Angiopoietin-1 Inhibits Endothelial Cell Apoptosis via the Akt/Survivin Pathway*

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A productive angiogenic response must couple to the survival machinery of endothelial cells to preserve the integrity of newly formed vessels. Angiopoietin-1 (Ang-1) is an endothelium-specific ligand essential for embryonic vascular stabilization, branching morphogenesis, and post-natal angiogenesis, but its contribution to endothelial cell survival has not been completely elucidated. Here we show that Ang-1 acting via the Tie 2 receptor induces phosphorylation of the survival serine-threonine kinase, Akt (or protein kinase B). This is associated with up-regulation of the apoptosis inhibitor, survivin, in endothelial cells and protection of endothelium from death-inducing stimuli. Moreover, dominant negative survivin negates the ability of Ang-1 to protect cells from undergoing apoptosis. The activation of anti-apoptotic pathways mediated by Akt and survivin in endothelial cells may contribute to Ang-1 stabilization of vascular structures during angiogenesis, in vivo.

During angiogenesis, endothelial cells receive cues from growth factors to initiate mitosis, migration, and organization of endothelial cells into primitive angiotubes and patent vascular networks (1, 2). These processes critically depend on preservation of endothelial cell viability. Disruption of endothelial cell-matrix contacts or interference with extracellular survival signals is sufficient to initiate caspase-dependent apoptosis in endothothelium, culminating with rapid involution of vascular structures (3, 4). Unlike most angiogenic regulators, including fibroblast growth factor or vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1) does not stimulate late endothelial cell growth but rather promotes stabilization of vascular networks and branching morphogenesis in vivo and in vitro (5–8). Little is known about the signaling requirements of these responses, and the mechanism(s) of Ang-1-induced cytoprotection are unknown (7, 9).

The major goal of this paper was to elucidate a potential link between endothelial cell viability and maintenance of angiogenesis by examining the ability of Ang-1 to activate the anti-apoptotic serine-threonine kinase, Akt (or protein kinase B). Moreover, we examined the relationship between Ang-1, Akt activation, and the expression of the anti-apoptotic genes, bel-2 and survivin, in cultured microvascular endothelial cells (MVECs).

MATERIALS AND METHODS

Cell Culture and Reagents—Bovine MVEC (Vec Technologies, Rensselaer, NY) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1-glutamine, and antibiotics (penicillin and streptomycin). Cells (up to passage 12) were used for the experiments. In experiments examining endogenous survivin expression, human umbilical vein endothelial cells (HUVEC) were used, because the survivin antibody recognized human survivin better than bovine survivin. HUVEC were cultured on gelatin-coated tissue culture flasks in M199 medium containing 20% fetal bovine serum, 50 μg/ml endothelial cell growth supplement (a commercial preparation that contains mainly acidic fibroblast growth factor), 100 μg/ml porcine heparin, 10 units/ml penicillin, and 100 μg/ml streptomycin. Two to three individual donors were pooled at passage one and used up to passage three. Both MVEC and HUVEC cultures had typical cobblestone morphology and stained uniformly for von Willebrand factor, as assessed by indirect immunofluorescence. Angiopoietin-1 and -2 and soluble recombinant Tie 1 and 2 receptors were provided by Regeneron. A recombinant form of Ang-1 was used in all of the experiments. This form of Ang-1 differs from the native Tie 2 ligand in that it possesses a modified NH2-terminal sequence and a mutation in Cys264 that make it easier to produce and purify.

Akt Phosphorylation and Activity—Cells were washed twice with PBS and lysed with cell lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 2 mM Na3PO4, 1 mM mercaptoethanol, 10 mM MgCl2, 10 mM MnCl2). Cells were washed twice with PBS and lysed with cell lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 2 mM Na3PO4, 1 mM mercaptoethanol, 10 mM MgCl2, 10 mM MnCl2). Cells were pooled at passage one and used up to passage three. Both MVEC and HUVEC cultures had typical cobblestone morphology and stained uniformly for von Willebrand factor, as assessed by indirect immunofluorescence. Angiopoietin-1 and -2 and soluble recombinant Tie 1 and 2 receptors were provided by Regeneron. A recombinant form of Ang-1 was used in all of the experiments. This form of Ang-1 differs from the native Tie 2 ligand in that it possesses a modified NH2-terminal sequence and a mutation in Cys264 that make it easier to produce and purify.

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solution containing 500 \( \mu \)g/ml RNase H and 50 \( \mu \)g/ml propidium iodide and analyzed by using an FACS. At least 5000 events were analyzed, and the percentage of cells in the sub-\( G_1 \) population was calculated. Aggregates of cell debris at the origin of the histogram were excluded from the analysis of sub-\( G_1 \) cells as indicated in the legends to Figs. 2 and 4.

**Virul Infection of MVEC—**MVEC were infected with 50–100 multiplicities of infection of herpes simplex viruses expressing \( \beta \)-galactosidase or the dominant negative \( \Delta \)p85 subunit of PI3 kinase as described (10). Alternatively, MVEC were infected with similar multiplicities of infection of adenoviruses containing the \( \beta \)-galactosidase or the hemagglutinin-tagged activation-deficient phosphorylation mutant Akt (AA-Akt). After 4 h, the virus was removed, and the cells were left to recover overnight in complete medium. In preliminary experiments with the \( \beta \)-galactosidase virus, these conditions were optimal for infecting 95% of the cultures. Infected cells were either plated in bacteriological dishes or lysed in lysis buffer for immunoblotting.

**Northern Blotting—**MVEC were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and serum-starved for 24 h followed by challenge with Ang-1 as described above. Total RNA was extracted from cell pellets with TRI reagent (10\(^6\) cells/0.2 ml, Molecular Research Center, Inc., Cincinnati, Ohio). For Northern analysis, 10–20 \( \mu \)g of total RNA were separated on 1% agarose gels with formaldehyde, transferred to nylon filters (Hybond-N, Amersham Pharmacia Biotech), UV cross-linked, and hybridized with the corre-responding \( ^{32} \)P-labeled cDNA probes (survivin, Pharmacia Biotech), UV cross-linked, and hybridized with the corre-sponding \( ^{32} \)P-labeled cDNA probes (survivin, Pharmacia Biotech), UV cross-linked, and hybridized with the corre-sponding \( ^{32} \)P-labeled cDNA probes (survivin, Pharmacia Biotech), UV cross-linked, and hybridized with the correspondence32P-labeled cDNA probes (survivin, Pharmacia Biotech), UV cross-linked, and hybridized with the correspond-32P-labeled cDNA probes (survivin, Pharmacia Biotech), UV cross-linked, and hybridized with the correspond-32P-labeled cDNA probes (survivin, Pharmacia Biotech), UV cross-linked, and hybridized with the correspondence

**Survivin Promoter Studies—**pLuc-cyc1.2 (1 + 1 to 268) was generated by polymerase chain reaction with the human survivin promoter sequence as a template and confirmed by DNA sequencing. pLuc-42 was generated by inserting the first 42-base pair fragment of the 5’-end of the human survivin promoter upstream of the luciferase gene and confirmed by sequencing. Transient transfection of MVEC was performed using Lipofectin reagent (Life Technologies, Inc.) as described previously (16). Briefly, MVEC were seeded in a 12-well plate (1 – 2 \( \times 10^5\) cells/well) in 1 ml of medium and grown to 50–80% confluence. 50 \( \mu \)l of Opti-MEM I (Life Technologies, Inc.) containing 1 \( \mu \)g of various plasmid DNAs was combined with 50 \( \mu \)l of Opti-MEM I containing 4 \( \mu \)l of Lipofectin reagent. The combined mixture was overlaid onto cells that were preincubated with serum-free medium for 20–30 min. The transfected cells were then incubated at 37 \( ^\circ\)C for 4–6 h. The DNA-liposome complex was replaced with complete medium, and luciferase activity/\( \beta \)-gal expression (internal control) were measured within 36–48 h post-transfection.

**Transfection of MVEC with GFP Survivin Constructs—**MVEC were transfected with the cDNAs for GFP, GFP-survivin (survivin), or GFP-C84A survivin (C84A survivin) for 24 h. Fusion of survivin with GFP does not interfere with its biological activity or localization. The survivin-GFP (Cys\( ^{86-87}\)-Ala) construct is a mutation in the Bir1 domain that is targeted to the mitotic spindle but is devoid of anti-apoptosis function. In experiments using GFP-survivin, approximately 50% of the cells were transfected, and apoptosis, under the various conditions tested, was determined by propidium iodide staining and flow cytometry. The percentage of cells with hypodiploid DNA content quantified in the GFP-expressing population is shown in each histogram. Aggregates of cell debris at the origin of the histogram were excluded from the analysis of sub-\( G_1 \) cells. In some experiments, cells were imaged on an inverted microscope (Zeiss, Axiovert) using DIC optics. **RESULTS AND DISCUSSION**

Stimulation of MVEC with Ang-1 increased Akt phosphorylation on Ser\(^{473}\) and Thr\(^{308}\) (not shown) and in a reaction suppressed by the PI3 kinase inhibitor, wortmannin (WM; Fig. 1A). Ang-1 also increased Akt activity in a wortmannin-sensitive manner (6.7 ± 0.6, 13.2 ± 1.8, and 6.1 ± 0.7 counts per min of \( ^{32} \)P \( (\times 10^5) \) incorporated into histone H2B for control; Ang-1 and Ang-1 plus wortmannin-treated cells, \( n = 3 \), \( p < 0.05 \)). To directly test the role of PI3 kinase in Ang-1-stimulated Akt activation, MVEC were infected with a replication-deficient herpes simplex virus encoding \( \beta \)-gal or a dominant negative construct for the \( \Delta \)p85 subunit of PI3 kinase (\( \Delta \)p85; 10). As seen in Fig. 1B, Ang-1 increased Akt phosphorylation in \( \beta \)-gal-transduced cells, whereas infection with the virus encoding \( \Delta \)p85 abrogated basal and Ang-1-stimulated Akt phosphorylation.

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Stimulation of MVEC with Ang-1 (250 ng/ml) for 15 min and analyzed for Akt phosphorylation (\( \alpha \)-Akt, upper panel) or total Akt expression (lower panel) by Western blotting. B, MVEC transduced with viruses for \( \beta \)-gal or \( \Delta \)p85 and Akt phosphorylation and total Akt examined as above. C, time-dependent activation of Akt by Ang-1. MVEC were incubated with Ang-1 for increasing amounts of time, and Akt activation was determined as above. D, Ang-1-induced Akt phosphorylation is blocked by soluble Tie 2 and Ang-2 but not by soluble Tie 1. MVEC were incubated with vehicle (TBS plus CHAPS) or the various indicated combinations of Ang-1 (250 ng/ml), Ang-2 (2.5 \( \mu \)g/ml), and soluble Tie 1 or Tie 2 receptors (rTie2-Fc or rTie1-Fc, 2.5 \( \mu \)g/ml) for 15 min before determination of Akt phosphorylation or total Akt expression by Western blotting. For all panels, data are representative of at least three experiments.
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Ang-1 stimulates Akt phosphorylation in a time-dependent manner with maximal activation occurring within 15–30 min and sustained phosphorylation lasting for up to 2 h (Fig. 1C). Ang-1-stimulated phosphorylation of Akt on Ser473 was antagonized by preincubation of Ang-1 with soluble Tie 2 receptor but not by incubation with soluble Tie 1 receptor bodies (Fig. 1D). In addition, Ang-1-induced Akt phosphorylation was partially blocked by the physiological antagonist of Ang-1, angiopoietin-2 (Fig. 2). Interestingly, Ang-2 alone weakly activated Akt in MVEC. Our results are consistent with data from heterologous expression systems and transformed endothelial cells documenting that Ang-1 activation of PI3 kinase is important for cell migration and survival (9, 12). Therefore, Ang-1 via the Tie 2 receptor stimulates Akt activation through a PI3 kinase/Akt-dependent mechanism.

Next, we examined a potential link between Ang-1 and expression of two known anti-apoptotic genes, survivin and bcl-2 (15, 16). Treatment of MVEC with Ang-1 rapidly induced a time-dependent increase in survivin mRNA levels (17), which peaked 12 h after stimulation and remained sustained for up to 24 h (Fig. 3A). In contrast, Ang-1 did not up-regulate bcl-2 mRNA expression in MVEC (Fig. 3A). Consistent with a receptor-mediated response, preincubation of Ang-1 with soluble Tie 2 receptor abolished Ang-1 induction of survivin RNA in MVEC (Fig. 3B). When MVEC were transfected with a survivin-luciferase construct (18), Ang-1 stimulated up-regulation of survivin transcriptional activity, which persisted for up to 24 h after stimulation (Fig. 3C). Ang-1-induced expression of survivin protein in HUVEC, an effect abrogated by WM, or by transduction with adenoviral AA-Akt (Fig. 3D). In contrast, Ang-1 did not increase the expression of bcl-2 protein expression in MVEC (Fig. 3D). These data demonstrate that Ang-1 stimulates survivin expression in endothelial cells via a PI3 kinase/Akt-dependent mechanism.

To determine whether survivin can mediate the anti-apoptotic function of Ang-1, we transfected MVEC with cDNAs containing GFP fused to wild-type survivin (GFP-survivin) or to a dominant negative Cys84Ala survivin (GFP-C84A survivin) and determined cytoprotection in response to apoptosis-inducing stimuli (19). Treatment with Ang-1 or expression of GFP-survivin, alone or in combination with Ang-1, suppressed the appearance of MVEC with hypodiploid DNA content induced by TNFα/cycloheximide or by anoikis (Fig. 4, A–C).

**Fig. 3. Ang-1 induces survivin expression via a PI3 kinase/Akt pathway.** A, time-dependent expression of survivin mRNA. Serum-starved MVEC were treated with Ang-1 for the indicated time intervals and survivin, GAPDH, and bcl-2 RNA expression were examined by Northern hybridization. B, soluble Tie 2 prevents Ang-1 induction of survivin RNA. The experimental conditions are the same as for A except that Ang-1 was preincubated in the absence or presence of a soluble Tie 2 receptor before addition to MVEC for 24 h and determination of survivin, GAPDH, or bcl-2 RNA expression. C, Ang-1 stimulates survivin promoter activity. MVEC were co-transfected with plasmids encoding a promoterless luciferase cassette (pLUC-42) or a 1.2-kilobase survivin promoter fragment (pLUC-cyc1.2) with β-galactosidase, and relative luciferase activity was determined. D, Ang-1 increases survivin protein expression. HUVEC treated with Ang-1 (250 ng/ml) under the various conditions tested were harvested after 18 h and analyzed for survivin, actin, and bcl-2 protein expression by Western blotting. Numbers below the survivin panel indicate relative levels based on densitometry. For all panels, data are representative of two to four experiments.

**Fig. 4. Survivin mediates the anti-apoptotic effect of Ang-1.** A, TNFα-cycloheximide. B, anoikis. C, morphology. MVEC were transfected with GFP vector, GFP-survivin (survivin), or GFP-C84A survivin (C84A survivin) for 24 h followed by treatment with TNFα (5 ng/ml) plus cycloheximide (5 μg/ml) for 9 h (A, TNF/CHX) or by plating in serum-free medium on bacteriological dishes (B) in the absence or presence of Ang-1 (250 ng/ml). Data are representative of two experiments in duplicate. Surv, survivin.
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A and B). In contrast, transfection of MVEC with GFP-C84A survivin abrogated the cytoprotective effect of Ang-1 against TNFα-cycloheximide- or anoikis-induced cell death (Fig. 4, A and B). Consistent with the above analysis, Ang-1 alone or in combination with transfected GFP-survivin resulted in healthier morphology of any adherent cells, in contrast to cells transfected with GFP alone or GFP-C84A survivin plus Ang-1 (Fig. 4C). These data identify survivin as a novel PI3 kinase/Akt-dependent target gene for Ang-1 and demonstrate that survivin is necessary for the anti-apoptotic effect of Ang-1 in endothelial cells.

In summary, Ang-1 prevents endothelial cell apoptosis by activating a critical survival messenger, Akt, and by up-regulating a broad spectrum apoptosis inhibitor, survivin. Although Akt activation is required for survivin expression and interference with survivin function by the C84A survivin mutant abolishes Ang-1 cytoprotection, activated Akt may also execute parallel anti-apoptosis pathways through phosphorylation of caspase-9 and/or Bad (20, 21). Recent studies have suggested parallel anti-apoptosis pathways through phosphorylation of Akt and by up-regulation of VEGF, our study with Ang-1, a non-mitogenic survival factor, that VEGF increases survivin expression in endothelial cells (22, 23). Complementing and extending these findings with VEGF, our study with Ang-1, a non-mitogenic survival factor, may have far-reaching implications for angiogenesis when endothelial cell morphology of any adherent cells, in contrast to cells transfected with GFP alone or GFP-C84A survivin plus Ang-1. These data identify survivin as a novel PI3 kinase/Akt-dependent target gene for Ang-1 and demonstrate that survivin is necessary for the anti-apoptotic effect of Ang-1 in endothelial cells.

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