

Vascular Endothelial Growth Factor Regulates Endothelial Cell Survival through the Phosphatidylinositol 3'-Kinase/Akt Signal Transduction Pathway

REQUIREMENT FOR Flk-1/KDR ACTIVATION*

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Vascular endothelial growth factor (VEGF) has been found to have various functions on endothelial cells, the most prominent of which is the induction of proliferation and differentiation. In this report we demonstrate that VEGF or a mutant, selectively binding to the Flk-1/KDR receptor, displayed high levels of survival activity, whereas Flt-1-specific ligands failed to promote survival of serum-starved primary human endothelial cells. This activity was blocked by the phosphatidylinositol 3'-kinase (PI3-kinase)-specific inhibitors wortmannin and LY294002. Endothelial cells cultured in the presence of VEGF and the Flk-1/KDR-selective VEGF mutant induced phosphorylation of the serine-threonine kinase Akt in a PI3-kinase-dependent manner. Akt activation was not detected in response to stimulation with placenta growth factor or an Flt-1-selective VEGF mutant. Furthermore, a constitutively active Akt was sufficient to promote survival of serum-starved endothelial cells in transient transfection experiments. In contrast, overexpression of a dominant-negative form of Akt blocked the survival effect of VEGF. These findings identify the Flk-1/KDR receptor and the PI3-kinase/Akt signal transduction pathway as crucial elements in the processes leading to endothelial cell survival induced by VEGF. Inhibition of apoptosis may represent a major aspect of the regulatory activity of VEGF on the vascular endothelium.

Angiogenesis and vascular remodeling occur throughout growth and development and involve both proliferation and regression of vascular endothelial cells. Although extensive research has been dedicated to the elucidation of the factors that induce endothelial cell proliferation and differentiation (for review, see Refs. 1, 2), surprisingly little is known about the mechanisms that regulate regression of blood vessels. Rapid degeneration of vascular structures occurs under various physiological or pathological circumstances. Apoptosis of endothelial cells probably contributes to the maturation of vascular granulation tissue into avascular scar tissue (3) and to the pruning of retinal vessels during development (4). Apoptosis-like changes in endothelial cells have been also observed in the

involving corpus luteum, breast (5), parotid glands undergoing pressure atrophy (6), and fibrotic lung lesions (7). However, experiments designed to study blood vessel regression *in vivo* have not been very successful so far at detecting apoptotic endothelial cells lining the blood vessels. This may be attributable, at least in part, to the fact that endothelial cells undergoing apoptosis tend to lose their attachment to the basement membrane. In addition, apoptotic endothelial cell may become the target of the immune system and thus are eliminated by phagocytes as soon as they manifest early signs of apoptosis (8). *In vitro*, apoptosis often follows withdrawal of a critical trophic factor. Accordingly, human umbilical vein endothelial (HUVE)¹ cells undergo apoptosis after loss of adhesion or serum deprivation (9, 10). Fibroblast growth factor and phorbol esters reduce the apoptosis of serum-deprived HUVE cells (11), and heat shock and endotoxin act together to increase apoptosis in porcine endothelial cells (12).

It has recently been shown that the survival signal mediated by various growth factors and cytokines may be dependent on the phosphatidylinositol 3'-kinase (PI3-kinase)/Akt signal transduction pathway (13–19). However, not all survival signals require PI3-kinase activity, and Akt (also referred as protein kinase B α or Rac α)-independent survival pathways exist (20–22). It was shown previously that vascular endothelial growth factor (VEGF) can induce PI3-kinase activity in a variety of endothelial cell (23–25), but so far a specific biological function of PI3-kinase in endothelial cells has not been demonstrated.

The endothelial cell-specific mitogen VEGF has been shown to be a key positive regulator of normal and abnormal angiogenesis (2, 26). The critical role of VEGF in the development of the vascular system is emphasized by embryonic lethality after loss of a single VEGF allele. A growing body of evidence indicates that VEGF may also act as a survival factor for newly formed blood vessels. In the developing retina, vascular regression in response to hyperoxia has been correlated with inhibition of VEGF release by glial cells (4). Furthermore, administration of anti-VEGF monoclonal antibodies results in regression of already established tumor-associated vasculature in xenograft models (27). More recently, using a tetracycline-regulated VEGF expression system in xenografted C6 glioma cells, it has been shown that decreased levels of VEGF produc-

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¹ The abbreviations used are: HUVE, human umbilical vein endothelial; PI3-kinase, phosphatidylinositol 3'-kinase; VEGF, vascular endothelial growth factor; rh, recombinant human; PIGF, placenta growth factor; PBS, phosphate-buffered saline; PI, propidium iodide; bFGF, basic fibroblast growth factor; sel, selective; KDR, kinase domain region.

tion lead to detachment of endothelial cells from the walls of preformed vessels in the tumor, and detached apoptotic endothelial cells were identified by means of double staining for Van Willebrand factor and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling staining (28).

VEGF exerts its biological effects by binding to its respective transmembrane receptors VEGF receptor 1 (Flt-1) and VEGF receptor 2 (Flk-1/KDR), both of which are expressed on endothelial cells specifically and contain a cytoplasmic tyrosine kinase domain. Knockout studies in mice revealed that both receptors are essential for the development of the embryonic vasculature, and mouse embryos null for either receptor died *in utero* between days 8.5 and 9.5 (29, 30). Stimulation of endothelial cells with VEGF demonstrated that VEGF induces phosphorylation of a variety of proteins, including phosphatidylinositol 3-kinase, Ras GTPase-activating protein, p190-rhoGAP, p62, phospholipase C- γ , the oncogenic adaptor protein NcK, p125 focal adhesion kinase, paxilin, and several others (23, 31–37; for review, see Ref. 38). Strong experimental evidence links Flk-1/KDR activation to VEGF-induced mitogenesis and angiogenesis (2). In contrast, the maintenance and survival function of VEGF has been associated with the Flt-1 receptor, because the Flt-1 mRNA is highly expressed in quiescent endothelium (39), whereas Flk-1/KDR is primarily expressed in proliferating vessels (40).

In the present study, we investigated the survival role of VEGF in HUVE cells cultured in serum-free conditions. We found that VEGF potently prevents apoptosis and promotes survival in this system. Using several approaches, we demonstrate the critical role of the PI3-kinase-Akt pathway in such effects. We also show that Flk-1/KDR, not Flt-1, is the primary mediator of such a signal.

EXPERIMENTAL PROCEDURES

Materials—Primary cultures of HUVE cells and human dermal microvascular endothelial cells were purchased from Cell Systems (Kirkland, WA). Wortmannin and dimethylsulfoxide were purchased from Sigma. ZVAD-fmk was purchased from Enzyme Systems Products (Dublin, CA). Cytomegalovirus-luciferase was purchased from Promega, and pGREEN LANTERN was from Life Technologies, Inc.

The KDR receptor-selective VEGF₁₆₅ mutant D63A, E64A, E67A has been previously described (41). The triple mutant R82E, K84E, H86E is a derivative of the Flt-1-selective R82A, K84A, H86A VEGF mutant (41), with ~10-fold higher selectivity, which results in an overall 1000-fold selective binding of Flt-1 over KDR. Wild-type recombinant human (rh) VEGF₁₆₅ and receptor-selective VEGF mutants were expressed in *Escherichia coli* and purified to homogeneity as described previously. The endotoxin content of the final purified material did not exceed 0.3 endotoxin units/mg of protein. rhPIGF₁₅₂ was expressed and purified as described previously (42). Recombinant proteins were quantified by amino acid analysis. Medium 199 was purchased from Life Technologies, Inc. CS-C medium was from Cell Systems. EB medium was from Clonetics (San Diego, CA). Flag-Akt179 was generated by mutation of the lysine 179, which is critical for ATP binding to alanine, which resulted in a kinase death Akt construct (43). Flag-Akt 2D was generated by mutating threonine 308 and serine 473 to asparagine, which results in a constitutively active Akt form (44). PIGF used in the Akt phosphorylation assay was purchased from R & D Systems, Inc (Minneapolis, MN).

Cell Culture—HUVE cells were maintained in CS-C complete medium containing 10% fetal bovine serum and mitogens, according to the instructions of the supplier (Cell Systems). Twenty-four hours before serum starvation, cells were trypsinized and plated to a density of $20\text{--}25 \times 10^3$ cells/cm² in 10- or 6-cm dishes for fluorescence-activated cell sorting analysis and in 6-cm dishes for biolistic transfection experiments. Immediately before the experiment, cells were washed twice with phosphate-buffered saline (PBS). For serum starvation, CS-C medium without serum and without mitogen, complemented with 0.1% bovine serum albumin, was added during the indicated length of time.

After biolistic transfection, cells were allowed to recover for 24 h in medium containing 10% serum. After washing the cells twice with Tris-buffered saline, minimum medium (without mitogen and serum)

was added, and the levels of apoptosis were analyzed 9 h after induction of serum starvation.

For survival assays, HUVE cells were seeded at the density of 2×10^4 cells per well in 48-multiwell plates in the presence of EB medium plus 10% FBS in 0.5 ml of M199 medium containing 10% serum. After 8–12 h, cells were washed twice with serum-free M199, and cells were incubated in serum-free M199 complemented with 0.1% bovine serum albumin, with or without growth factors. After 4 or 5 days, 25 μ l of alamar blue was added to each well. After a 2-h incubation time, plates were read at 405 nm in an enzyme-linked immunosorbent assay plate reader. Alternatively, cells were dissociated by exposure to trypsin, and the cell number was determined.

Gene Transfer of HUVE Cells by Microprojectile Bombardment—Gene transfer was performed using the Bio-Rad 500 optimization kit in the Biolistic PDS-1000/He* particle delivery system (Bio-Rad). In a series of pilot experiments using luciferase reporter gene DNA, parameters were established to achieve the highest levels of luciferase activity in primary HUVE cell cultures (data not shown): particle size, 1.6 μ m; rupture disc, 1100 p.s.i. bombardment chamber vacuum, 25 mm Hg; the microcarrier launch assembly was positioned in shelf position 1, the target shelf was at position 3 (from the top). Gold particles were prepared according to the manufacturer's recommendations and stored as 50- μ l aliquots for 3–4 months at -20°C . Aliquots were coated with DNA immediately before gene transfer following the manufacturer's recommendations. Briefly, for five samples, a 50- μ l aliquot of gold particles was vortexed for 3 min. and 15 μ l of plasmid DNA (1 mg/ml) was added. Vortexing was continued for 2 min. Thereafter, 50 μ l of 2.5 M CaCl₂ solution was added to the mix. After 2 min of additional vortexing, 20 μ l of a freshly prepared 0.1 M spermidine solution was added, followed by 2 min of constant vortexing. The mixture was allowed to settle for 1 min before tubes were centrifuged for 5 s in a table-top microcentrifuge. The supernatant was removed, and the gold pellet was washed first with 140 μ l of 70% ethanol followed by 140 μ l of 100% ethanol. After removing the supernatant, the gold particles were taken up in 55 μ l of 100% ethanol, and 10 μ l was used per biolistic transfection. Immediately after bombardment, 5 ml of complemented medium was added.

Analysis of Endothelial Cell Apoptosis—For fluorescence-activated cell sorting analysis, cells were stained by fluorescein isothiocyanate-conjugated annexin V and by the fluorescent dye propidium iodide (PI). Cells negative for both PI and annexin V staining are live cells; PI-negative, annexin V-positive staining cells are early apoptotic cells; PI-positive, annexin V-positive staining cells are primarily cells in a late stage of apoptosis (45). Cells of one 6- or 10-cm dish were harvested and incubated on ice in annexin- and PI-containing binding buffer for 10 min and further processed as described previously (46).

Time lapse video recording was done with an Optronics DEI-470T charge-coupled device video camera hooked up to a Panasonic AG-6730 time lapse cassette recorder and a Nikon Diaphot-300 inverted microscope at a magnification of 20 \times . The graphics program Adobe Photoshop 4.0 was used to generate the picture.

Akt Phosphorylation Assay—HUVE cells were grown essentially as described in cell culture. Cells were split to a density of 14×10^3 cells/cm² 24 h before start of serum starvation. Cells were washed twice with PBS, and 5 ml of CS medium without serum and mitogen (Cell Systems), complemented with 0.1% bovine serum albumin, was added. 18–20 h after start of serum starvation, cells were washed with PBS, and 5 ml of fresh starvation medium was added, containing wortmannin (30 nM) where indicated. 2–3 h later, growth factors were added at the indicated amounts, and incubation occurred for 15 min in a tissue culture incubator before lysing the cells with 100 μ l of SDS gel loading buffer. For detection of the levels of Akt phosphorylation, whole-cell extracts were treated essentially as described in the instruction manual included in the PhosphoPlus Akt (Ser⁴⁷³) antibody kit (New England Biolabs, Beverly, MA). Blocking of the membrane was done by incubation overnight at 4 $^\circ\text{C}$; incubation with the phospho-specific Akt (Ser⁴⁷³) antibody (rabbit polyclonal IgG, affinity purified) occurred at room temperature for 3 h; and the incubation with the secondary antibody (anti-rabbit secondary antibody conjugated to horseradish peroxidase) was for 1 h at room temperature. The immune complexes were detected with the enhanced chemiluminescence detection system. The chemiluminescent detection was provided by the manufacturer (New England Biolabs, Beverly, MA).

RESULTS

Growth Factor Removal Induces Apoptotic Endothelial Cell Death—To investigate the survival activity of VEGF relative to

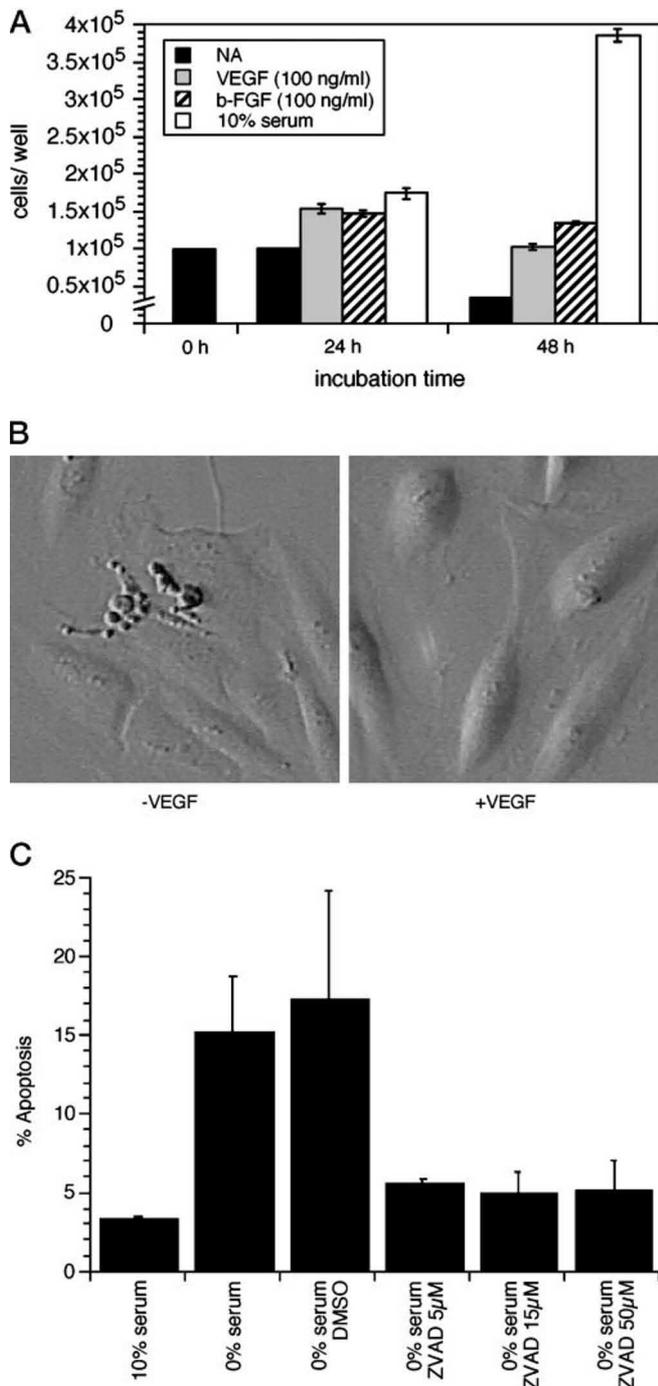


FIG. 1. Primary endothelial cells in culture undergo apoptotic cell death after serum starvation. *A*, HUVE cells were plated at the density of 1.5×10^5 cells per well in six-well dishes and cultured for 24 h in the presence of 10% serum. Cells were then washed twice with PBS before adding serum-free medium containing 0.1% bovine serum albumin complemented with either VEGF (100 ng/ml) or bFGF (100 ng/ml). 10% serum was added to control wells. 24 or 48 h after changing to serum-free medium, cells were trypsinized and counted in a cell counter. Error bars indicate the S.D. of triplicate plates. *B*, HUVE cells were plated at the density of 20,000 cells/cm² and cultured for 24 h in medium containing 10% serum. Immediately after changing to serum-free medium, rh-VEGF₁₆₅ was added (100 ng/ml), and a photograph was taken after 24 h. Photographs were taken with a computerized video imaging system. *C*, HUVE cells were plated at the density of 200,000 cells/cm² and grown for 24 h in the presence of 10% serum. Two hours before switching to serum-free conditions, dimethylsulfoxide (control) or ZVAD was added. Cells were washed twice in PBS, and medium containing 10 serum, 0% serum, dimethylsulfoxide (DMSO), or ZVAD in the indicated amounts was added 24 h after initiation of serum starvation. Cells were then harvested, and apoptotic cell death was determined by flow cytometry by annexin V

that of basic fibroblast growth factor (bFGF), which was previously found to be a survival factor for endothelial cells (11), we incubated HUVE cells in serum-free medium in the presence of VEGF (100 ng/ml) or bFGF (100 ng/ml). Control cells were cultured in the presence of 10% serum, which stimulated proliferation. As illustrated in Fig. 1A, the number of cells decreased significantly after 24 h of serum starvation, whereas cell numbers remained relatively constant over 48 h of incubation in the presence of VEGF or bFGF. These findings indicated that VEGF can induce a survival response in HUVE cells to similar levels as bFGF. In the absence of VEGF, extensive membrane blebbing, loss of contacts with neighboring cells, shrinking of the cytoplasm, disintegration into small vesicles, also called apoptotic bodies, and ultimately detachment of cells from the culture dish were observed by means of time lapse video recording (Fig. 1B). These features are indicative of apoptotic cell death. Under such conditions, we did not observe any obvious morphological differences between VEGF-treated cells and cells exposed to 10% serum (data not shown). To determine whether the endothelial cell loss was a consequence of apoptotic cell death, we analyzed HUVE cells for binding to annexin V, which serves as a marker for early apoptosis (46). As early as 16 h after growth factor removal, we observed a significant increase in the number of apoptotic, annexin V-positive cells, which continued over time, and after 48 h, 50–80% of the cells had undergone apoptotic cell death (Fig. 2B and data not shown).

To test whether the observed induction of endothelial cell apoptosis was mediated by the universal cell death machinery, we added ZVAD-fmk, an inhibitor of caspases 1 (interleukin-1 β -converting enzyme) (ICE) and 3 (CPP32) (48) to HUVE cells. Apoptosis induced by serum starvation was completely blocked after 24 h by ZVAD-fmk at concentrations as low as 2 μ M. These findings indicated that endothelial cells grown in culture underwent caspase-dependent, programmed cell death on serum withdrawal.

VEGF Is a Survival Factor for Primary Human Endothelial Cells: Requirement for Flk-1/KDR Activation—VEGF reduced the serum starvation-induced apoptosis of HUVE cell with a bell-shaped dose-response curve, with a maximal effect between 10 and 100 ng/ml (Fig. 2A). Higher concentrations resulted in lower reduction of apoptosis. Human dermal microvascular endothelial cells responded at even lower VEGF levels (0.3 ng/ml; data not shown). Similar bell-shaped dose-response curves in response to VEGF in cell-based assays have been reported before (49). Although the cause of this effect is not entirely clear, a current thought is that a vast excess of ligand over receptor results in a reduced effectiveness of receptor dimerization and signaling, such that an increasing number of receptors remain in a monomeric form after ligand binding. However, the percentage of apoptotic cells detected in the presence of 10% serum was usually lower compared with cells grown in the presence of VEGF, at all concentrations tested, suggesting that additional factors present in the serum render the cells more resistant to apoptosis (9). Whether this is a consequence of increased survival activity or is attributable to the ongoing proliferation, or both, remains to be analyzed.

We next attempted to determine the role of the two VEGF receptors in mediating the survival activity induced by VEGF. We first tested placenta growth factor (PlGF), a member of the VEGF family of proteins, which binds Flt-1 with high affinity but fails to bind Flk-1/KDR. Previous studies have shown that

binding and propidium iodide staining, as described under "Experimental Procedures." Bars represent means \pm S.E. of two independent experiments conducted with different preparations of endothelial cells.

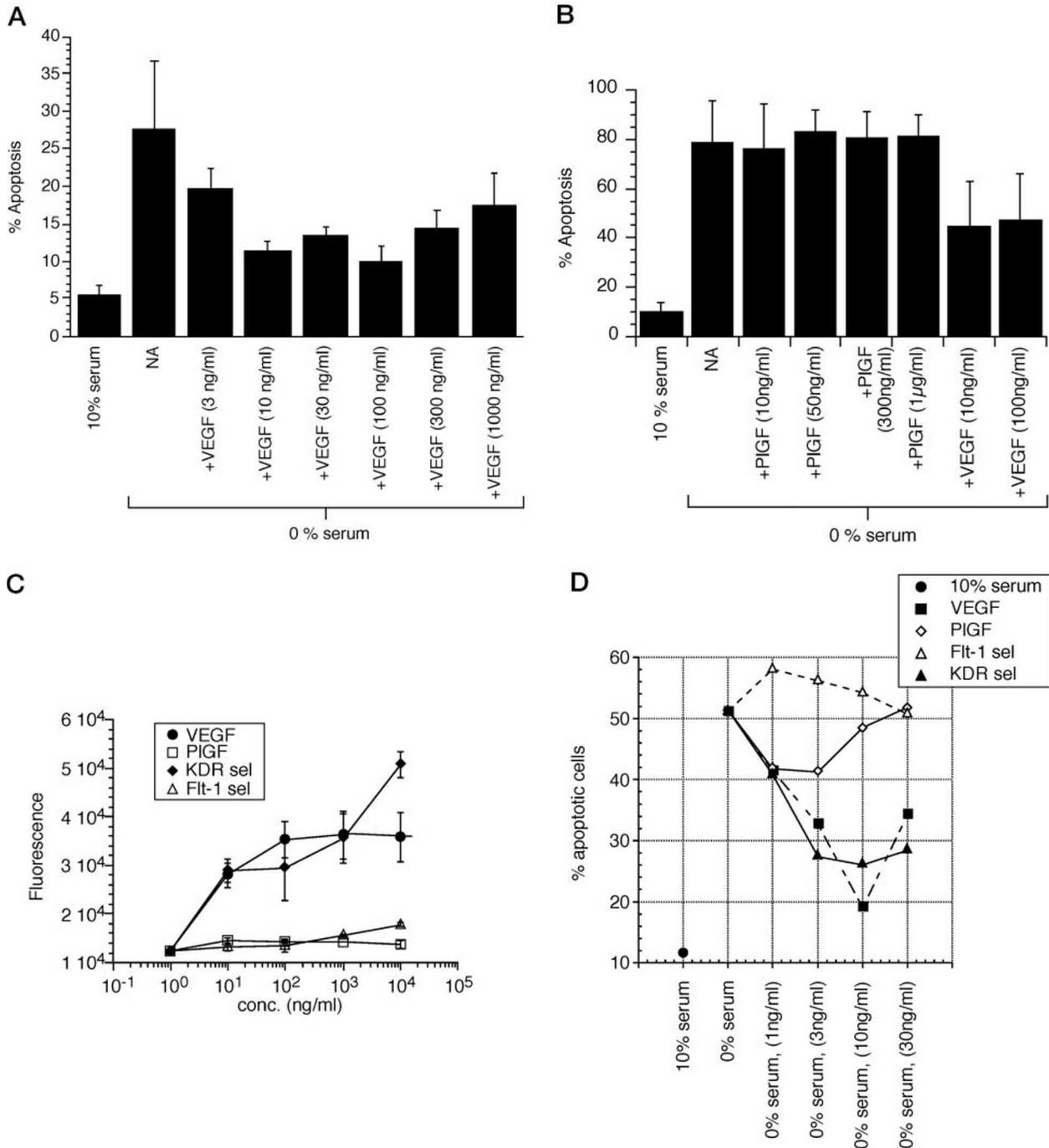


FIG. 2. VEGF is a survival factor for primary HUVE cells in culture. *A*, HUVE cells were grown for 24 h in the presence of 10% serum and rhVEGF₁₆₅ was added immediately after changing to serum-free conditions. Apoptotic cell death was determined after 24 h by flow cytometry for annexin V and propidium iodide, as described under “Experimental Procedures.” Bars represent means ± S.E. of two independent experiments conducted with different preparations of endothelial cells. *B*, HUVE cells were cultured for 48 h in the presence of the indicated amounts of rhVEGF₁₆₅ or PIGF. Apoptotic cell death was determined as described above. *C*, After 8–12 h in serum-containing media, HUVE cells were washed twice and incubated in 0% serum in the presence of rhVEGF₁₆₅ (100 ng/ml), Flt-1-sel (100 ng/ml), which is an Flt-1-selective mutant VEGF₁₆₅ form, or KDR-sel (100 ng/ml), which binds Flk-1/KDR 100-fold more selective than VEGF (for descriptions see “Materials” under “Experimental Procedures”). The relative number of cells was quantified after 4 days in culture by alamar blue staining, as described under “Experimental Procedures.” Error bars represent the S.E. of triplicate analysis of one representative experiment. *D*, HUVE cells were cultured for 36–48 h in the presence of the indicated concentrations of VEGF, PIGF, and the receptor-selective mutant VEGF forms Flt-1-sel and KDR-sel. The number of apoptotic cells was determined as described in *A*. Data points represent the average of three independent experiments with different HUVE cell preparations.

PIGF induces little or no mitogenic activity on HUVE cells, nor does it induce tyrosine phosphorylation of Flt-1 (42). In our survival assay, PIGF did not reduce the number of apoptotic HUVE cells at all concentrations tested, as assessed by binding to annexin V (Fig. 2, *B* and *D*) or alamar blue staining of cells (Fig. 2*C*). To further confirm these findings, we tested receptor-

selective VEGF mutants, which bind preferentially to either one of the VEGF receptors, KDR/Flk or Flt-1, respectively. Previous studies have shown that mutants defective in KDR binding lack the ability to induce proliferation, whereas Flt-1 binding-deficient mutants are effective mitogens (41). We found that the KDR-selective VEGF₁₆₅ mutant (KDR-sel) ex-

erted a similar survival activity as wild-type VEGF in the 4-day survival assay. However, we could not detect any significant survival effect by the Flt-sel mutant (Fig. 2C).

In agreement with these findings, neither PlGF nor the Flt-sel VEGF mutant led to a reduction in the amount of apoptotic HUVE cells after 36–48 h, as assessed by annexin V binding (Fig. 2D). In contrast, the KDR-sel mutant was as potent as wild-type VEGF₁₆₅. From these findings we conclude that activation of Flk-1/KDR is necessary to mediate the survival-promoting activity of VEGF.

The Antiapoptotic Activity of VEGF Is Mediated by PI3-kinase—The PI3-kinase-specific inhibitor wortmannin is known to block the protective action of nerve growth factor (50) and platelet-derived growth factor in serum-deprived PC12 cells (14). These findings implicated an important role of the PI3-kinase pathway in apoptosis prevention by growth factors. To examine the involvement of PI3-kinase in VEGF-mediated protection, we incubated HUVE cells with the two specific inhibitors of PI3-kinase, wortmannin and the structurally unrelated synthetic compound LY294002 (51). As assessed by the numbers of apoptotic cells, wortmannin (30 nM) and LY294002 (100 nM) almost completely blocked the protective effect of VEGF (Fig. 3, A and B). Both agents slightly enhanced the degree of apoptosis observed in the absence of VEGF, possibly resulting from the inhibition of basal PI3-kinase activity present in serum-starved cells, similarly to what has been observed in other systems (16, 18, 52). Our results support the conclusion that the antiapoptotic activity of VEGF on endothelial cells is mediated via PI3-kinase.

The Flk-1/KDR-selective VEGF Mutant but Not Flt-1-selective Ligands Triggers Akt Phosphorylation—To test whether the PI3-kinase effector protein Akt is involved in the survival response, we analyzed whole-cell lysates of HUVE cells treated with VEGF for activation of Akt by phosphorylation at serine 473 by means of a phospho-specific antibody. Akt is activated by phospholipid binding, and phosphorylation occurs within the activation loop at threonine 308 and within the carboxyl terminus at serine 473. In initial time course experiments we found Akt to be maximally activated 15–30 min after exposure to VEGF. The response gradually decreased after prolonged incubation (data not shown). Incubation of HUVE cells with increasing concentrations of VEGF led to a dose-dependent activation of Akt, with highest levels between 1 and 10 ng/ml (Fig. 4A). Densitometric analysis of the signals displayed in Fig. 4 revealed a maximal 5.7-fold stimulation by VEGF at 1 ng/ml. At the same concentration, KDR-sel-stimulated cells displayed a 3.6-fold stimulation of Akt phosphorylation. Neither PlGF nor Flt-sel led to any increase in Akt activity at all concentrations tested (Fig. 4, C and D). Interestingly, the PI3-kinase inhibitor wortmannin (30 nM) completely abolished the Akt activation in response to VEGF or KDR-sel. In fact, Akt levels fell below unstimulated background. This latter finding correlates well with the slight increase in the amount of apoptotic cells observed in the presence of wortmannin alone, as shown in Fig. 3A. The reduction of background Akt activity in the presence of wortmannin or LY has been observed in other systems (17, 53) and is probably attributable to the low constitutive PI3-kinase background activity in HUVE cells cultured in serum-free conditions.

The Serine-Threonine Kinase Akt/Protein Kinase B Is Necessary and Sufficient to Transduce the Survival Signal Induced by VEGF—To verify that Akt, the downstream effector of PI3-kinase, is responsible for the survival response, we transiently transfected HUVE cells with expression vectors coding for wild-type Akt (Flag-Akt) or two mutant forms. Flag-Akt 179A represents a catalytically inactive mutant that contains a point

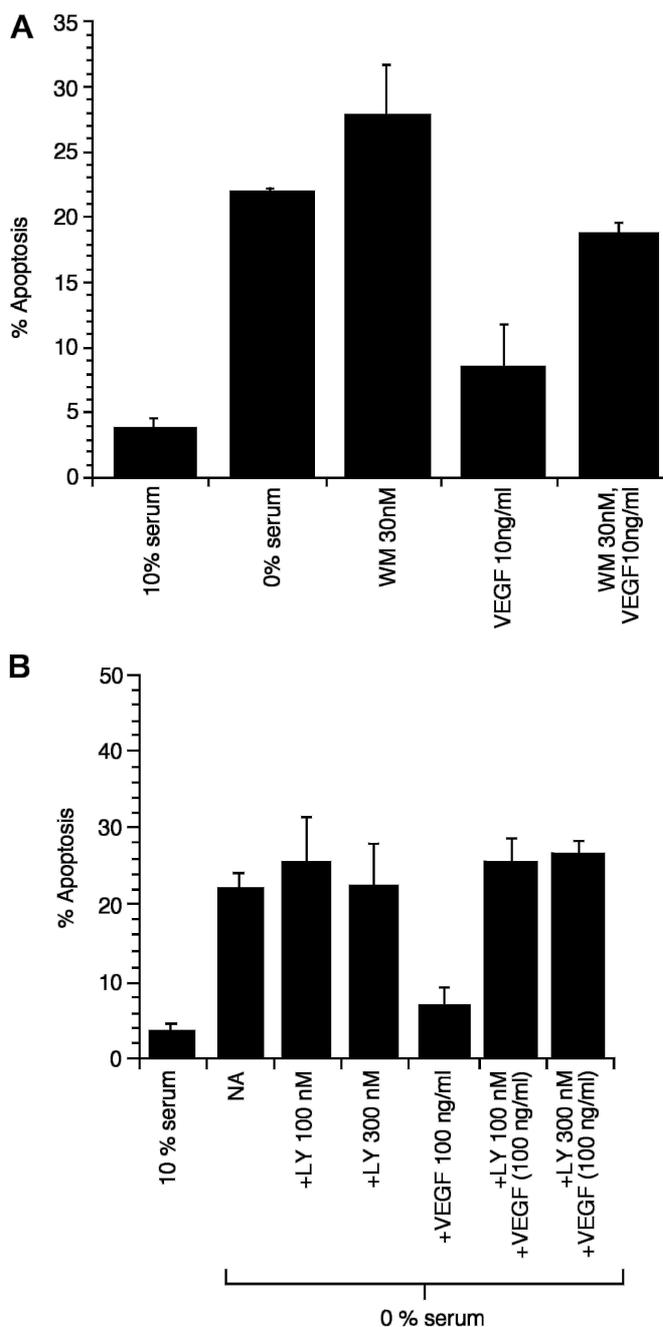


FIG. 3. The survival activity of VEGF is inhibited by the PI3-kinase inhibitors wortmannin and LY294002. A 2 h before serum starvation, wortmannin (WM) was added to HUVE cells grown in the presence of 10% serum for 24 h. After washing the cells twice with PBS, media containing 0% serum and the indicated amounts of VEGF and wortmannin were added. Cells were harvested after 24–36 h, and apoptotic cell death was determined by flow cytometry for annexin V and propidium iodide. Bars represent means \pm S.E. of two independent experiments conducted with different preparations of endothelial cells. B, immediately after exposing HUVE cells to serum starvation conditions, the PI3-kinase inhibitor LY294002 (LY) was added to the cells. Two hours later, VEGF was added to the medium, and cells were cultured for additional 24–36 h before harvest. Apoptotic cell death was determined as described above. Bars represent means \pm S.E. of two independent experiments conducted with different preparations of endothelial cells.

mutation in the ATP binding site (43) and Flag-Akt 2D encodes a constitutively active Akt mutant (44). Transfected endothelial cells were identified after co-transfection with an expression vector for green fluorescent protein in a fluorescence microscope and classified by their morphology. Healthy

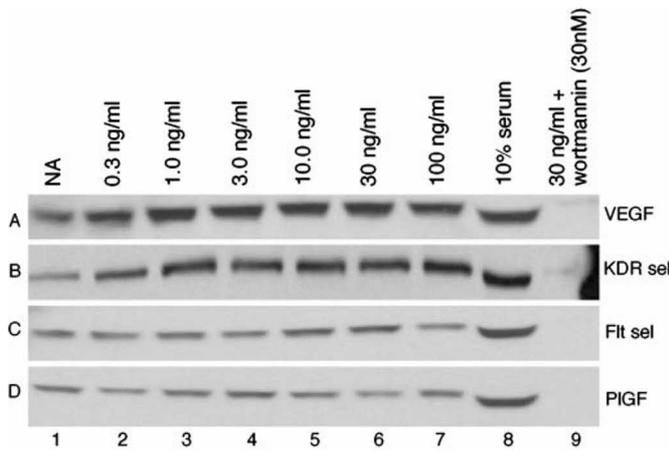


FIG. 4. Akt is activated by Flk-1/KDR but not by Flt-1-specific ligands. Akt activation assay was performed as described under "Experimental Procedures." *A*, blot of HUVE cell extracts treated with various amounts of rhVEGF₁₆₅ for 15 min (lanes 2–7). In lane 8, cells were exposed to 10% serum, reflecting maximal Akt activation. In lane 9, HUVE cells were exposed to VEGF (30 ng/ml) in presence of wortmannin (30 nM). *B*, blot of HUVE cell extracts treated with various concentrations of KDR-sel VEGF mutant for 15 min (lanes 2–7). In lane 9, HUVE cells were exposed to a KDR-specific VEGF mutant (*KDR sel*, 30 ng/ml), in the presence of wortmannin (30 nM). *C*, blot of HUVE cell extracts treated with various concentrations of Flt-selective VEGF mutant (*Flt sel*), for 15 min (lanes 2–7). In lane 9, HUVE cells were exposed to Flt-sel (30 ng/ml) in the presence of wortmannin (30 nM). In lane 9, HUVE cells were exposed to PIGF (30 ng/ml) in the presence of wortmannin (30 nM). *D*, Western blot of HUVE cell extracts treated with various concentrations of PIGF for the duration of 15 min (lanes 2–7). NA, cells grown in the absence of serum and growth factors.

endothelial cells appear flat and well attached to the plate, forming the classical cobblestone pattern. Apoptotic endothelial cells are rounded, and some are fragmenting with small cytoplasmic blebs (Fig. 1B; Refs. 54, 55). When we stained cells with the DNA dye bisbenzimidazole (Hoechst 33258), apoptotic endothelial cells could be easily identified by the pronounced nuclear condensations (data not shown). We first examined whether the constitutively active Flag-Akt 2D form was sufficient to mediate survival of serum-starved endothelial cells. As shown in Fig. 5, biolistic transfection of the Flag-Akt 2D construct prevented endothelial cell apoptosis in the absence of VEGF and promoted survival to similar levels as seen when cells were grown in the presence of VEGF or 10% serum. Moreover, transfection of endothelial cells with the dominant-negative Flag-Akt 179A construct blocked VEGF survival activity completely. In the absence of VEGF, a weak increase in apoptosis was observed in cells transfected with Flag-Akt 179A, which probably reflects inhibition of constitutive Akt activity in serum-starved cells. Our findings indicate that Akt is critically involved in mediating the VEGF effects on endothelial cell survival.

DISCUSSION

The elucidation of the mechanisms responsible for blood vessel regression is critical to address several fundamental biological questions. This process is prominent not only in the course of organogenesis and organ remodeling during embryonic life but also in a variety of physiological situations in the adult, such as corpus luteum regression (56) and uterine or breast involution after delivery (5). Over the last few years, several inhibitors of angiogenesis have been identified, including angiostatin (57) and endostatin (58). These agents are able to inhibit tumor growth by suppressing angiogenesis in xenograft models. However, it is presently unknown whether these molecules participate in the physiological regulation of blood vessel regression. Recently, members of the family of

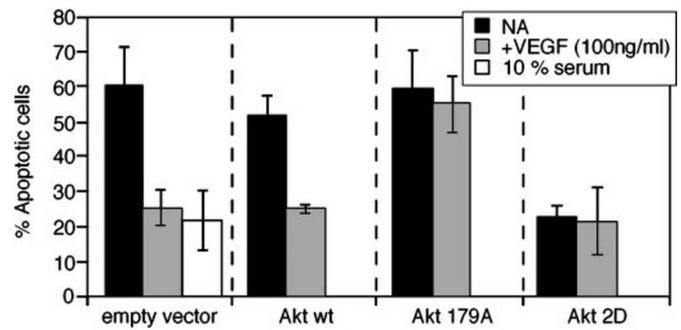


FIG. 5. Akt is necessary and sufficient to mediate the survival signal induced by VEGF. HUVE cells were plated at the density of 7×10^5 per 6-cm culture dish and cultured in the presence of 10% serum 24 h before biolistic transfection. Per dish, 3 μ g of expression vector encoding cytomegalovirus-driven green fluorescent protein (pGREEN LANTERN) and 3 μ g of plasmid DNA encoding the indicated Akt form or empty vector (cytomegalovirus-luciferase) were used per dish. Cells were allowed to recover for 24 h in presence of 10% serum after transfection. Serum starvation was induced by changing the medium to 0% serum, and cells were then cultured in the presence of VEGF (100 ng/ml) or in 10% serum for 12 h. The number of green fluorescent protein-positive and apoptotic cells was assessed phenotypically by the characteristic nuclear condensation and membrane morphology in a defined area of the culture dish (54). The percentage of apoptotic endothelial cells was calculated by dividing the total amount of green fluorescent protein-positive cells by the amount of apoptotic, green fluorescent protein-positive cells. Data shown are the means \pm S.E. of two independent experiments using different passage number endothelial cells. NA, cells grown in the absence of serum and growth factors.

Tie-2 ligands such as angiopoietin-2 have been shown to be involved in blood vessel remodeling and pruning, at least during embryonic development (59).

Increasing evidence supports the concept that the withdrawal of a positive effector such as VEGF is sufficient to result in blood vessel regression in various *in vivo* systems, including tumors (60) and developing organs (4, 28, 56). These studies demonstrated the presence of an inverse correlation between VEGF expression and the levels of vessel regression, implicating VEGF as an important survival factor for endothelial cells present in newly formed vasculature.

Using an *in vitro* model system of serum-starved HUVE cells, we attempted to elucidate the signal transduction pathways resulting in VEGF-mediated survival. The HUVE cells used in our experiments consisted of pooled primary isolates from 300 individual donor umbilical veins and thus are likely to represent an average population of endothelial cells. Both VEGF receptors, Flk-1/KDR and Flt-1, were previously found to be expressed on HUVE cells by cell binding and cross-linking studies with VEGF or the Flt-1-specific ligand PIGF (35, 42) or by real-time reverse transcription-polymerase chain reaction analysis (61). We first verified that serum removal indeed resulted in apoptotic cell death. This was confirmed by a series of morphological changes consistent with apoptosis, as evidenced by time lapse video recording. Furthermore, the finding that the caspase inhibitor ZVAD-fmk completely prevented apoptosis and mimicked VEGF activity demonstrates that this process is mediated by the universal cell death machinery.

In our survival assay, we found decreased levels of VEGF-dependent survival when cells were exposed to wortmannin or LY 294002, two potent inhibitors of PI3-kinase. Activation of PI3-kinase increases the intracellular amounts of phosphatidylinositol-3,4,5-bisphosphate and phosphatidylinositol-3,4,5-triphosphate, which positively regulates Akt by binding to the pleckstrin homology domain of Akt. Akt activation by growth factors requires PI3-kinase activity (62). In our experiments we found that Akt becomes phosphorylated after VEGF stimulation, and such activation could be inhibited by the PI3-kinase-

specific inhibitor wortmannin. In a previous report, PI3-kinase was found to be tyrosine phosphorylated after exposure of endothelial cells to VEGF (23). However, another report failed to detect induction of PI3-kinase after stimulation of HUVE cells by VEGF (34). These latter findings do not necessarily conflict with our data, because those experiments were conducted in presence of 2% fetal bovine serum, which may induce the PI3-kinase/Akt pathway to high levels, which cannot be further increased by VEGF. Interestingly, Xia *et al.* (24) recently reported that PI3-kinase is activated 2.1-fold by VEGF in cultured endothelial cells but is not required for VEGF-dependent proliferation.

The serine-threonine protein kinase Akt (63) is one of the major targets of PI3-kinase-generated signals. The ability of activated Akt to promote survival was found in fibroblasts (18) and PC12 pheochromocytoma cells (64) and neuronal cells (16). A number of different growth factors have been shown to rapidly activate Akt via PI3-kinase activation, such as platelet-derived growth factor, epidermal growth factor, bFGF, insulin, and insulin-like growth factor 1 (43, 65–69). In our experiments, VEGF and a mutant VEGF form, which binds to the Flk-1/KDR receptor specifically, could strongly induce Akt phosphorylation, and this induction was PI3-kinase-dependent. Moreover, in transient transfection experiments, the kinase-inactive Akt mutant, which has been suggested to act in a dominant-negative manner (43), was found to block VEGF survival activity on endothelial cells. These findings constitute, to the best of our knowledge, the first direct demonstration of a role played by the PI3-kinase/Akt pathway in mediating a VEGF biological activity.

Although the significance of VEGF and its endothelial cell-specific receptors in angiogenesis is well established, the signal transduction cascades initiated by the two known VEGF receptors resulting either in proliferation or in survival of endothelial cells are incompletely characterized.

Based on gene knockout experiments in mice and several other studies, it can be deduced that Flk-1/KDR is critical for VEGF-mediated angiogenesis, both in the developing and in the adult animal (29, 70). In contrast, Flt-1 appears to be primarily involved in endothelial cell morphogenesis, at least during embryonic development (30). Although the role of Flt-1 in the adult animal is less clearly defined, there is increasing evidence that Flt-1 is able to transduce a signal and mediate a biological response. Flt-1, but not Flk-1/KDR, has been implicated in monocyte migration in response to VEGF or PlGF via a GTP-dependent signaling pathway (71, 72). Furthermore, the finding that Flt-1 mRNA is expressed in both proliferating and quiescent endothelial cells suggests a role for this receptor in the maintenance of endothelial cells (39).

The motif YXXM required for binding of the SH2-domain of the p85 regulatory subunit of PI3-kinase is absent from the noncatalytic regions of the intracellular domain of Flt-1 as well as from Flk-1/KDR. Using the yeast two-hybrid system, two groups reported independently an association between tyrosine 1213 of the Flt-1 carboxyl terminus, present within the tyrosine kinase domain and the p85 subunit of phosphatidylinositol 3-kinase. (47, 73). However, when we tested PlGF or an Flt-1-specific VEGF mutant form in different assay systems, we could not detect any significant survival effect. The same mutants did not induce Akt phosphorylation, as assessed by Western blotting using an antibody that specifically recognizes the phosphorylated Akt. Likewise, we were unable to detect any change in the percentage of annexin V-positive, apoptotic endothelial cells in response to the Flt-1-selective ligands. These findings lead us to the conclusion that the Flt-1 receptor, at least when present in the homodimeric form, is not sufficient to

mediate survival activity. In contrast, a KDR-selective VEGF mutant completely mimicked the antiapoptotic and survival-promoting effects of VEGF and led to strong activation of Akt via PI3-kinase. Given the absence of a consensus binding site on the cytoplasmic domain of Flk-1/KDR, we speculate on the existence of an adaptor molecule mediating the activation of p85 by Flk-1/KDR. We consider the possibility that only heterodimeric VEGF receptors consisting of Flt-1 and Flk-1 subunits would allow for p85 binding and activation to be very unlikely, because the KDR-selective mutant was sufficient to induce survival and Akt activation.

In conclusion, our findings provide evidence that VEGF survival signals in endothelial cells are mediated by the Flk-1/KDR receptor through the PI3-kinase/Akt signal transduction pathway. Further delineation of this signaling pathway may yield attractive targets for cancer therapy aimed to induce endothelial cell apoptosis and blood vessel regression.

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