 20 in PBS. The immune complexes were detected by enhanced chemiluminescence methods (ECL, Amersham).

Cell Viability Assays

HUVECs were grown on gelatin-coated 2-well chamber slides (Nalge Nunc International) at a density of 2×10^5 cells per well. At the conclusion of the treatment period, cells were washed with cold PBS twice and then fixed with 3.7% formaldehyde in PBS for 20 minutes. After fixation, the cells were washed with PBS twice and stained with Hoechst 33342. Cells were analyzed for the appearance of pyknotic nuclei by using a Nikon Diaphot microscope. Alternately, apoptosis was monitored by measuring hypodiploid DNA content. After various treatments, attached and floating endothelial cells were harvested and fixed in cold 90% ethanol for 20 minutes and then resuspended in staining buffer consisting of 1 mg/mL RNaseA, 20 μg/mL propidium iodide, and 0.01% NP40. DNA content was analyzed by flow cytometry on an FL-2 channel, and gating was set to exclude debris and cellular aggregates. Ten thousand events were counted for each analysis.

X-Gal Staining for β-Gal Expression

HUVECs were cotransfected with AdCMV-rtTA and AdTet-LacZ with or without doxycycline (Dox, 300 ng/mL) at a multiplicity of infection (MOI) of 2 or 10 for 24 hours. After transfection, cells were washed with PBS twice and fixed with 2% formaldehyde and 0.2% glutaraldehyde for 30 minutes and then stained with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) solution for 2 hours at 37°C.

Results

VEGF Promotes FLIP Expression via PI 3-Kinase Signaling

Endothelial cells, such as HUVECs, are normally resistant to Fas-mediated apoptosis.7,14–16 HUVECs express high levels of FLIP protein compared with human VSMCs or Jurkat cells (Figure 1A), which are more susceptible to Fas-mediated apoptosis.15 As shown in Figure 1B, serum deprivation of HUVECs for 12 hours results in decreased FLIP protein expression. Exposure to vascular endothelial growth factor (VEGF, 100 ng/mL) restores FLIP expression levels to those seen in cultures incubated in complete media, whereas coinubcation with the PI 3-kinase inhibitor wortmannin (200 nmol/L) abolishes the ability of VEGF to promote FLIP expression (Figure 1B). Wortmannin treatment in the absence of VEGF or serum did not lead to a further reduction in FLIP expression.
Akt Signaling Regulates FLIP Expression and Susceptibility to Fas-Mediated Apoptosis

To elucidate the role of Akt signaling on FLIP expression, HUVECs were transfected with adenovirus vectors expressing either the constitutively active Akt (Adeno-myrAkt) or the dominant-negative Akt mutant (Adeno-dnAkt). Western immunoblots were performed to assess levels of FLIP, adenovirus-encoded Akt construct (anti-hemagglutinin [HA]), and tubulin. Under conditions in which FLIP expression is reduced by incubation with wortmannin, FLIP levels were restored when cells were infected with Adeno-myrAkt (Figure 2A). Similarly, myrAkt restored FLIP levels in HUVECs exposed to serum-free medium or the PI 3-kinase inhibitor LY294002 (Figure 2B). In contrast, suppression of Akt signaling by infection with Adeno-dnAkt led to a decrease in basal FLIP expression, whereas the control vector expressing β-gal had no effect (Figure 2C). In the absence of wortmannin, infection with Adeno-myrAkt increased FLIP expression above basal levels (Figure 2C).

To determine the role of PI 3-kinase/Akt signaling in endothelial cell sensitivity to Fas-mediated apoptosis, HUVEC survival after treatment with agonistic anti-Fas antibody was assessed (Figure 3). In these experiments, cultures were treated with IFN-γ to upregulate endogenous levels of cell surface Fas expression. This cytokine alone does not affect endothelial FasL expression (data not shown), nor does it promote Fas-mediated apoptosis under normal culture conditions (Figure 3). In contrast, treatment with wortmannin induced apoptosis, and the frequency of apoptosis was further increased when cells were incubated with wortmannin and anti-Fas antibody. Similarly, transduction of dominant-negative Akt sensitized the HUVECs to apoptosis in the presence of anti-Fas antibody (data not shown). Transduction with myrAkt protected cells from apoptosis induced by wortmannin and anti-Fas antibody (Figure 3). Suppression of PI 3-kinase/Akt also sensitized the HUVECs to Fas-mediated apoptosis in the absence of IFN-γ, but the overall level of apoptosis was less (data not shown). In contrast, the MAPK inhibitor PD98059 did not promote cell death, nor did it synergize with anti-Fas antibody to induce apoptosis (Figure 3). Collectively, these data suggest that inhibition of PI 3-kinase/Akt signaling sensitizes endothelial cells to Fas-mediated apoptosis.

Tetracycline-Regulated FLIP Expression System

To examine the role of FLIP in the Fas-mediated apoptosis of endothelial cells, a tetracycline-inducible FLIP expression system was developed by using a binary-adenovirus strategy (Figure 4A). The first replication-defective adenovirus encodes the transgene, either FLIP-L or LacZ, under the transcriptional control of seven tetracycline operator sites (AdTet-FLIP). The second vector expresses a chimeric transcription factor composed of a mutant tetracycline repressor fused to the VP16 trans-activator domain from the CMV promoter/enhancer (AdCMV-rtTA). This factor does not
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Figure 3. PI 3-kinase signaling regulates endothelial cell susceptibility to Fas-mediated apoptosis. HUVECs were mock-infected or preinfected with Adeno-myrAkt (Ad-myrAkt) at an MOI of 50 for 12 hours. Then the cells were transferred to fresh medium with or without MEK inhibitor PD98059 (3 μmol/L) or PI 3-kinase inhibitor wortmannin (wort, 200 nmol/L). After 12 hours, IFN-γ (200 U/mL) was added to promote Fas expression, and agonistic antibody (CH11, 500 ng/mL) was added to the indicated cultures. After 24 hours, cells were fixed and stained with Hoechst 33342 to assess the frequency of cells with pyknotic nuclei (left). The percentage of cells with pyknotic nuclei was quantified (right). Data are shown as the mean±SD (n=4).

Figure 4. Tetracycline-regulated adenovirus constructs. A, Structures of Tet-regulated adenovirus vectors. AdCMV-rTA expresses a mutation of the tetracycline-dependent regulator fused to the VP16 trans-activator from the full CMV promoter/enhancer. This hybrid transcription factor is activated by Dox. AdTet vectors express FLIP-L or LacZ genes under the control of the TRE and a minimal CMV promoter. B, Endothelial cells were cotransfected with AdCMV-rTA (MOI 0 or 10) and AdTetFLIP (MOI 0, 2, or 10) in the absence or presence of Dox (10, 30, or 300 ng/mL) for 24 hours. FLIP expression was analyzed by Western immunoblotting of 10 μg of lysate. Tubulin expression shows quantitative uniformity of protein loading in each lane. C, β-Gal expression in AdTet-LacZ-transfected endothelial cells in the presence or absence of Dox. Endothelial cells were cotransfected with AdCMV-rTA (MOI 0 or 10) and AdTet-LacZ (MOI 0, 2, or 10) in the presence or absence of Dox (300 ng/mL) for 24 hours and then analyzed by X-Gal staining.

FLIP Restoration Protects Endothelial Cells Against Fas-Mediated Apoptosis Under Conditions of Suppressed PI 3-Kinase Signaling

To examine the role of PI 3-kinase signaling in the control of Fas-mediated apoptosis in endothelial cells, HUVEC cultures were assessed for the number of pyknotic nuclei present after incubation with wortmannin (200 nmol/L) or the agonist anti-Fas antibody (CH11) (Figure 5). In these experiments, all cultures were pretreated with INF-γ because this factor increases cell surface Fas expression.15,16 As can be seen in the control lanes of Figure 5, agonistic anti-Fas antibody did not promote apoptosis in HUVECs, consistent with previous reports.15,16 whereas treatment with wortmannin produced levels of cell death that were significantly higher than those observed in control cultures. Of particular importance, coinubation of cultures with wortmannin plus anti-Fas antibody induced higher levels of apoptosis than were observed in the presence of wortmannin alone. Similarly, higher levels of apoptosis were detected in the presence of anti-Fas antibody when HUVECs were infected with Adeno-dnAkt (data not shown). Collectively, these data show that inhibition of PI 3-kinase/Akt signaling will potentiate Fas-mediated death signals in endothelial cells.
To examine the functional significance of FLIP downregulation under conditions of suppressed PI 3-kinase and Akt signaling, HUVECs were infected with AdTet-FLIP or AdTet-LacZ (MOI 2) in the presence of AdCMV-rtTA (MOI 10) and Dox (Figure 5). Restoration of FLIP expression had no effect on the frequency of cell death induced by wortmannin alone. However, exogenous FLIP completely blocked the increased apoptosis observed when cells were incubated with anti-Fas antibody under conditions of suppressed PI 3-kinase signaling. Parallel experiments with AdTet-LacZ did not affect apoptosis frequencies in the presence of wortmannin with or without anti-Fas antibody relative to control, demonstrating that FLIP expression, and not the viral expression system itself, was responsible for inhibiting Fas-mediated apoptosis in HUVECs under conditions of suppressed PI 3-kinase signaling.

FLIP Restoration Inhibits Depletion of Procaspase-8 When Cells Are Treated by Wortmannin and Fas Agonist

Cleavage of procaspase-8 is essential for Fas-mediated apoptosis. Therefore, depletion of procaspase-8 was assessed to corroborate that FLIP restoration inhibits the Fas-mediated apoptosis pathway under conditions of suppressed PI 3-kinase signaling. As shown in Figure 6A, treatment with wortmannin or anti-Fas antibody alone did not substantially change procaspase-8 levels, as assessed by Western blot analysis. In contrast, the simultaneous treatment of HUVECs with wortmannin and anti-Fas antibody led to a marked decrease in procaspase-8 levels, consistent with the propagation of a Fas-mediated death signal. However, prior infection of HUVECs with AdenoTet-FLIP (MOI 2) and AdenoCMV-rtTA (MOI 10 for each) blocked the depletion of procaspase-8 by the combination of wortmannin and anti-Fas antibody. The caspase-8 cleavage data were confirmed by a colorimetric assay of caspase-8 activity (Figure 6B). In this assay, wortmannin sensitized the cells to caspase-8 activation by anti-Fas antibody, and this activation was blocked when cells were transduced with FLIP.

Discussion

The PI 3-kinase/Akt signaling pathway is of central importance in endothelial cell biology. Recent reports have shown that this pathway is essential for endothelial cell differentiation, migration and NO production, key features of the angiogenic response. In addition, PI 3-kinase/Akt signaling also confers survival to endothelial cells in response to angiogenic cytokine stimulation, fluid shear stress, and matrix attachment signals. It is generally assumed that PI 3-kinase/Akt signaling promotes endothelial cell survival by suppressing the mitochondrial pathway of apoptosis via Akt-mediated phosphorylation of Bad and procaspase-9. In the present study, it is shown that this signaling pathway also blocks Fas-mediated apoptosis in endothelial cells. It is well established that endothelial cells are normally resistant to Fas-mediated apoptosis. The present study demonstrates that Akt signaling is an important determinant of
endothelial sensitivity to Fas-mediated death signals through its ability to modulate the expression of the caspase-8 inhibitor FLIP. It was shown that FLIP is downregulated under conditions that lead to diminished PI 3-kinase/Akt signaling in endothelial cells, including serum deprivation, wortmannin, and LY294002 treatment, or transduction with dominant-negative Akt. Importantly, the downregulation of FLIP induced by treatment with PI 3-kinase inhibitors could be reversed by transduction of constitutively active Akt. In contrast, modulation of Akt signaling had no detectable effect on cell surface Fas expression on HUVECs, whereas FasL expression was modestly upregulated and downregulated by dominant-negative and constitutively active Akt, respectively (data not shown). The functional significance of PI 3-kinase/Akt signaling in endothelial cell sensitivity to Fas-mediated apoptosis was indicated by the observation that cells become sensitive to killing by a Fas agonist antibody in the presence of wortmannin or dominant-negative Akt. Similarly, the functional significance of FLIP downregulation under these conditions was demonstrated by experiments showing that FLIP transduction prevented caspase-8 activation and endothelial cell apoptosis under conditions of PI 3-kinase inhibition.

There has been considerable confusion and controversy regarding the function of cellular FLIP. Confusion results, at least in part, from the isolation of multiple FLIP isoforms that arise from alternative splice patterns of a single gene. Moreover, the functions of these proteins has been controversial, with some groups reporting that they act as death activators and others finding that they inhibit apoptosis. Most of these studies have evaluated FLIP function by overexpressing FLIP by transient transfection experiments, in which levels of FLIP overexpression in the individually transduced cells were not assessed. To address this issue, we established a tetracycline-regulated adenoviral expression system that allows us to assess the effects of systematically varying FLIP expression levels on cell viability. In this system, one adenoviral vector expresses FLIP downstream from seven tetracycline operator sites, whereas the second adenoviral vector expresses the tetracycline-dependent trans-activator. Under optimal induction conditions, this system can produce far more robust transgene expression than is produced by conventional adenoviral vectors that express transgenes from the CMV promoter, yet it is relatively silent under noninduction conditions. Relative low levels of FLIP overexpression, achieved with a viral titer of MOI 2, protected endothelial cells from Fas-mediated apoptosis under conditions of suppressed PI 3-kinase signaling. Under these conditions, the majority of the cells were transduced with adenovirus, and levels of exogenous FLIP were similar to endogenous levels. Furthermore, exogenous FLIP did not induce toxicity at these viral titers. However, higher levels of FLIP expression (AdTet-FLIP MOI ≥10) produced apoptosis in both HUVECs and human VSMCs (data not shown). Under conditions of superphysiological FLIP expression, toxicity was associated with cleavage of procaspase-8 (data not shown). Taken together, these data suggest that excessive FLIP overexpression may lead to activation of caspase-8 because of forced aggregation and cleavage of procaspase-8 molecules, which display a low level of intrinsic caspase activity.

These data provide a mechanistic rationale that may explain previous observations of antagonism between Akt/PI 3-kinase and Fas signaling pathways. For example, Gibson et al have shown that epidermal growth factor stimulation protects epithelial cell lines from Fas-mediated caspase activation and apoptosis. In addition, PTEN heterozygous mutant mice display impaired Fas-mediated apoptosis and develop a polyclonal autoimmune disorder. Because PTEN encodes a phosphatase that opposes the PI 3-kinase reaction, these data implicate PI 3-kinase/Akt signaling as a negative regulator of Fas-mediated apoptosis. Further investigation is required to determine whether the regulation of FLIP can account for the PI 3-kinase/Akt-dependent suppression of Fas-induced apoptosis in these systems, although it has recently been shown that Akt signaling regulates FLIP expression in tumor cells.

Our observations are also relevant for endothelial cell biology because multiple angiogenic factors activate PI 3-kinase/Akt signaling, and endothelial cells are normally resistant to Fas-mediated apoptosis, although they express functional FasL on their cell surface. Thus, decreases in FLIP expression, which are due to diminished PI 3-kinase/Akt signaling, may lead to blood vessel regression. For example, it has been shown that the matrix-derived angiogenesis inhibitor canstatin specifically induces apoptosis in endothelial cells, and this toxicity has been correlated with a downregulation of FLIP. Furthermore, FLIP expression is diminished when endothelial cells are exposed to oxidized lipids, a condition that is associated with impaired blood vessel growth. Therefore, the regulatory mechanism described in the present study may be significant for maintenance of the endothelium and blood vessel growth.
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