Vascular Endothelial Growth Factor Induces Expression of the Antiapoptotic Proteins Bcl-2 and A1 in Vascular Endothelial Cells*

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We examined the role of vascular endothelial growth factor (VEGF) in preventing apoptosis in primary human umbilical vein endothelial (HUVE) cells. VEGF was capable of preventing serum starvation-induced apoptosis at concentrations between 10 and 100 ng/ml. The addition of VEGF to serum-starved HUVE cells led to a 5.2-fold induction of Bcl-2 after 36 h and to a transient, 2.4-fold induction of A1 after a 7-h incubation, as quantitated by real time reverse transcriptase-polymerase chain reaction analysis. Western blot analysis demonstrated a 2–3-fold induction of Bcl-2 protein after 18–36 h of exposure to VEGF and a transient induction of A1 after 7 h of VEGF stimulation. Moreover, overexpression of Bcl-2 by means of transient biolistic transfection experiments of HUVE cells was sufficient to prevent endothelial cells from apoptotic cell death in the absence of VEGF. These findings indicate that Bcl-2 plays an important role in mediating the survival activity of VEGF on endothelial cells.

Bcl-2 belongs to a growing family of apoptosis regulatory gene products, which may either be death antagonists (Bcl-2, Bcl-XL, Bcl-w, Bcl-1, Bax, Bad, Bcl-2, and A1) or death agonists (Bax, Bak, Bcl-Xs, Bad, Bid, Bik, and Hrk; for review, see Ref. 1). Bcl-2 is an intracellular protein that localizes to mitochondria, endoplasmic reticulum, and the nuclear envelope (2, 3) and has been shown to block apoptosis without inducing cellular proliferation (4, 5). In vitro, many growth factors and cytokines have been shown to promote survival in different cell lines tested by activation of the Ras–Raf–mitogen-activated protein kinase module (for review, see Refs. 6 and 7). However, the mechanisms by which growth factors control proliferation and regression of endothelial cells remain poorly understood.

In human umbilical vein endothelial (HUVE) cells, increased levels of apoptosis and necrosis were observed when cells were incubated with lipopolysaccharide. Vitamins C and E reduced the levels of apoptosis, which was paralleled by an increase in Bcl-2 expression and a decrease in Bax protein levels (8). Upon incubation of HUVE cells with transforming growth factor-β, decreased levels of Bcl-2 correlated with increased levels of apoptotic cell death (9). 2-Methoxyestradiol was found to induce apoptosis of cultured bovine pulmonary endothelial cells (10). A1, a homolog of the Bcl-2 family of antiapoptotic proteins originally cloned from phorbol ester-stimulated endothelial cells, was shown to be induced by the inflammatory cytokines tumor necrosis factor and interleukin-1 (11). Infection of microvascular endothelial cells with retroviral constructs encoding A1 led to inhibition of tumor necrosis factor-α-induced cell death in the presence of actinomycin D (11). Similar findings were reported when Bcl-2 was overexpressed by means of retroviral infection of murine aortic endothelial cells. Enforced expression of Bcl-2 prevented apoptosis of these cells when cultured in fibroblast growth factor-depleted medium (12).

The endothelial cell-specific mitogen vascular endothelial growth factor (VEGF) has been shown to be a key positive regulator of normal and pathological angiogenesis (13). 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 (14). Furthermore, administration of anti-VEGF monoclonal antibodies results in regression of established tumor-associated vasculature in xenograft models (15). More recently, using a tetracycline-regulated VEGF expression system in xenografted C6 glioma cells, it has been shown that decreased levels of VEGF production lead to detachment of endothelial cells from the walls of preformed vessels in the tumor (16).

We have found recently that VEGF can counteract endothelial cell apoptosis induced upon serum starvation of HUVE cells in culture (see Fig. 3). This survival activity was critically dependent on the phosphatidylinositol 3-kinase/Akt pathway.

In this report, we found increased expression of the antiapoptotic proteins A1 and Bcl-2 but not Bax or Bcl-XL upon challenging primary HUVE cells with VEGF. Thus Bcl-2 and A1 are two novel VEGF target genes.

EXPERIMENTAL PROCEDURES

Cell Culture—HUVE cells, human microvascular endothelial cells, and CS-C medium were purchased from Cell System (Kirkland, WA). HUVE cells are pooled primary isolates from 300 individual donor umbilical veins. Cells were maintained in CS-C complete medium containing 10% fetal bovine serum and mitogens, according to the recommendations of the supplier. 24 h before initiation of serum starvation, cells were treated with trypsin and plated to a density of 20–25 × 10^5 cells/cm^2 on six-well dishes (reverse transcriptase-polymerase chain reaction (RT-PCR) analysis) or 6-cm dishes (biolistic transfection experiments). Immediately before the experiment, cells were washed twice with phosphate-buffered saline. For serum starvation, CS-C medium without serum and mitogen, complemented with 0.1% bovine serum albumin, was added for the indicated amount 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, minimal medium (without mitogen and serum) complemented with 0.1% bovine serum albumin was added, and the levels of

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§ The abbreviations used are: HUVE cells, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; RT-PCR, reverse transcriptase-polymerase chain reaction; GFP, green fluorescent protein.

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apoptosis were analyzed 24 h after induction of serum starvation.

Real Time RT-PCR Analysis—HUVE cells were initially expanded for 8–10 days in the presence of completed medium. Routinely, cells between passages 4 and 10 were used in the different experimental procedures. 24 h before serum starvation, cells were split and seeded in complete medium at a density of 200,000 cells/well in six-well plates, following the procedures recommended by the manufacturer. Cells were washed for 5 min before adding Cell System basic medium supplemented with 0.1% bovine serum albumin. After incubations of various duration, cells were harvested by the STAT 60 method (TEL-TEST “B”, Inc., Friendswood, TX), and total RNA was prepared according to the manufacturer’s recommendations. The RNA was dissolved in 50 µl of H2O, and the concentration was determined by spectrophotometer (A260/280 nm).

To monitor gene expression we used real time RT-PCR analysis. This novel approach has been described previously (18). Briefly, 100 ng of total RNA was added to a 50-µl RT-PCR (PCR-Access, Promega). The reaction master mix was prepared according to the manufacturer’s protocol to give final concentrations of 1 × avian myeloblastosis virus/Tfl reaction buffer, 0.2 mM dNTPs, 1.5 mM MgSO4, 0.1 unit/ml avian myeloblastosis virus reverse transcriptase, 0.1 unit/µl Tfl DNA polymerase, a 250 nM concentration of the primers, and a 200 nM concentration of the corresponding probe. Primers and probes for real time PCR analysis Bcl-2, A1, Bax, and Bad genes were designed by the Primer Express Program according to Heid et al. (19). For sequence information of all oligonucleotides, see Table I. The primers for the human Bcl-2 gene were HUMBCL-2 555.F and HUMBCL-2 639.R, and the probe was HUMBCL-2 598.FP. For A1 analysis, the following primers were used: HUMA1 14.F and HUMA1 131.R; the probe was HUMA1 59.FP. For Bcl-XL analysis we used HSBCXLX 408.F and HSBCXLX 851.R primers, the probe was HSBCXLX 585.FP. For Bax-A analysis, we used HUMBAXA 155.F and HUMBAXA 301.R as primers and HUMBAXA 235.FP as probe. Primers and probes were synthesized at Genentech using conventional nucleic acid synthesis chemistry. The β-actin primer and probe (TaqMan β-actin detection reagents) were purchased from Perkin-Elmer.

RT-PCRs and the resulting relative increase in reporter fluorescent dye emission were monitored in real time by the 7700 sequence detector (Perkin-Elmer). Signals were analyzed by the sequence detector 1.6 program (Perkin-Elmer). Conditions were as follows: one cycle at 48 °C for 45 min; one cycle at 94 °C for 2 min; 40 cycles at 94 °C for 30 s, 60 °C for 1 min, and 68 °C for 2 min. Data were generated as indicated in the legend to Fig. 1.

Western Blotting—Cells cultured in 10-cm dishes were washed twice in Tris-buffered saline, and 0.6 ml of RIPA buffer (1 × phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml phenylmethylsulfonyl fluoride, 30 µg/ml aprotinin, and 1 µg/ml sodium orthovanadate) containing freshly added protease inhibitors was added. Mouse monoclonal antibody directed against human Bcl-2 (Bcl-2(100)) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified goat polyclonal antibody against human A1 (A1(N-20)) was from the same manufacturer. The secondary antibody to Bcl-2(100) was goat anti-mouse Ig (biotin-labeled) from Southern Bio-technologies (Birmingham, AL). The secondary antibody to A1(N-20) was biotinylated anti-goat IgG from Vector Laboratories (Burlingame, CA). Total endothelial cell extract, polyaerylamide gel electrophoresis, blotting, and immunodetection were performed according to the Santa Cruz Biotechnology protocol. 60 µg of whole cell extract was loaded on each lane of a 4–16% polyaerylamide gel. The ECL reaction kit was purchased from Amersham Pharmacia Biotech, and horseradish peroxidase streptavidin from Vector Laboratories was used.

Gene Transfer of HUVE Cells by Microprojectile Bombardment—

| TABLE I Sequences of oligonucleotide primers and real time RT-PCR probes |
|-----------------------------|-----------------------------|-----------------------------|
| HUMBCL-2 598.FP             | 5′(FAM)–GTG CGC GTA TAA ATT GCC GA–(TAMRA)p3′ |
| HUMBCL-2 555.F              | AAG CGG TCC CGT GGA TAG A |
| HUMBCL-2 639.R              | TCC GTG ATT CCC AGA AGT CC |
| HSBCXLX 585.FP              | 5′(FAM)–TGC GGG GAA AGC GTA GAC AAG GAG ATG C–(TAMRA)p3′ |
| HSBCXLX 408.F               | GAG GCA GCC GGC GAG TTT GAA |
| HSBCXLX 851.R               | GGG GTG GGA GGG TAG AGT GGA |
| HUMA1 59.FP                 | 5′(FAM)–TGC TCT CCA CCA GCC AGA AGA TGA CA–(TAMRA)p3′ |
| HUMA 14.F                   | CAG CAC ATT GCC TCA ACA GC |
| HUMA1 131.R                 | TGC AGA TAG TCC TGA GCC AGC |
| HUMBAXA 235.FP              | 5′(FAM)–ATG ATT GCC GCC GTG GAC ACA GAC TCC–(TAMRA)p3′ |
| HSBSAXA 155.F               | AGG ATG CTT CCA CCA AGA AG |
| HSBSAXA 301.R               | CCA GTT GAA GTT GCC GTC AGA |

Fig. 1. Quantitative analysis of A1 and Bcl-2 expression in HUVE cells by real time RT-PCR. Panel A, analysis of total RNA (100 ng) isolated from HUVE cells cultured under serum starvation conditions (% serum) in the absence of growth factors (black bars) or in the presence of 100 ng/ml VEGF (gray bars) for the indicated length of time. The relative A1 expression levels were calculated by dividing the A1 signals by the signals obtained for the β-actin gene. Linear regression of the β-actin standard curve was used to normalize the result for the A1 signals to 100 ng of total RNA (17). Data shown are the means ± S.E. of triplicate analysis of three independent experiments. A paired t test was performed separately at each time point using Statview Software (Abacus Concepts, Berkeley, CA). For the 7 h time point, we observed a 2.4-fold induction of A1 (p = 0.032). *, p < 0.05. Panel B, analysis of total RNA isolated from HUVE cells treated as described in panel A, for the expression of the Bcl-2 gene. Data analysis was performed as outlined in panel A. For the 18 h time point we observed a 4.3-fold induction (p = 0.0123) and after 33 h, a 5.2-fold (p = 0.0218) increase in the Bcl-2 RNA levels. * indicates p < 0.05.

Experiments were done 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, conditions were established which yield the highest levels of luciferase activity in HUVE cells (data not shown). The settings used are as follows: particle size, 1.0 µm; rupture disc, 1,100 p.s.i.; bombardment chamber vacuum,
25 mm Hg; and target shelf position 3 (from the top). Gold particles were prepared according to the manufacturer’s recommendations and stored as 50-μl aliquots for several months at −20 °C. Aliquots were coated with DNA immediately before gene transfer following the manufacturer’s recommendations. Briefly, for five samples, a 50-μl aliquot of gold particles was vortex mixed for 3 min, and plasmid DNA (1 mg/ml) was added to a total of 10 μl. Vortexing was continued for 2 min. Thereafter, 100 μl 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 tabletop microcentrifuge. The supernatant was removed, and the gold pellet was washed first with 140 μl of 70% EtOH followed by 140 μl of 100% EtOH and 10 μl was used per biolistic transfection. Immediately after bombardment, 5 ml of complemented medium was added.

RESULTS

VEGF Induces Expression of Bcl-2 and A1 When Added to Serum-starved Endothelial Cells—To find putative VEGF target genes mediating the VEGF survival activity, we analyzed total RNA of HUVE cells treated with VEGF by quantitative RT-PCR (TaqMan). We designed primer probe sets for quantifying the expression of genes mediating the VEGF survival activity, we analyzed total RNA from HUVE cells cultured under the same conditions as those employed for the RT-PCR analysis. We found a direct correlation between the mRNA and the protein levels (Fig. 2) of those genes employed for the RT-PCR analysis. We found a direct correlation between the mRNA and the protein levels (Fig. 2) of those genes employed for the RT-PCR analysis. To verify if increased expression of A1 message in response to VEGF may be caused by the decreased sensitivity of the Northern blot analysis, or because the cells were exposed to a mixture of basic fibroblast growth factor (4 ng/ml) and VEGF (10 ng/ml), or because of the different incubation length with VEGF (3 h versus 7 h).

When we analyzed total RNA from HUVE cells for Bcl-XL and Bax expression, we could not detect any significant change in their expression levels in response to VEGF under serum starvation conditions (data not shown).

To verify if increased expression of A1 and Bcl-2 in response to VEGF stimulation is also reflected in increased protein levels, we performed Western blot experiments with whole cell extracts of HUVE cells cultured under the same conditions as those employed for the RT-PCR analysis. We found a direct correlation between the mRNA and the protein levels (Fig. 2) of Bcl-2 and A1 in human endothelial cells in response to VEGF stimulation. These findings suggest that VEGF exerts its up-regulatory activity on the Bcl-2 and A1 genes primarily at the transcriptional level.

Bcl-2 is Sufficient to Mediate Survival of Serum-starved Endothelial Cells—We found decreased levels of Bcl-2 and A1 proteins in endothelial cells cultured in the absence of VEGF, suggesting that these genes may be involved in mediating survival activity of VEGF on endothelial cells (Fig. 3). It has been shown previously that A1 levels are induced upon incubation of human endothelial cells to inflammatory mediators such as tumor necrosis factor-α and interleukin-1β, and a possible role for the A1 gene in inflammation was suggested.
transiently transfect primary human endothelial cells, we wanted to test whether Bcl-2 was sufficient to mediate survival in our assay conditions. Transfected endothelial cells were identified by cotransfection of an expression vector for green fluorescent protein (pEGFP, CLONTECH, Palo Alto, CA) 24 h after the beginning of serum starvation and identified by fluorescence microscopy. To assess the effects of Bcl-2, we scored transfected cells in a blinded manner as healthy or apoptotic by morphology (21). Healthy endothelial cells are flat and well attached to the plate. Apoptotic endothelial cells are rounded, and some are fragmenting with the small cytoplasmic blebs that are characteristic of apoptosis. When we stained the cells with the DNA dye bisbenzimide (Hoechst 33258), apoptotic endothelial cells showed pronounced nuclear condensations. As shown in Fig. 3, Bcl-2 was sufficient to inhibit endothelial cell apoptosis in the absence of VEGF. We have observed lower levels of apoptosis when cells were transfected with Bcl-2 compared with untransfected cells grown in the presence of VEGF. We have observed lower levels of Bcl-2 are critical for endothelial cell survival when compared with untransfected cells grown in the presence of VEGF (100 ng/ml) or 10% fetal calf serum. These findings suggest that the levels of Bcl-2 are critical for endothelial cell survival when cultured under serum starvation conditions.

**DISCUSSION**

VEGF exerts its biological effects by binding to its respective transmembrane receptors Flt-1 and Flk-1/KDR, which are expressed mainly on endothelial cells. Stimulation of endothelial cells with VEGF and subsequent immunoblot analysis demonstrated that VEGF induces phosphorylation of phosphatidylinositol 3-kinase, Ras GTPase-activating protein, p190-rhoGAP, p62, PLCy, the oncogenic adapter protein NeK, p125 focal adhesion kinase (p125 FAK), paxillin, and several others (22–30; for review see Ref. 31). The signal transduction pathways involved in mediating the various biologic functions of VEGF on endothelial cells such as migration, proliferation, or survival remain to be characterized. Both VEGF receptors, Flk-1/KDR and Flt-1, were found previously to be expressed on HUVE cells by cell binding and cross-linking studies with VEGF or the Flt-1-specific ligand PGF (32) or by RT-PCR analysis (18). The HUVE cells used in our experiments were pooled primary isolates from 300 individual donor umbilical veins and thus are likely to represent an average population of endothelial cells.

Upon incubation of primary human endothelial cells with VEGF, we found increased expression of two antiapoptotic proteins, Bcl-2 and A1. The increase in Bcl-2 mRNA as well as the transcriptional level as well as an increase in RNA stability. The overall increase of the Bcl-2 protein observed in these cells may represent higher levels of Bcl-2 mRNA as well as the result of an increase in the protein stability.

Recently, several reports identified altered Bcl-2 levels in endothelial cells engaged in physiologic as well as pathologic angiogenesis. In human corpora lutea, Bcl-2 was found to be expressed in the vascular endothelium of some luteal arterioles and venules (33). During normal luteal phase or after treatment with chorionic gonadotrophin, the corpus luteum undergoes a rapid and massive increase in size and vasculature. This process was completely inhibited by administration of a truncated soluble Flt-1 receptor in a rat model of hormonally induced ovulation (34). These findings indirectly suggested a correlation between the levels of VEGF and Bcl-2 in endothelial cells. However, the numbers of blood vessels exhibiting Bcl-2 staining showed little variation throughout the luteal phase implying that other mechanisms are involved in luteal maintenance (33). An increase in the levels of Bcl-2 expression within the vascular endothelial spindle-shaped cells in Kaposi sarcoma lesions in humans was observed, indicating that up-regulation of Bcl-2 may be important in the pathogenesis of both classical and AIDS-associated Kaposi sarcoma (35).

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