

Short Communication

Control of Apoptosis during Angiogenesis by Survivin Expression in Endothelial Cells

Daniel S. O'Connor,* Jeffrey S. Schechner,*†
Colette Adida,* Mehdi Mesri,*
Annette L. Rothermel,* Fengzhi Li,*
Anjali K. Nath,* Jordan S. Pober,*† and
Dario C. Altieri*

From the Boyer Center for Molecular Medicine of the Department of Pathology* and the Department of Dermatology,† Yale University School of Medicine, New Haven, Connecticut

Mechanisms controlling endothelial cell survival during angiogenesis were investigated. Stimulation of quiescent endothelial cells with mitogens, including vascular endothelial growth factor and basic fibroblast growth factor, induced up to ~16-fold up-regulation of the cell cycle-regulated apoptosis inhibitor survivin. Mitogen stimulation rapidly increased survivin RNA expression in endothelial cells, which peaked after 6 to 10 hours in culture and decreased by 24 hours. Inflammatory cytokines, tumor necrosis factor α , and interleukin-1 did not induce survivin expression in endothelial cells. Formation of three-dimensional vascular tubes *in vitro* was associated with strong induction of survivin in endothelial cells, as compared with two-dimensional cultures. By immunohistochemistry, survivin was minimally expressed in endothelium of nonproliferating capillaries of normal skin, whereas it became massively up-regulated in newly formed blood vessels of granulation tissue *in vivo*. Recombinant expression of green fluorescent protein survivin in endothelial cells reduced caspase-3 activity and counteracted apoptosis induced by tumor necrosis factor α /cycloheximide. These findings identify survivin as a novel growth factor-inducible protective gene expressed by endothelial cells during angiogenesis. Therapeutic manipulation of survivin expression and function in endothelium may influence compensatory or pathological (tumor) angiogenesis. (*Am J Pathol* 2000, 156:393–398)

Apoptosis, the genetic control of cell death and viability, preserves tissue and organ homeostasis by eliminating senescent or damaged cells.¹ This process involves dif-

ferent gene families of inhibitors and stimulators of cell death and culminates with activation of intracellular cysteine proteases, caspases.² Aberrations of apoptosis are known to contribute to human diseases, including cancer³ and vascular disorders.⁴ Specifically, aberrantly increased cell death has been shown to influence atherosclerotic plaque instability,⁵ congestive heart failure,⁶ coronary disease,⁷ and ischemic neuronal loss.⁸

The endothelium is one of the most critical sites for the control of apoptosis in vascular injury and vascular remodeling.⁹ In inflammation, a heterogeneous group of protective genes activated by nuclear factor κ B opposes cell death and proinflammatory changes in endothelial cells (EC) induced by cytokines, ie, tumor necrosis factor α (TNF α).¹⁰ Inhibition of apoptosis may also be required during vascular remodeling and new blood vessel formation, angiogenesis.¹¹ In this context, EC-specific mitogens, including vascular endothelial cell growth factor (VEGF) or basic fibroblast growth factor (bFGF), transduce survival signals critically maintaining EC viability *in vivo*.^{12–14} However, the downstream effector genes coupling mitogen-dependent survival to the anti-apoptotic machinery in EC have not been completely elucidated.

In this study, we sought to investigate a potential role of the cell cycle-regulated apoptosis inhibitor survivin^{15,16} on EC viability. We found that mitogen stimulation strongly induced survivin expression in endothelium during vascular remodeling and angiogenesis, *in vitro* and *in vivo*, and that this pathway counteracted caspase-3 activity and apoptosis induced by TNF α .

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Address reprint requests to Dario C. Altieri, M.D., Yale University School of Medicine, BCM436B, 295 Congress Avenue, New Haven, CT 06536. E-mail: dario.altieri@yale.edu.

Materials and Methods

Cells and Cell Culture

Human umbilical vein EC were maintained in M199 medium supplemented with 20% fetal calf serum (FCS), 50 $\mu\text{g/ml}$ endothelial cell growth supplement (ECGS), 100 $\mu\text{g/ml}$ heparin, 100 $\mu\text{g/ml}$ penicillin, and 100 $\mu\text{g/ml}$ streptomycin (all from Life Technologies, Grand Island, NY) in 5% CO_2 at 37°C. Bovine aortic EC were isolated and maintained in culture as described.¹⁷ Subconfluent EC were rendered quiescent by 24 hours' culture in M199 plus 0.1% FCS. Cells were detached with 0.05% trypsin/0.02% EDTA, seeded in C6-well plates (Costar Corp., New Bedford, MA), grown to 70% confluency, and used between passages 2 and 3.

Modulation of Survivin Expression in EC

Quiescent subconfluent EC were incubated with VEGF (Collaborative Biomedical Products, Bedford, MA; 10–100 ng/ml), basic fibroblast growth factor (bFGF; Calbiochem Corp., La Jolla, CA; 5 ng/ml), 10% FCS, or recombinant interleukin-1 (IL-1; R&D Systems, Minneapolis, MN; 2 ng/ml, 200 U/ml) or $\text{TNF}\alpha$ (10 ng/ml, Endogen, Woburn, MA) for 14 hours at 37°C in M199 plus 0.1% FCS. Cells were washed, harvested by trypsin/EDTA, and extracted in 4% sodium dodecyl sulfate plus protease inhibitors. Protein-normalized aliquots of cell extracts were electrophoresed on a 13.5% sodium dodecyl sulfate polyacrylamide gel, transferred to nylon membranes (Millipore Corp.) for 1 hour at 1 A, and immunoblotted with 1 $\mu\text{g/ml}$ of a rabbit antibody to survivin followed by chemiluminescence (Amersham, Arlington Heights, IL).¹⁸ Samples were analyzed for equal protein loading by immunoblotting with a mouse antibody to β -actin. For Northern blot hybridization, serum-deprived EC were stimulated with 100 ng/ml VEGF and harvested at increasing time intervals between 1.5 and 24 hours culture at 37°C. Total RNA was extracted using the TRI Reagent (10⁶ cells/0.2 ml, Molecular Research Center, Cincinnati, OH), and further processed for Northern blot hybridization with a ³²P α -dCTP random-primed labeled survivin cDNA or control β -actin probe, as described.¹⁵

Three-Dimensional EC Culture

EC were suspended at a density of $3 \times 10^6/\text{ml}$ in a liquefied matrix of rat-tail type I collagen (1.5 mg/ml) and human plasma-derived fibronectin (0.15 mg/ml) in M199, pH 7.5. One milliliter of the EC suspension was transferred into each well of rat-tail type I-coated C6 wells and warmed to 37°C to allow polymerization of the matrix. After a 24 hour incubation at 37°C in M199 plus 20% FCS, 50 $\mu\text{g/ml}$ ECGS, 100 $\mu\text{g/ml}$ heparin, 100 $\mu\text{g/ml}$ penicillin, and 100 $\mu\text{g/ml}$ streptomycin, the three-dimensional culture was placed in OCT and paraffin-embedded for immunohistochemical analysis. Alternatively, two- or three-dimensional EC cultures were homogenized in a tissue grinder and immunoblotted for survivin expression. Dur-

ing the incubation period, EC throughout the gel were observed to elongate and form multicellular tubular structures, as described.¹⁹

Immunohistochemistry

Four skin biopsies, containing granulation tissue or normal, non-inflamed skin by hematoxylin-eosin staining, were collected from the archives of Yale-New Haven Hospital. Five-micron sections were prepared from paraffin-embedded tissues, deparaffinized in xylene, and rehydrated in graded alcohol with quenching of endogenous peroxidase in 2% H_2O_2 in methanol. Immunolocalization of survivin was carried out as described previously,¹⁵ after antigen retrieval by pressure cooking for 5 minutes in 0.01 mol/L citrate buffer, pH 6.0. Binding of the primary antibody was revealed by addition of 3,3'-diaminobenzidine, or, alternatively, 3-amino-9-ethylcarbazole (AEC, Vector), as a substrate. Control experiments were carried out in the absence of primary antibody or in the presence of preimmune rabbit IgG.

EC Protection by Survivin

The cDNA of wild-type survivin¹⁵ was inserted in-frame in the *EcoRI* site of green fluorescence protein (GFP)-encoding plasmid, pEGFPc1 (Clontech, San Francisco, CA). The correct orientation and reading frame of pEGFPc1 fusion plasmid were confirmed by DNA sequencing. Bovine aortic EC were seeded in C6-well plates at 40 to 50% confluency and transfected with GFP vector or GFP survivin by lipofectin for 6 hours at 37°C. After removal of the DNA-lipid mixture, the EC monolayer was placed in complete growth medium for 35 hours at 37°C and incubated with 5 ng/ml $\text{TNF}\alpha$ plus 5 $\mu\text{g/ml}$ cycloheximide for an additional 8 hours at 37°C. Cells (floaters plus attached cells) were fixed in 70% ethanol, stained with 10 $\mu\text{g/ml}$ propidium iodide plus 100 $\mu\text{g/ml}$ RNase A and 0.05% Triton X-100 in phosphate-buffered saline, pH 7.4, and GFP-expressing cells were analyzed for DNA content by flow cytometry. In other experiments, bovine EC transfected with GFP vector or GFP survivin were treated with control medium or 5 to 10 ng/ml $\text{TNF}\alpha$ plus 10 $\mu\text{g/ml}$ cycloheximide for 8 hours at 37°C. Cells were harvested and analyzed for caspase-3 activity by hydrolysis of the fluorogenic substrate Ac-DEVD-AMC (N-Acetyl-Asp-Glu-Val-Asp-aldehyde, Pharmingen, San Diego, CA), in the presence or in the absence of the caspase-3 inhibitor Ac-DEVD-CHO. Fluorescence emissions were quantitated on a spectrofluorometer with excitation wavelength of 360 nm and emission wavelength of 460 nm.

Results

Mitogen-Stimulated Induction of Survivin in EC

Expression of ~16.5-kd endogenous survivin in quiescent, serum-deprived endothelium was minimally detectable by immunoblotting (Figure 1A), in agreement with

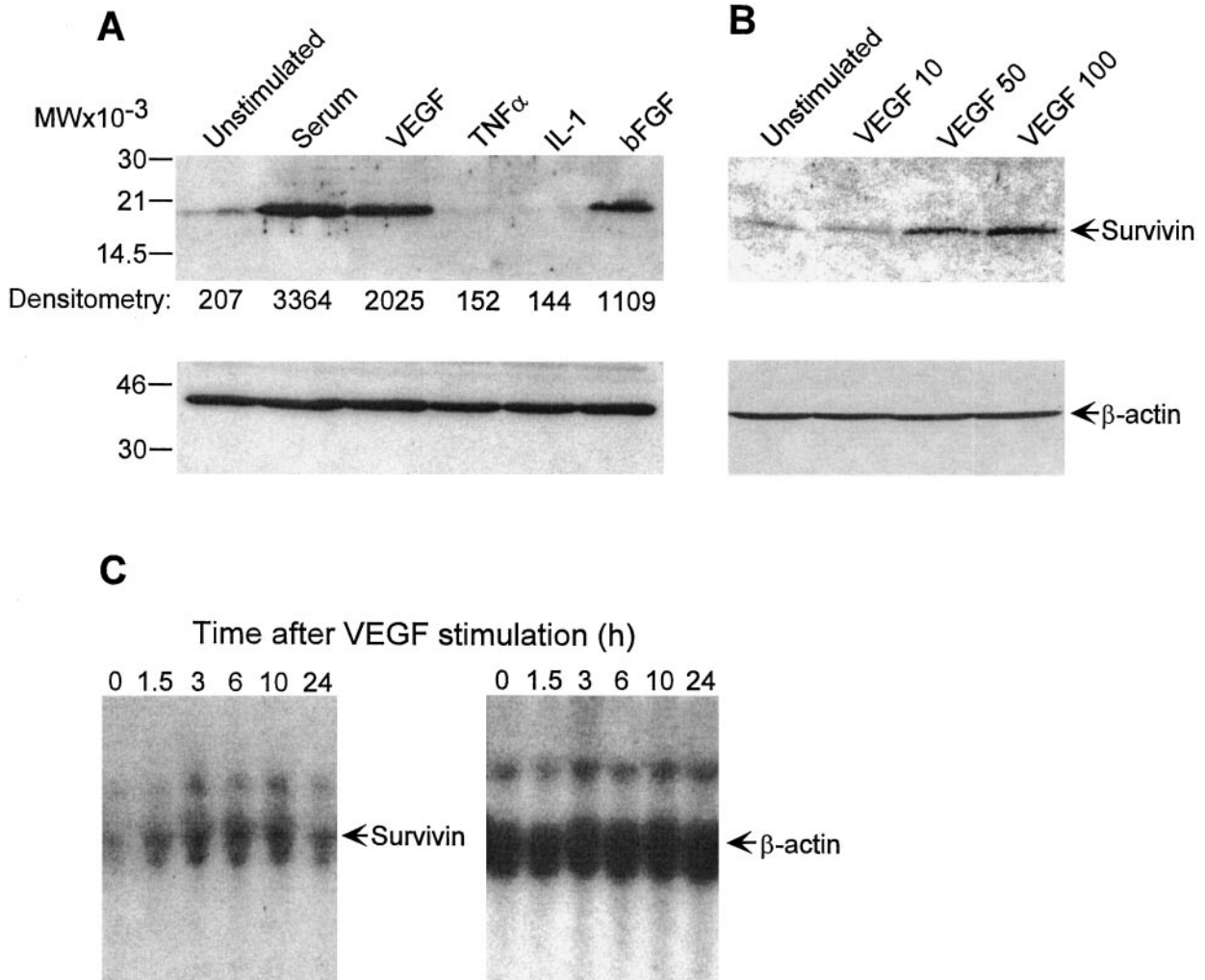


Figure 1. Modulation of survivin expression in EC. **A:** Quiescent EC were incubated with medium or serum (10% FCS), VEGF (100 ng/ml), bFGF (5 ng/ml), TNF α (10 ng/ml), or IL-1 (2 ng/ml) for 16 hours at 37°C. Cells were harvested, sodium dodecyl sulfate-extracted, and analyzed for expression of survivin or β -actin by immunoblotting. **B:** Control or EC were stimulated with the indicated increasing concentrations of VEGF for 16 hours at 37°C and analyzed for expression of survivin or β -actin by immunoblotting. **C:** Total RNA was extracted from EC stimulated with 100 ng/ml VEGF at the indicated time intervals, separated on agarose-formaldehyde denaturing gels, and hybridized with probes to survivin or control β -actin.

previous observations.¹⁵ EC stimulation with serum or the specific mitogens VEGF or bFGF resulted in an 8- to 16-fold up-regulation of survivin expression, by immunoblotting (Figure 1A). Survivin induction by VEGF was concentration-dependent and maximal at ~50 ng/ml (Figure 1B). EC stimulation with cytokines TNF α or IL-1 did not increase survivin expression, which was reduced below background levels of untreated cells (Figure 1A). In control experiments by flow cytometry, TNF α or IL-1 stimulated strong up-regulation of intercellular adhesion molecule-1 in EC, whereas VEGF was ineffective (not shown). By Northern blot hybridization, a main 1.9-kb survivin message and a fainter 3.4-kb survivin transcript were minimally detected in quiescent EC (Figure 1C). VEGF treatment resulted in rapid up-regulation of survivin RNA in EC, in a response that peaked 6 to 10 hours after stimulation and decreased to approach background levels 24 hours after treatment (Figure 1C).

Survivin Expression in Three-Dimensional EC Cultures

Survivin was expressed at very low levels in two-dimensional EC cultures, by immunohistochemistry (Figure 2A). In contrast, formation of three-dimensional vascular tubes in collagen/fibronectin matrix resulted in strong expression of survivin in EC (Figure 2B). No staining of three-dimensional EC cultures was observed with control non-binding antibody (Figure 2C). By immunoblotting, a prominent ~16.5-kd survivin band was prominently induced in EC extracts of three-dimensional vascular tubes, as compared with two-dimensional EC cultures (Figure 2D).

Survivin Expression in Proliferating EC in Vivo

In four out of four cases, survivin was strongly expressed in the cytoplasm of EC of newly formed capillaries of skin

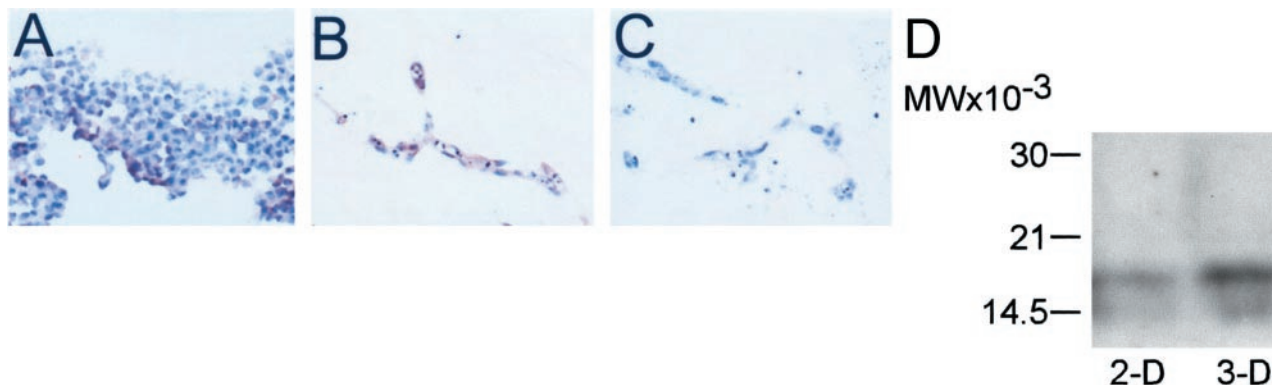


Figure 2. Expression of survivin in three-dimensional EC culture. EC were grown in three-dimensional fibronectin-collagen gels, paraffin-embedded, and analyzed for survivin expression (red staining) by immunohistochemistry. **A:** Survivin expression in control, two-dimensional EC culture. **B:** Survivin expression in three-dimensional EC culture. **C:** Control staining of three-dimensional EC culture with preimmune antibody. **D:** Two- (2-D) or three-dimensional (3-D) EC cultures were harvested, homogenized in a tissue grinder, and analyzed for survivin expression by immunoblotting.

granulation tissue, by immunohistochemistry (Figure 3A). Abundant expression of survivin was also demonstrated in EC of large vessels of granulation tissue at the dermis/hypodermis junction (Figure 3C). By contrast, no staining of granulation tissue was observed in the absence of primary antibody (Figure 3, B and D), or with control

preimmune antibody (not shown). Analysis of nonproliferating capillaries of noninflamed normal skin revealed minimally detectable expression of survivin in EC (Figure 3E), as compared with control staining with preimmune IgG (Figure 3F).

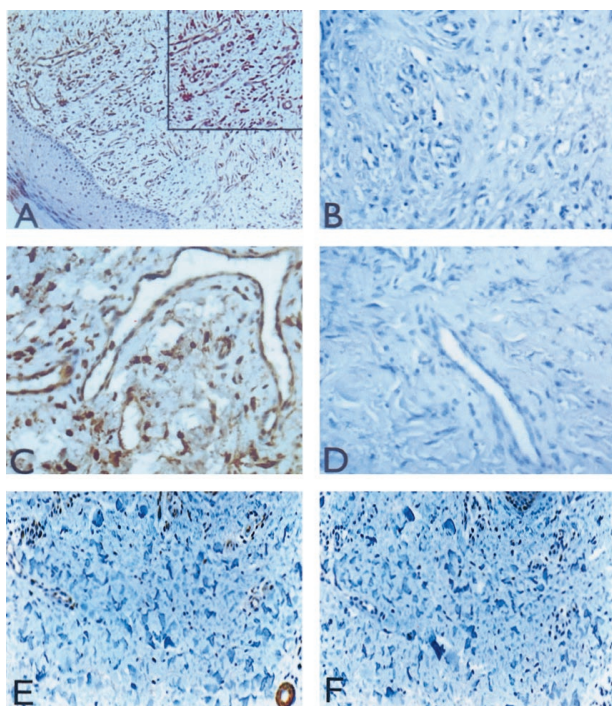


Figure 3. Expression of survivin in proliferating and nonproliferating skin capillaries. Five-micron sections of formalin-fixed, paraffin-embedded skin biopsies containing granulation tissue and normal skin were analyzed for survivin expression by immunohistochemistry after antigen retrieval by pressure-cooking. **A:** Strong cytoplasmic expression of survivin in EC of dermal capillaries in granulation tissue. **Inset:** Detail representation of dermal capillaries stained for survivin expression. **C:** Expression of survivin in endothelium of large vessel in granulation tissue at the dermis/hypodermis junction. **B** and **D:** Control staining for **A** and **C**, respectively, in the absence of primary antibody. **E:** Expression of survivin in nonproliferating capillaries of noninflamed normal skin. **F:** Control incubation for panel **E** in the presence of preimmune antibody. Original magnifications, $\times 200$ (**A**, **E**, **F**) and $\times 400$ (**B**, **C**, **D**).

Anti-Apoptotic Effect of Survivin in EC

Treatment with $\text{TNF}\alpha$ /cycloheximide induced EC apoptosis and generation of a hypodiploid population by propidium iodide staining and flow cytometry (Figure 4A). Expression of GFP survivin inhibited $\text{TNF}\alpha$ -induced apoptosis in EC and reduced the percentage of hypodiploid cells to control levels of untreated cultures (Figure 4A). In contrast, transfection of GFP vector alone did not affect $\text{TNF}\alpha$ -induced EC apoptosis (Figure 4A). Moreover, expression of GFP survivin in EC strongly inhibited caspase-3 activity in $\text{TNF}\alpha$ -treated EC, as determined by DEVD hydrolysis, whereas GFP vector alone was ineffective (Figure 4B). In control experiments, preincubation of $\text{TNF}\alpha$ -treated EC extracts with the caspase-3 inhibitor DEVD-CHO abrogated DEVD hydrolysis (Figure 4B).

Discussion

In this study, we have shown that mitogen stimulation of EC results in strong up-regulation of the cell cycle-regulated apoptosis inhibitor, survivin.^{15,16} From minimally detectable levels in quiescent endothelium, survivin became abundantly expressed in EC of three-dimensional vascular tubes *in vitro* and in newly formed capillaries during angiogenesis *in vivo*. Recombinant expression of survivin efficiently reduced caspase-3 activity in EC and blocked EC apoptosis induced by $\text{TNF}\alpha$ /cycloheximide.

Recent experimental evidence has suggested that inhibition of EC apoptosis may be an essential prerequisite to maintain angiogenesis *in vivo*.¹¹ Accordingly, disruption of $\alpha_v\beta_3$ integrin-matrix interaction²⁰ or interference

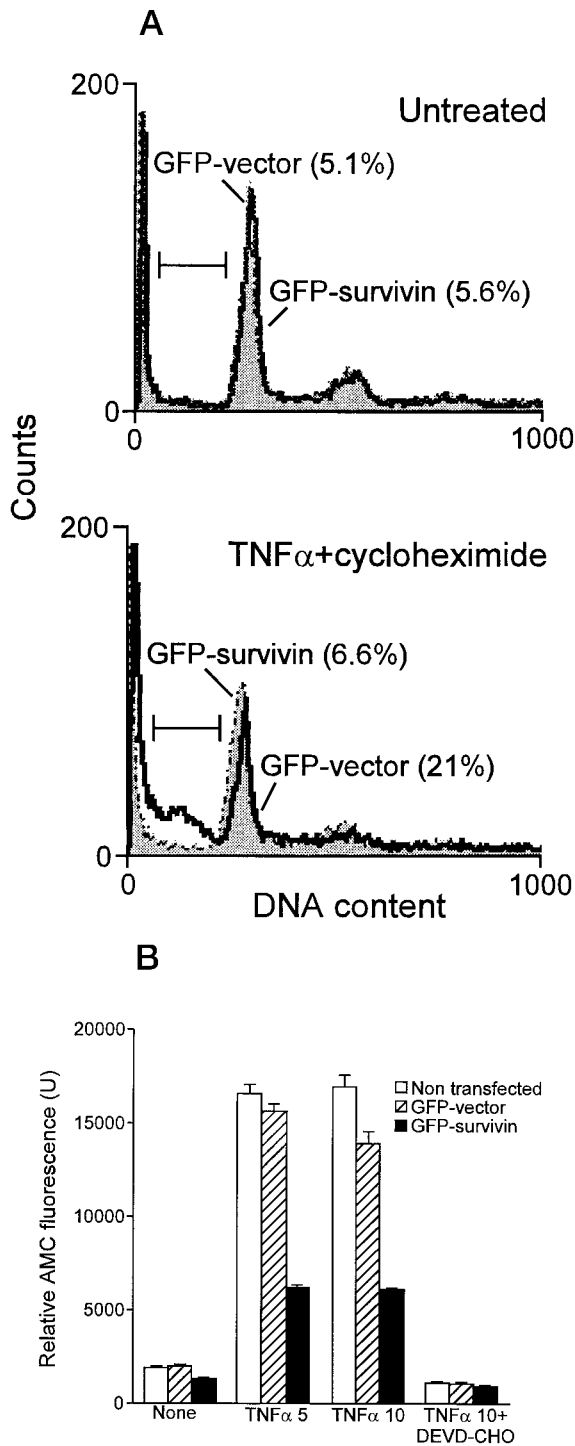


Figure 4. Anti-apoptotic function of survivin in EC. **A:** Subconfluent bovine aortic EC were transfected with GFP vector or GFP survivin by lipofectin, cultivated for 35 hours at 37°C, and treated with 5 ng/ml TNF α /5 μ g/ml cycloheximide for additional 8 hours at 37°C. GFP-expressing cells (green fluorescence) were analyzed for DNA content by propidium iodide staining (red fluorescence) and flow cytometry. The percentage of cells with hypodiploid DNA content (sub-G1 fraction) is indicated in parentheses for each condition tested. **B:** EC, untreated or transfected with GFP vector or GFP survivin, were incubated with the indicated concentrations of TNF α /10 μ g/ml cycloheximide, harvested, and analyzed for caspase-3 activity by hydrolysis of the fluorogenic substrate DEVD-AMC in the presence or absence of the caspase-3 inhibitor DEVD-CHO. Data are the mean \pm SD of replicates of a representative experiment.

with VEGF-dependent survival signals¹²⁻¹⁴ triggered EC apoptosis and involution of newly formed capillaries *in vivo*. Despite the up-regulation of anti-apoptotic *bcl-2* and A1 molecules in VEGF-stimulated endothelium,^{21,22} alternative mechanisms of cytoprotection have been postulated,²³ prompting the search for additional effector genes contributing to VEGF-dependent EC survival. Here, the dramatic up-regulation of survivin in mitogen-stimulated endothelium is consistent with the cell cycle-dependent expression of the survivin gene in G2/M¹⁶ and suggests that this pathway may maintain a critical anti-apoptotic threshold at cell division. Consistent with the spontaneous induction of apoptosis resulting from survivin targeting in model cell types,^{18,24} these data suggest that VEGF induction of survivin may provide a critical prerequisite to maintain EC viability during angiogenesis, whereas loss of survivin may facilitate involution of newly formed capillaries *in vivo*.¹²⁻¹⁴ On the other hand, survivin expression in VEGF-stimulated endothelium may not be simply a consequence of cell proliferation, since survivin was not detected in other normal proliferating tissues, including the basal layer of epidermis.¹⁸ This suggests that VEGF induction of survivin may provide a unique paradigm of regulation of this anti-apoptotic pathway in a normal, terminally differentiated cell type.

The suppression of caspase-3 activity in survivin-expressing EC shown here is consistent with the general function of IAP proteins as caspase inhibitors, either directly or through interference with caspase-9 processing.^{25,26} In EC, active caspase-3 has been directly implicated in proteolysis of p125^{FAK},^{27, 28} and p27/p21 cyclin-dependent kinase inhibitors,²⁹ thus disassembling cell-to-matrix interactions and dysregulating cell division control mechanisms. In this context, VEGF induction of survivin is expected to provide a broad anti-apoptotic spectrum, counteracting a variety of death-inducing stimuli converging on caspase-3 activation as the executioner phase of apoptosis.² It is also intriguing that both *bcl-2* and survivin become up-regulated in VEGF-stimulated EC.²¹ This suggests that inhibition of EC apoptosis during angiogenesis may occur simultaneously through parallel and non-overlapping pathways, involving preservation of mitochondrial integrity by *bcl-2*³⁰ and suppression of caspase activity by survivin.^{25,26}

The findings reported here may have potentially far-reaching therapeutic implications. First, targeted inhibition of apoptosis in endothelium may be exploited to limit tissue damage in vascular diseases.³¹ Accordingly, caspase antagonists³² or overexpression of anti-apoptotic *bcl-2*³³ afforded increased neuronal viability in ischemia/hypoxia models. In this context, survivin gene transfer may result in improved EC viability during VEGF-stimulated compensatory angiogenesis in ischemic vascular diseases.³⁴ Conversely, for the selective expression of survivin in cancer,¹⁵ molecular antagonists of this pathway may not only sensitize tumor cells to therapy-induced apoptosis, but also remove a critical EC cytoprotective mechanism exploited during tumor angiogenesis.

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