REGULATION OF ENDOTHELIAL CELL APOPTOSIS BY VASCULAR ENDOTHELIA GROWTH INHIBITOR (VEGI) AND DEATH RECEPTOR 3 (DR3)

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Bachelor of Science, Humboldt State University 2002

Submitted to the Graduate Faculty of

The School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2008

UNIVERSITY OF PITTSBURGH

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Sammy Grimaldo, BS, PhD

University of Pittsburgh, 2008

Vascular Endothelial Growth Inhibitor (VEGI) is an endothelial cell autocrine factor and a member of the tumor necrosis family of ligands. VEGI is able to specifically inhibit endothelial cell growth and is an efficient inhibitor of angiogenesis. The molecular mechanisms of VEGI activity on endothelial cells remain undefined. Here we focused on two important steps in the signal transduction of VEGI. We first determined a role of NF-κB in VEGI-induced apoptosis. We found that inhibition of the NF-κB pathway resulted in an increased apoptotic potential of VEGI. We conclude that the NF-κB pathway plays a role in suppressing the apoptotic potential of VEGI. We next investigated the receptor responsible for VEGI-induced endothelial cell apoptosis. DR3 is a receptor for VEGI and thus we first focused on confirming if DR3 is the receptor responsible for VEGI-mediated endothelial cell death. We determined VEGI had diminished apoptotic activity in endothelial cells that are depleted of DR3 by siRNA. However, it was determined that the apoptotic stimuli, LPS and TNFα, were also unable to mediate cell death in DR3-depleted endothelial cells. We conclude that DR3 is mediating an intracellular event that is involved in controlling the apoptotic pathway. This is a novel role of DR3 that is yet to be described. However, this role of DR3 interferes with our analysis of the ligand/receptor relationship and therefore we were unable to confirm that DR3 is the receptor responsible for VEGI-induced apoptosis. We also provide preliminary evidence that VEGI is utilizing an

unknown receptor to mediate NF-κB activation. We therefore provide several mechanisms to control VEGI-mediated endothelial cell death, one being the activation of NF-κB to suppress the apoptotic potential of VEGI and the needed presence of DR3 for VEGI to initiate apoptosis, a role that is possibly independent of ligand binding.

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PREFACE

First I would like to thank my mentor Dr. Lu-Yuan Li, who has been instrumental in my career and giving me the independence and guidance for my development as researcher. Special thanks goes to my thesis committee for their guidance in my scientific endeavor. I would also like to thank Dr. Shi-Yuan Cheng for his excellent career advice.

Next, I would like to thank all the members of the Li Lab throughout the years. They have always been there when I needed their help.

Lastly I would like to thank my family for their continued support throughout the years. This dissertation is dedicated and in loving memory of my mother Lorraine E. Grimaldo. She has been the most influential person in my life and her early guidance has given me the gift of philosophy. She was always there to encourage my education and will always be in my thoughts at each succession in my life.

1.0 INTRODUCTION

The realization that tumor growth is dependent on angiogenesis has significantly changed the approach to cancer therapy. Understanding the molecular and cellular mechanisms that mediate angiogenesis is an area of intensive biomedical research. An extensive effort in drug development is now underway dedicated towards developing inhibitors of endothelial cell growth. Due to the natural and inherent capabilities of endogenous angiogenesis inhibitors to modulate endothelial cell growth there is focus to use these molecules as anti-angiogenic drugs.

1.1 ENDOTHELIUM

The endothelium is a type of simple squamous epithelium consisting of the largest organ in the body with an aggregated mass of ~1kg and a large surface area ~7000 m^2 (Pries et al., 2000). The endothelium consists of approximately $6x10^{13}$ polarized endothelial cells, with a luminal side in contact with the plasma and the abluminal side bathed by the interstitial fluid (Gimbrone, 1986). The endothelium is distributed throughout the body and therefore in a favorable position to mediate multiple functions and maintain homeostasis throughout the body. Once thought as a simple barrier between blood and tissue, the endothelium is now known to play an active role in vital functions including the maintaining the equilibrium of body fluids, the regulation of vascular tone, coagulation, and activation of inflammation (Gimbrone, 1986). The majority of

mature endothelial cells in the endothelium are mainly in a quiescent state with a cell-turnover marked in years and (Tobelem, 1990).

1.2 ANGIOGENESIS AND TUMOR PROGRESSION

Angiogenesis refers to the process of the formation of new capillary sprouts from pre-existing capillary vessels. Angiogenesis occurs during normal physiological processes such as embryonic development and the ovarian cycle. Angiogenesis is also associated with pathological conditions such as tumor growth, macular degeneration, and rheumatoid arthritis (Klagsbrun and Moses, 1999).

The angiogenic process is well studied and the major steps in the formation of new vasculature have been summarized (Bergers and Benjamin, 2003). The process begins with vasodilation and the increased permeability of pre-existing capillaries. The surrounding pericytes of the capillaries then loosen and become detached from the capillaries. The endothelial cell basement membrane is degraded by matrix metalloproteinase allowing for endothelial cell migration towards a chemotactic signal. Endothelial cells are able to interact with the extracellular matrix during the migration through interactions of the cell-surface intergrins such as $\alpha v\beta 3$ and $\alpha v\beta 5$. Endothelial cells then proliferate to form a migration column differentiate, change shape and adhere to form the lumen. The newly formed vessel then deposits a new basement membrane and pericytes are then recruited to stabilize the vessel wall.

A correlation between angiogenesis and tumor growth was first reported in the early 1900s. In 1904, Ribbert postulated that tumors establish a superior vascular supply to that of other

tissues (Ribbert, 1904). In 1908, Bowen raised the notion the obliteration of the vascular supply as a potential site for therapy (Bowen 1908). However, the medical value of angiogenesis was not fully appreciated until 1971 when Judah Folkman hypothesized that the growth of a primary tumor was angiogenesis-dependent and proposed the use of anti-angiogenic therapy as an approach to treat human cancer (Folkman, 1971). Over the next 30 years at least 18 anti-angiogenic inhibitors have been placed in clinical trials for the use as cancer therapeutics (McCarty et al., 2003; Folkman, 2007). The first angiogenesis inhibitor, bevacizumab, was approved by the Food and Drug Administration in 2004 for the treatment of metastatic colon cancer and later approved in 2006 for first line treatment of patients with advanced nonsquamous nonsmall cell lung cancer.

The progression of cancer is a multistep process as shown in figure 1, in which a growth of normal and neoplastic tumor mass must activate oncogenes for continued growth signaling and inactivate tumor suppressors to become irresponsive to growth inhibition and apoptotic signaling (Davis, 2007). One of the crucial steps involved in the conversion of cancer to a deadly disease is the ability of the tumor to obtain and manipulate a vasculature for its growth and dissemination of cells to secondary sites (Folkman, 1990).

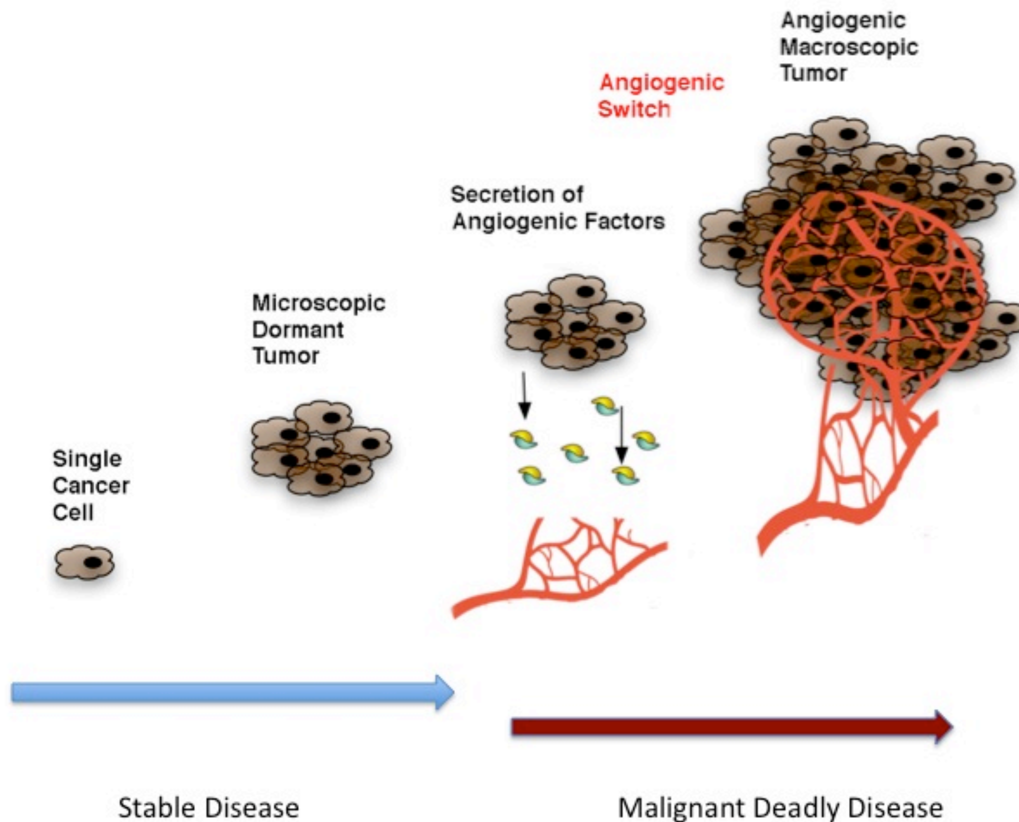

Figure 1. The progression of a tumor to a malignant phenotype is angiogenesis-dependent. Genetic abnormalities lead to the growth of a microscopic tumor to a ~1mm mass where it remains as a dormant tumor. The balance of pro- and anti-angiogenic factors are in an equilibrium resulting in a stable disease. Tumor progression at this stage is marked by the ability of the tumor to secrete pro-angiogenic factors shifting the balance of factors towards an angiogenic state thereby resulting in the recruitment of blood vessels from the surrounding stroma. The increased vasculature of the tumor provides increased nutrients allowing for the growth of the tumor to a macroscopic mass. The new bloods vessels allow for metastasis eventually leading to the death of the host.

1.3 REGULATORS OF THE ANGIOGENIC BALANCE

Under normal conditions the endothelium is thought to be in a state controlled by a balance between pro- and anti- angiogenic factors. The process of converting the quiescent endothelium into a state of angiogenesis is known as the “angiogenic switch”, in which there is a shift in the balance towards the pro-angiogenic factors. Since the angiogenic switch is necessary for the onset of tumor progression and several other pathological conditions there is extensive research directed towards understanding the factors involved in the switch. A prominent pro-angiogenic factor is vascular endothelial growth factor (VEGF). VEGF is a potent endothelial cell mitogen and key regulator of both physiological and pathological angiogenesis. VEGF (VEGF-A) belongs to a family of growth factors consisting of placenta growth factor, VEGF-B, VEGF-C and VEGF-D (Ferrara, 2002). In addition to its proliferative activities VEGF is a pro-survival factor able to induce a variety of anti-apoptotic proteins (Alon et al., 1995). VEGF is potent permeability factor able to induce vascular leakage, which is important in inflammation (Kirk and Karlik, 2003). The previously mentioned anti-angiogenic drug, bevacizumab, is directed towards blocking the action of VEGF. Another endothelial-specific growth factor is angiopoietin -1 (Ang-1) is known to be important in the stabilization and maturation of blood vessels by the recruitment of pericytes to the blood vessel (Sato et al., 1995). Basic fibroblast growth factor (bFGF) is another important factor in regulating angiogenesis and is responsible for mediating and maintaining endothelial cell survival (Fuks et al., 1994; O'Connor et al., 2000).

The endogenous anti-angiogenic factors are important in stabilizing and maintaining the vasculature in a quiescent state. During normal physiological angiogenesis the anti-angiogenic factors oppose the actions of the pro-angiogenic factors in order to bring the angiogenic process

to an end.   A number of endogenous inhibitors have been identified (Figure 2). However, their mechanisms of action are not as understood as compared to what is known about the mechanisms of the pro-angiogenic factors. One of the first endothelial-cell specific inhibitors identified was thrombospondin-1 (TSP-1) (Good et al., 1990). TSP-1 is a large multifunctional ECM glycoprotein that mediates its activity by binding to CD36, a class B scavenger receptor, and triggering apoptosis (Jimenez et al., 2000). TSP-1 is an effective inhibitor of tumor growth and metastasis (Streit et al., 1999; Rodriguez-Manzaneque et al., 2001).

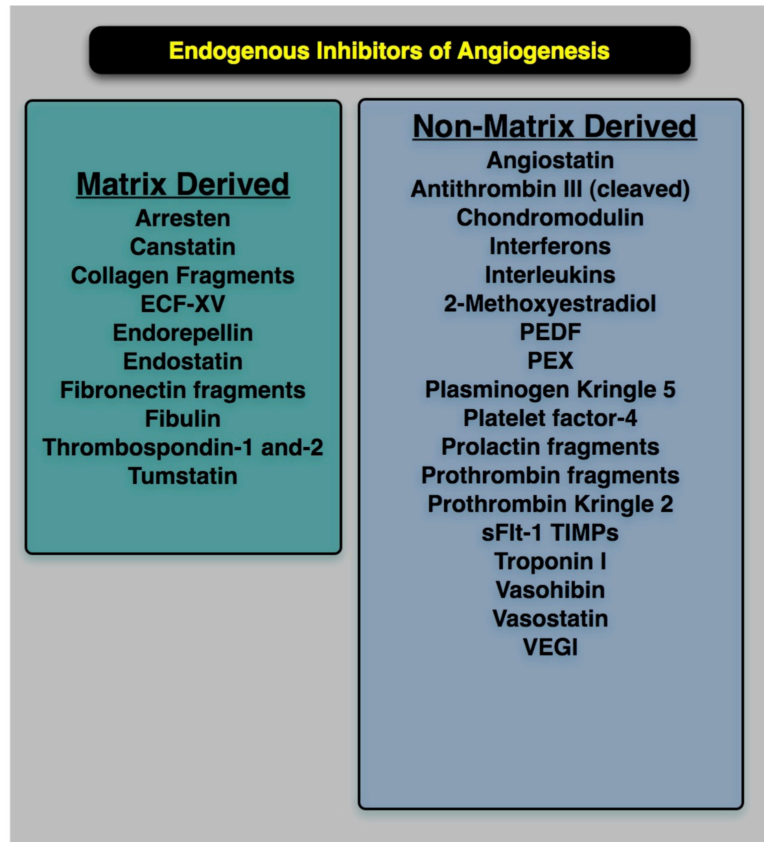

Figure 2. Endogenous inhibitors of vascular growth.

Two other well-known endogenous inhibitors are angiostatin and endostatin. Angiostatin, is a 38-kD to 45 kd cryptic fragment of plasminogen containing kringles-1 and -4 to kringle-1 and 3 (Cornelius et al., 1998). Angiostatin is able to mediate endothelial cell apoptosis (Lucas et al., 1998) and is able to bind and block ATP-synthase on the surfaces of endothelial cells, thereby inhibiting proliferation (Moser et al., 1999). Endostatin is a 20-kDa C-terminal fragment of collagen XVIII is a broad-spectrum anti-angiogenic effect and shown to be a potent inhibitor of tumor angiogenesis (Folkman, 2006; O'Reilly et al., 1997). Endostatin inhibits endothelial cell migration, induce apoptosis in endothelial cell, bind to ATP on the endothelial cell surface, and block the actions of the metalloproteinase (Dhanabal et al., 1999; Shichiri and Hirata, 2001; Nyberg et al., 2003).

1.4 ANGIOGENESIS-DEPENDENT DISEASES

As mentioned above, angiogenesis is a necessary step in the progression of a tumor. It should be noted that angiogenic therapy might not only benefit cancer therapy but also be helpful to unrelated disease states angiogenesis-dependent. Autoimmune disorders, age-related macular degeneration and atherosclerosis are known to require angiogenesis in order for disease progression (Miller, 1997; Smolen and Lipsky, 2003). Anti-angiogenic therapy has been referred to as an “organized principle” for drug discovery (Folkman, 2007). As there are clinical advantages for angiogenesis inhibitors approved for one disease such as cancer, can be used for another disease. One such example is Ranibizumab a variant of the drug Bevacizumab that is successful in recovering eyesight of patients with age-related macular degeneration (Rosenfeld et al., 2006). Thus, the development of anti-angiogenic drug development can benefit not only

cancer research but also a broader spectrum of benefits towards multiple diseases.

1.5 TUMOR NECROSIS SUPERFAMILY

The necrosis factor (TNF) family of ligands consists of 19 type II transmembrane proteins characterized by a conserved C-terminal domain required for receptor binding known as the TNF homology domain (THD) (Bodmer et al., 2002). Most members are membrane-bound proteins that are made into soluble ligands following proteolytic cleavage by metalloproteases of the ADAM (a disintegrin and metalloproteinase domain) family (Black et al., 1997). TNF ligands exist as trimers, with a hallmark structure of anti-parallel beta-pleated sheet sandwich in a standard jellyroll topology (Jones et al., 1989).

The TNF family of receptors consist of 29 type I transmembrane proteins with the exception of BCMA, TAC1, BAFFR, and XEDAR, which are type III transmembrane proteins. The extracellular portions are characterized by the presence of cysteine-rich domains which are pseudo-repeats typically containing six cysteine residues engaged in the formation of three disulfide bonds (Bodmer et al., 2002). Four members of the TNF receptor family are produced as soluble proteins known as the decoy receptors; DCR1, DCR2, DCR3 and osteoprotegrin, that are generally thought of as negative regulators of cell signaling. TNF receptors are classified into two groups depending on whether or not the receptor contains an intracellular domain, known as the death domain. The death domains are responsible for activating the caspase pathway and deletion of this domain abolishes ligand-induced apoptosis (Itoh and Nagata, 1993). Death-domain containing TNF receptors are referred to the death receptors consisting of TNFR1, CD95, DR3, DR4, DR5, DR6, EDAR, and NFGR.

It was initially thought that TNF ligands are able to induce receptor trimerizaiton leading to their activation. However, studies with the prototypic death receptor, TNFR1, reveal that TNFR1 exists as a pre-formed trimer at the cell-membrane (Chan et al., 2000). Formation of the pre-formed trimer requires an extracellular domain called the pre-ligand domain. Thus, TNFα does not mediate aggregation of TNFR but rather induces a conformational change in the pre-formed receptor allowing for the recruitment of downstream adaptors. Homologous pre-ligand domains are also found in other TNF receptors such as DR3 and DR5 and thus is predicted other TNF receptors exist as pre-formed trimers on the cell-surface (Chan et al., 2000).

TNF ligands upon binding to their cognate receptor can activate several signaling pathways to mediate their cellular responses including NF-κB, JUN N-terminal kinase (JNK), p42/p44 mitogen-activated protein kinase (MAPK), and p38 MAPK. Activation of these pathways can initiate a host of biological functions involved in immune surveillance, haematopoiesis, protection against microbial infection, innate immunity, and tumor regression. Several of TNF ligands can expand their biological activities by binding to multiple TNF receptors (Figure 3) (Ashkenazi, 2002). Signaling activated by TNFα serves as a paradigm for most of the TNF superfamily members. In particular, studies with TNFα binding to TNFR1 are used to map out the proteins involved in the signaling cascade common to the death receptors. TNFα binding to TNFR1 first leads to the recruitment of the adaptor protein, TNFR-associated death domain protein (TRADD) and binds to the death domain of TNFR1 (Baud and Karin, 2001). TRADD serves as platform for further recruit of other adaptor proteins and it this point the adaptor proteins exhibit two distinct signaling arms of TNFR1. One arm involves activation of the pro-survival and inflammation cascades and the second arm consists of activating the caspase pathway leading to cell death (Hsu et al., 1996). One of the essential adaptor proteins in the pro-

survival signaling arm of TNFR signaling is TNF-receptor-associated factor 2 (TRAF2) belonging to the TRAF family of proteins. TRAF2 compromises a highly conserved C-terminal TRAF domain (C-domain) and a more variable N-terminal domain that contains a ring finger and several ZN fingers (Arch et al., 1998). TRAF2 and other TRAF family members are responsible for the activation of IKK, JNK, and p38 (Liu et al., 1996). The second signaling arm involves the recruitment of the Fas-associated death domain protein (FADD). Upon recruitment of FADD to

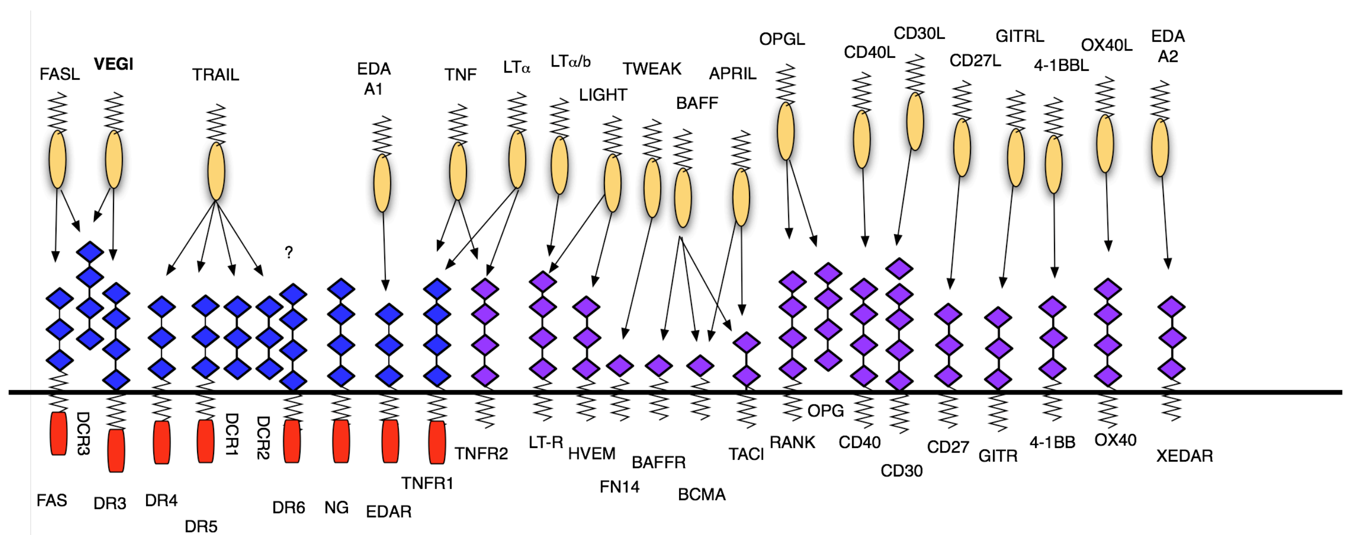

Figure 3. Members of the TNF family of ligands and their cognate receptors. Arrows indicate receptor binding. The ligands for several receptors are yet to be identified, indicated by question marks. Diamonds indicate cysteine-rich domains. Red bars indicate death domain capable of inducing apoptosis.

the receptor complex it can interact and activate the initiator caspases, caspase-8/10 (Muzio et al., 1996). The receptor-interacting serine/thereonine kinase (RIP) family of adaptor proteins are known to be involved in both the pro-survival and pro-apoptotic signaling arms of TNFR. RIP contains a C-terminal death domain and N-terminal serine/thereonine protein kinase domain (Stanger et al., 1995). The RIP1 family member is a key effector in the activation of NF-κB (Liu et al., 1996) and the RIP2 and RIP3 family members are involved in both NF-κB activation and

apoptosis (Yu et al., 1999; McCarthy et al., 1998). These adaptor proteins create a modularity of TNF receptors allowing for the flexibility of mediating multiple biological functions.

1.5.1 TNFSF15; Vascular endothelial growth inhibitor (VEGI); TL1A

VEGI is an endogenous inhibitor of endothelial cell growth (Figure 2) and a member of the TNF family of ligands (Figure 3). The crystal structure of VEGI has been determined (Jin et al., 2007a; Jin et al., 2007b). It was determined that there are three VEGI monomers in the asymmetric unit and they are related by a 3-fold non-crystallographic symmetry. These monomers form a trimer and assume a jellyroll β-sandwich typical of other members of the TNF family of ligands. A multiple structure alignment between VEGI and other members of the TNF reveals that most strands have a very similar length and their overall structures superimpose with one another revealing that there is a greater resemblance between VEGI and the TNF ligands as would be predicted from the sequence homology. A loop between strands C and D in VEGI is the longest among the TNF ligand members, while the AA' loop, part of which is disordered, in VEGI is the second longest after that in TRAIL. Similar loops in TNFβ/LTα are known to participate in receptor binding.

VEGI mRNA expression is detected predominantly in endothelial cells and VEGI gene transcripts are found in many human tissues, including placenta, lung, kidney, skeletal muscle, pancreas, spleen, prostrate, small intestine and colon (Tan et al., 1997; Zhai et al., 1999a). Figure 4 shows the vascular localization of VEGI found in placenta and bladder tissue. There are three isoforms of VEGI designated, VEGI-251, VEGI-192, and VEGI-174. The expression profiles of VEGI-251 and VEGI-174 have been described (Chew et al., 2002). The 7.5 kb transcript encoding VEGI-251 is expressed at high levels in the placenta, kidney, lung and liver, whereas

the 2 kb transcript corresponding to VEGI-174 is detected in liver, kidney, skeletal muscle and heart. The VEGI-174 mRNA is more abundant in heart, skeletal muscle, pancreas, adrenal gland, and liver, while VEGI-251 is more abundant in fetal kidney and fetal lung. Overlapping of the two transcripts is detected in prostate, salivary gland and placenta. When examined in cultured cells by using RNAse protection assay, all three isoforms are found in human endothelial cells, including coronary artery endothelial (HCAE), human umbilical-vein endothelial cells (HUVEC), and human microvascular endothelial (HMVE) cells. Although the isoforms are present simultaneously, VEGI-251 is the most abundant. These expression patterns suggest the possibility of tissue- or developmentally specific functions for the variants of VEGI.

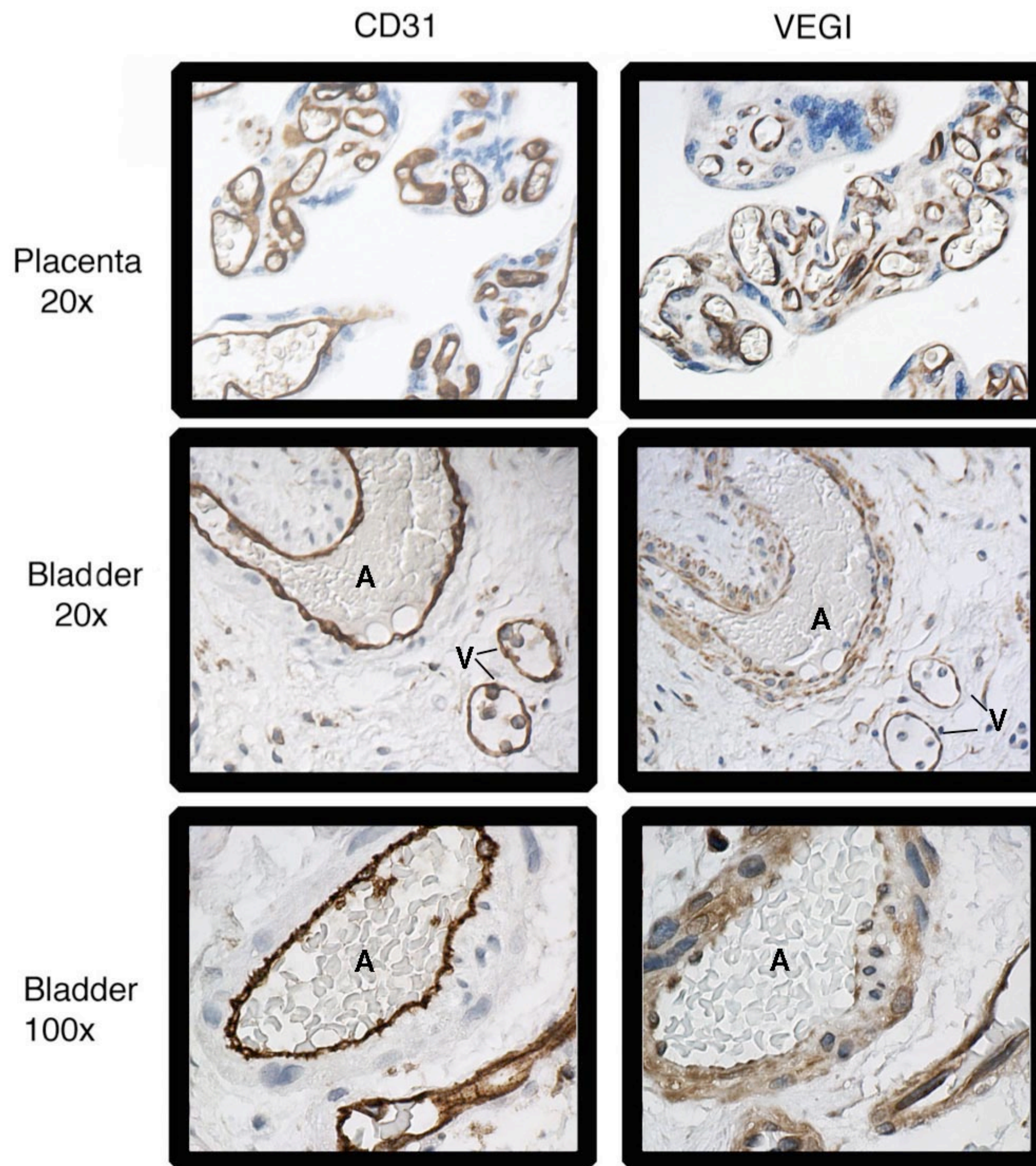

Figure 4. Localization of VEGI in the vasculature. Top, the microvessel-rich placenta shows a similar pattern of both endothelial-cell marker CD31 and VEGI. Lower panels, Low and High magnification of normal human bladder tissue. VEGI expression in blood vessels of the bladder is found in the endothelial lining of arteries (A) and veins (V). Note VEGI expression is found throughout the arterial wall.

The anti-angiogenesis activity of VEGI was assessed in two angiogenesis models. In an *in vitro* angiogenesis model, recombinant VEGI-174 was used to treat ABAE cells grown on a layer of collagen gel (Zhai et al., 1999b). When stimulated with fibroblast growth factor-2 (FGF2), untreated ABAE cells formed a network of capillary-like tubes in the gel. Addition of the VEGI-174 preparation completely inhibited the capillary formation but did not cause cell death. In another experiment, human vascular endothelial cell line transfected with a VEGI-expressing plasmid exhibited a decreased ability to form microtubules in Matrigel, a reconstituted basement membrane (Conway et al., 2007). When evaluated in the chick embryo chorioallantoic membrane (CAM) model, recombinant VEGI-174 inhibited about 50% of the neovascularization induced by either FGF or VEGF (Zhai et al., 1999b). These results suggest that VEGI can inhibit capillary formation regardless of the types of angiogenic stimuli. In a different approach, an adenovirus expressing a fusion protein consisting of endostatin and the C-terminal 151 residues of TNFSF15, termed AdhENDO-VEGI151, were shown to be able to prevent angiogenesis in both the CAM model and a rabbit corneal neovascularization model (Pan et al., 2004).

VEGI is an important factor in vascular biology. However, similar to other members of the TNF family of ligands VEGI can elicit a number of activities on immune cells. VEGI increases the responsiveness of T cells to IL-2, although VEGI alone does not induce proliferation of T cells (Migone et al., 2002). Pre-treatment with VEGI enhances T cell proliferation in response to IL-2, increases IL-2Rα and β expression, and induces secretion of interferon-γ (IFN-γ) and granulocyte-macrophage colony stimulating factor (GM-CSF). Additionally, IL-2 receptor IL-2Rα (CD25) and IL-2Rβ (CD122) expression increases in VEGI treated cells. Recombinant VEGI protein was shown to stimulate dendritic cells (DCs) maturation (Tian et al., 2007) VEGI-

stimulated DCs are able to facilitate the proliferation and differentiation of $CD4^{+}$ naive T cells in co-cultures. VEGI was also shown to be strongly up-regulated in human monocytes and dendritic cells when the FcγR signaling pathway is activated (Prehn et al., 2007).

VEGI is a ligand for two TNF receptors: DR3 and DcR3 (Migone et al., 2002). Using a 293F cell line stably expressing full-length VEGI on the cell surface, a Fc-fusion form of the extracellular domain of TNFR family members including TNFR1, Fas, HveA, DR3, DR4, DR5, DR6, DcR1, DcR2, DcR3, OPG, RANK, AITR, TACI, CD40, and OX40 were evaluated for binding. The results show that only DR3 or DcR3 can bind to the VEGI protein on the 293F cells. In another experiment, a FLAG-tagged soluble form of VEGI was applied to cells transiently transfected with various members of the TNFR family, including TNFR2, LT R, 4-1BB, CD27, CD30, BCMA, DR3, DR4, DR5, DR6, DcR1, DcR2, RANK, HveA, and AITR. The tagged VEGI was found to bind only to DR3-overexpressing cells. In addition, direct interaction between VEGI and DR3 was detected by co-immunoprecipitation. Another independent group also confirmed the binding of VEGI to DR3 and DcR3 in a flow cytometry-based assay (Bossen et al., 2006).

1.5.2 TNFRSF25; Death Receptor 3 (DR3)

DR3 is a death-domain containing receptor having 4 cysteine-rich domains in its extracellular region. Several groups indentified DR3 independently (Marsters et al., 1996; Bodmer et al., 1997; Kitson et al., 1996). Several of the approaches utilized EST database searching for homologous TNFR domains. One approach used a two-hybrid system (Kitson et al., 1996). In attempt to find interacting proteins with TNFR1, Kitson et al cloned the death domain of TNFR1 and constructed GAL4 fusion protein using the fusion protein in a HeLa cell two-hybrid library

Seven clones were indentified that interacted specifically with TNFR1 death domain, 6 of which encoded cytoplasmic regions of TNFR1. The seventh clone was indentified to be a new complementary DNA, later to be named DR3. The expression of DR3 is enriched in lymphocyte-rich tissues such as peripheral blood lymphocytes, thymus, colon, intestine, and spleen (Bodmer et al., 1997). $DR3^{-/-}$ mice were determined develop normally with no obvious abnormalities (Wang et al., 2001). It was revealed that $DR3^{-/-}$ mice are impaired in the negative selection of anti-CD3-induced apoptosis in the thymus suggesting a role of DR3 in the removal of self-reactive T cells in the thymus.

Ectopic overexpression of DR3 activates NF-κB and apoptosis (Kitson et al., 1996; Marsters et al., 1996). Following the identification of VEGI as a ligand for DR3 it was determined that VEGI-induced activation of DR3 recruited TRADD, TRAF2, and RIP and activated the NF-κB, ERK, JNK, and p38 mitogen-activated protein kinase pathways in human erythroleukemic TF-1 cells (Migone et al., 2002). Alternative splicing of DR3 results in at least 11 isoforms (Screaton et al., 1997; Warzocha et al., 1998). Eight of the isoforms lack transmembrane domains and thus are predicted to be secreted soluble variants of the receptor. Most isoforms contain premature stop codons and are predicted to be targeted for degradation by the mRNA surveillance mechanism, nonsense mediated RNA decay (Hillman et al., 2004). Alternative splicing of DR3 in peripheral blood lymphocytes (PBLs) results "on" and "off" state in state of DR3 (Screaton et al., 1997). In non-activated PBLs the DR3 isoforms mainly exist in the soluble state and unable to mediate cell signaling. Following the activation of PBLs, the DR3 isoforms undergo an isoform switch from the soluble to the membrane-bound isoform, the functional variant needed for cell signaling.

DR3 interacts with two endothelial-specific gene products. One, as previously mentioned,

is the TNF ligand VEGI. The other is the endothelial cell-surface protein, E-selectin (Gout et al., 2006). Gout et al studied the interactions of DR3 expressed on colon carcinoma cells HT29 and E-selectin expressed on human umbilical vein endothelial cells. Using siRNA to deplete DR3 or its neutralization by an antibody on HT29 cells impaired the adhesion, transendothelial cell migration, and activation of p38 and ERK by E-selectin. Thus, DR3 serves as a counter-receptor for E-selectin and may be involved in the metastatic process in which cancer cells must bind to the endothelium in order to extravasate to the secondary site. Also, the reverse signaling of E-selectin activation of DR3 conferred pro-survival signaling in the colon cancer cells thus fortifying the survival of cancer cells.

1.6 THE NF-κB PATHWAY

The Rel/NF-κB family is consists of the five members: c-REL, RelA, RelB, p1025/NF-κB1 and p100/NF-κB2. The Rel family members are able to form homo- or hetero- dimers with other family members and each member posses a 300 amino acid N-terminal domain, known as the Rel homology domain (RHD). The REL domain is responsible for dimerization, nuclear translocation and DNA binding. This family can be divided into two groups classified by whether the member is synthesized as a mature protein or produced as a pre-cursor protein that needs to be proteolytically cleaved in order to be active. The members Rel A (p65), c-Rel and RelB, are produced as mature products and do not require proteolytic processing. The second group is encoded by NF-κB1 (p50/105) and NF-κB2 (p52/100) genes are first produced as large precursors, p105 and p100 then proteolytically cleaved to form the mature p50 and p52 NF-κB proteins.

Shuttling to the nucleus and the cytoplasm modulates the activity of the NF-κB family

members. The IκB family of proteins are responsible for inhibiting NF-κB/Rel activity by binding and retaining the transcription factors in the cytoplasm. There are seven known mammalian IκB members, IκBα, IκBβ, IκBε, IκBγ, BCL-3 and the precursors REL proteins p100 and p105 (Ghosh et al., 1998). The IκBs proteins are able to sequester NF-κB by the presence of multiple ankyrin repeat, which are protein-protein interaction domains that interact with NF-κB via the RHD. The dimerization domain of the NF-κB dimers is the primary region of interaction with IκBs and differences in the interactions mounted by IκBα and IκBβ may play a role in regulating NF-κB activity (Ghosh and Karin, 2002).

Figure 5 shows the essential steps in the activation of the NF-κB leading to gene expression. Activation of the canonical NF-κB begins with a ligand stimulation and activation of the IKK complex. The mechanism that leads to the active IKK complex by TNF signaling is still unclear. IKK is a large complex (700-900 kDA) composed of IKKα (IKK1), IKKβ (IKK2) the catalytic subunits, and IKKγ (NEMO), the regulatory subunit (Rothwarf and Karin, 1999). The IKK complex activates NF-κB by phophorylating the inhibitor IκB family of proteins. IKKα and IKKβ both target two serines in the N-terminal regulatory domain of IκB proteins, with IKKβ being the more potent kinase (Zandi et al., 1997). The phoshporlyation of the IκBs tags the proteins for polyubiquitination by the ligase, beta-TrCP, belonging to the SCF family (Ben-Neriah, 2002). Following ubiquitination of the IκBs they are degraded thereby freeing NF-κB and allowing for nuclear translocation where binds to DNA, and activates transcription. DNA binding consists of NF-κB dimer making a complex with each contact on one half of the DNA binding site, slight variations in the 10 base pair consensus sequence 5’GGGGYNNCCY3’ confer preference to selected Rel members (Kunsch et al., 1992)

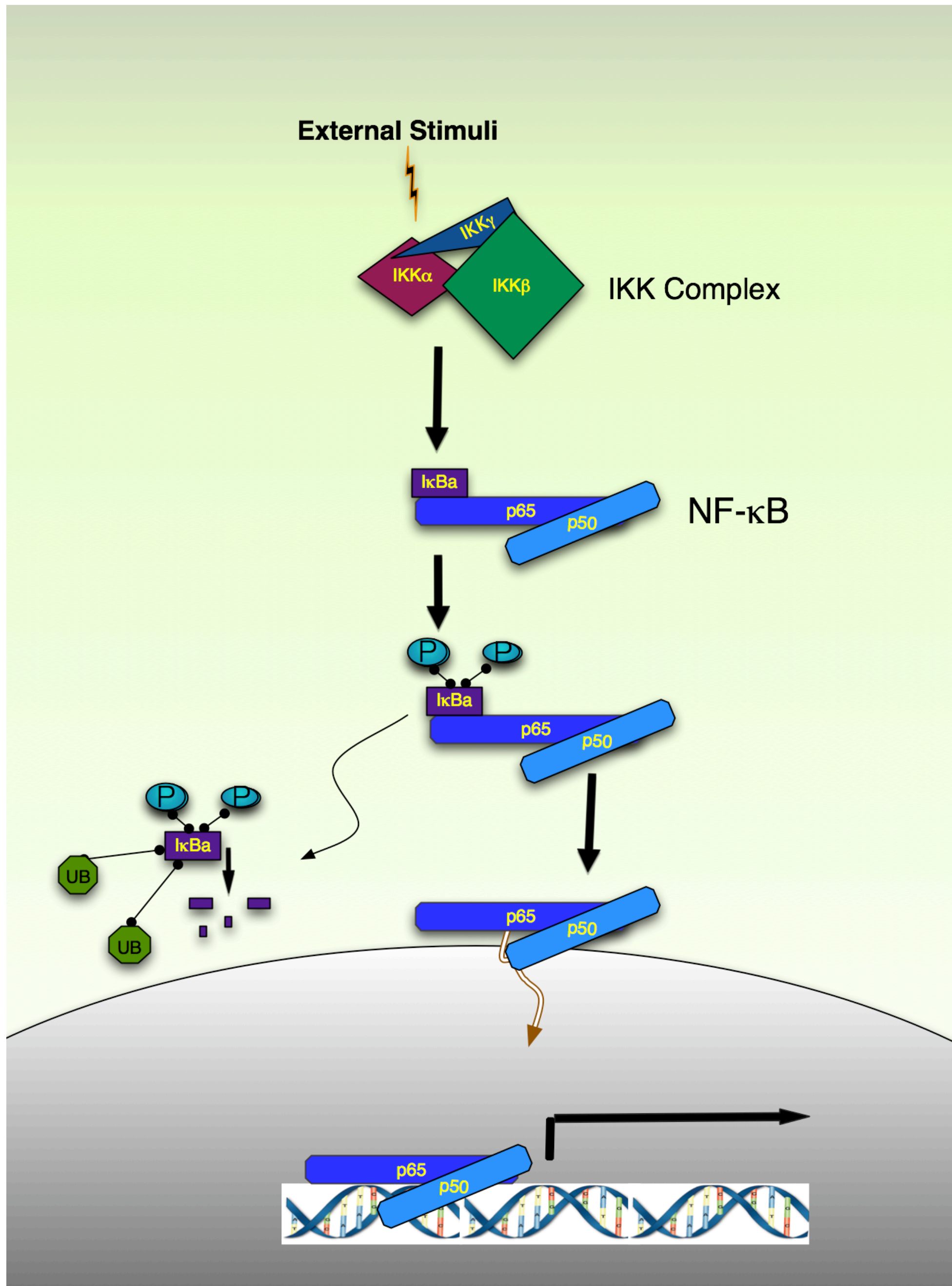

Figure 5. The canonical NF-κB pathway. NF-κB is sequestered in the cytoplasm by the inhibitor IκBα. Upon stimulation of the cell this leads to activation of the IKK complex. The IKK complex is able to phosphorylate IκB marking it for proteosome-mediated degradation. NF-κB is freed and able to translocate to the nucleus where it is able to initiate transcription. (See text for details)

1.7 THE CASPASE PATHWAY

Apoptosis or programmed cell death sometimes referred to as cell suicide is a tightly regulated process that removes unnecessary cells from an organism and plays an important role in tissue homeostasis. Apoptosis is distinguishable from necrosis, as it does not lead to inflammation and neighboring cells are unaffected.

Caspases are the central components of the apoptotic response. The involvement of these enzymes in apoptosis was first realized in studies with the characterization of nematode worm *Caenorhabditis elegans gene*, ced-3 (cell death abnormality-3) and determined to closely resemble the mammalian caspase-1 (Yuan et al., 1993). Caspases are a conserved family of proteins and to date 14 mammalian caspase have been indentified with 11 being in humans (Shi, 2002). Caspases are produced as zymogens and during the initiation of the apoptotic process must undergo proteolytic processing in order to activate the next sequence of caspases. Caspases can be divided into two groups: the initiator caspases (-2, -8, -9 and -10) or the effector caspases (-3, -6, and -7), the initiator caspase able to auto-activate and activate the effector caspases. The initiator caspases cleave specific aspartate residues found in the effector caspases resulting in the formation of two subunits the large (~p20) and small (~p10) subunits (Riedl and Shi, 2004). The caspase cascade is tightly regulated and often requires the assembly of a multi-component complex during apoptosis (Adams and Cory, 2002).

Depending on the origin of the apoptotic stimuli whether from inside the cell or from extracellular stimuli the pathways known as the intrinsic pathway or the extrinsic pathway, respectively. The intrinsic pathway is mediated by the mitochondria and can be triggered by such events such as DNA damage (Wang, 2001). Activation of the intrinsic pathway results in

the release of several proteins from the intermembrane space of the mitochondria into the cytoplasm. One important factor that is released is cytochrome c, which is responsible for activating the protein APAF1 leading to the formation of the apoptotsome and activates caspase-9 and the remaining caspase cascade (Li et al., 1997; Zou et al., 1999). The extrinsic pathway is initiated by the binding of extracellular death ligands, which leads to formation of the death inducing signaling complex and activation of caspase-8 (Rath and Aggarwal, 1999). Caspase-8 is activated through an induced proximity mechanism. Fadd and caspase-8 are necessary for TNFR1-, FAS-, and DR3- induced apoptosis (Yeh et al., 1998;Varfolomeev et al., 1998).

Caspase activation by either the intrinsic or extrinsic pathway will eventually lead to the degradation of cytoskeletal and nuclear proteins critical for maintenance of cell structure and cleavage of other enzymes necessary for cell survival and DNA fragmentation (Enari et al., 1998; Thornberry and Lazebnik, 1998).

1.8 SCOPE OF THIS DISSERTATION

Vascular endogenous growth inhibitor is an endogenous inhibitor of angiogenesis that belongs to the TNF superfamily of ligands. To date the molecular mechanisms underlying the regulation of VEGI signaling are not clearly described. We investigated two important mechanisms of VEGI action on endothelial cells while trying to determine the therapeutic potential of this unique cytokine.

Our first investigation was to determine the role of NF-κB in VEGI-mediated endothelial cell death. NF-κB is an anti-apoptotic factor known to be activated by VEGI. Since we observe cell death under normal signaling of VEGI we wanted to establish if in fact NF-κB is utilized as an anti-apoptotic factor in endothelial cells. We used either siRNA as well as NF-κB inhibitors in

order to prevent VEGI-induced NF-κB activation. We determined that inhibition of the NF-κB pathway resulted in an increased apoptotic potential of VEGI, suggesting that NF-κB suppresses VEGI-induced apoptosis. This study suggests a new approach to use VEGI with NF-κB inhibitors in order to improve its anti-angiogenic potential.

In addition we sought to determine the essential receptor required for VEGI in regulating endothelial cell death. We investigated if the known receptor for VEGI in TF-1 cells, DR3, is the major receptor responsible for mediating VEGI-induced apoptosis in endothelial cells. We were unable to clarify if DR3 is the major receptor because the removal of DR3 had a unique impact on the cells. However, we determined that DR3 is involved in regulating the apoptotic pathway of several apoptotic stimuli such as LPS and TNFα. Furthermore, this investigation also gives preliminary evidence of a novel receptor for VEGI in endothelial cells.

2.0 SENSITIZATION OF ENDOTHELIAL CELLS TO VEGI-INDUCED APOPTOSIS BY INHIBITING THE NF-KB PATHWAY

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2.1 ABSTRACT

Vascular endothelial growth inhibitor (VEGI) is a member of the tumor necrosis factor superfamily and endothelial-cell autocrine factor that induces apoptosis in proliferating endothelial cells. We investigated the relationship between VEGI-induced NF-κB activation and apoptosis in endothelial cells. NF-κB or its activator IKK2 was depleted from adult bovine aortic endothelial cells using short interfering RNA (siRNA). Depletion of these molecules prevented VEGI-induced NF-κB activation, evidenced from a reduced extent of NF-κB nuclear translocation and diminished expression of NF-κB-target genes such as interlueukins-6 and -1β. The siRNA-treated cells when exposed to recombinant VEGI exhibited markedly decreased cell viability and significantly increased apoptosis. Alternatively, inhibition of NF-κB activation with specific inhibitors, curcumin and BMS-345541, gave similar results. These findings suggest that VEGI-induced apoptosis in endothelial cells is accompanied by a concomitant activation of the pro-survival NF-κB pathway, which modulates the extent of cell death. VEGI is a potential anti-angiogenic therapeutic agent. Our findings indicate that sensitization of endothelial cells to VEGI-induced apoptosis by inhibiting the NF-κB pathway could be a useful strategy in anti-angiogenic and anti-cancer treatment.

2.2 INTRODUCTION

Vascular endothelial growth inhibitor (VEGI; also known as TL1A) belongs to the TNF superfamily of ligands (TNFSF15) (Tan et al., 1997). VEGI is expressed predominately by vascular endothelial cells and inhibits endothelial cell growth by an autocrine mechanism (Zhai et al., 1999a; Kim and Zhang, 2005; Yang et al., 2004). Uniquely, the inhibition effect of VEGI is selective towards vascular endothelial cells (Zhai et al., 1999a; Hou et al., 2005). VEGI

inhibits endothelial cell growth in two ways: it induces apoptosis in proliferating cells or enforces a growth arrest if the cells are in G0 or early G1 of the growth cycle (Yu et al., 2001). In murine tumor models recombinant VEGI is an effective inhibitor of neovascularization and tumor growth (Zhai et al., 1999a; Zhai et al., 1999b; Hou et al., 2005). The constitutive expression of VEGI in the vasculature and the modulation of endothelial cell growth suggest a role of this cytokine in the maintenance of vascular homeostasis.

The ligands of the TNF family are known to typically activate the NF-κB transcription factor. (Gaur and Aggarwal, 2003) NF-κB proteins are dimeric DNA binding proteins consisting of members of the Rel/NF-κB family of transcription factors. The predominant NF-κB complex is a heterodimer consisting of the p50 and p65 family members. (Baeuerle and Baltimore, 1989) In resting cells the NF-κB complex is sequestered in the cytoplasm by the inhibitor IκBα, which masks the nuclear localization signal of NF-κB. Ligand stimulation leads to activation of the IKK complex consisting of IKK1, IKK2, and IKKγ subunits. Upon activation, the IKK complex phosphorylates IκBα, leading to poly-ubiquitination and subsequent proteosome-mediated degradation. Degradation of IκBα thereby releases NF-κB, allowing the transcription factor to translocate to the nucleus and bind to specific DNA sequences to regulate expression of target genes.

VEGI interacts with the death receptor 3 (DR3), and determined to be a ligand for DR3 in human T cells (Migone et al., 2002). The death receptors are a subset of the TNF receptor family defined by the presence of an intracellular death domain in their primary structures capable of activating the caspase pathway leading to apoptosis (Baker and Reddy, 1998). VEGI treatment does not result in apoptosis of T cells, however activation of NF-κB is known to be responsible for the ability of cells to resist apoptosis by up-regulating the anti-apoptotic factor c-IAP2 (Wen

et al., 2003). Interestingly, unlike T cells, endothelial cells undergo apoptosis when treated with VEGI. The role of VEGI-induced NF-κB activation in endothelial cells remains undefined. Here, we report that the prevention of NF-κB activation by either silencing key components of the NF-κB pathway with siRNA or using small-molecule inhibitors sensitizes endothelial cells to VEGI-induced apoptosis. Our findings suggest that VEGI activation of NF-κB modulates the extent of VEGI-induced apoptosis in endothelial cells. The use of NF-κB inhibitors to improve the efficacy of VEGI could be a potential strategy for anti-angiogenic and anti-cancer therapy

2.3 MATERIALS AND METHODS

2.3.1 Cells and reagents

Adult bovine aortic endothelial (ABAE) cells were purchased from Lonza (Walkersville, MD). NF-κB p65 (F-6) monoclonal mouse antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). IKK2 (L570) polyclonal rabbit antibody was purchased from Cell Signaling Technology (Danvers, MA). Curcumin was purchased from Sigma-Aldrich (St. Louis, MO). BMS-345541 was purchased from Calbiochem (San Diego, CA). VEGI was produced from E.coli as previously described (Hou et al., 2005). One unit of VEGI activity is defined as the IC50 value (100-500 ng/ml) of the VEGI preparation (Tian et al., 2007).

2.3.2 siRNA transfection

Custom designed siRNA duplexes were synthesized by Sigma-Proligo (St. Louis, MO). siRNA sequences were designed against the bovine NF-κB p65 and IKK2 mRNA: 5'-GGACGTACGAG ACCTTCAA-3' (p65), and 5'-AGAAAGTGCGAGTGATTTA-3' (IKK2). A scrambled siRNA control was purchased from Ambion (Austin, TX). Transfection was carried out with RNAiMax

(Invitrogen, Carlsbad, CA) and opti-MEM reduced serum medium according to manufacturer's instructions overnight at 37 ºC , 5% CO_2. The cells were then rinsed and maintained in normal growth media for 48 hours before being exposed to VEGI.

2.3.3 Viability assay

ABAE cells were seeded in black 96-well well plates. Following the treatments, the cells were washed once with PBS. Calcein-AM (Invitrogen Carlsbad, CA) 1 μg/ml in PBS was then added to the cells and incubated for 30 minutes at room temperature. The cells were washed with PBS and fluorescent intensity, 485nm excitation: 520 nm emission was measured on a Tecan Safire fluorescence plate reader (Tecan, San Jose, CA)

2.3.4 Western blot analysis

Cells were subjected to lysis and total protein lysate was resolved using 12% SDS-PAGE. The proteins were transferred onto a Hybond-ECL nitrocellulose membrane, blocked with 5% nonfat dried milk powder for 1 h at room temperature, and incubated overnight at 4 ºC with the primary antibodies. The membrane was washed and incubated with an appropriate HRP-conjugated secondary antibody and developed with the ECL system (Amersham Biosciences, Pittsburgh, PA).

2.3.5 Nuclear translocation assay

Nuclear translocation of NF-κB was analyzed using an automated fluorescence imaging system. ABAE cells were cultured in a 96-well plate and following treatments the cells were fixed with 4% paraformaldehyde for 10 min, then permeabilized with PBS containing 1% Triton X-100 for 5 min. The cells were labeled with mouse anti-p65 antibody and Alexa Fluor 488 goat anti-mouse antibody (Invitrogen). Nuclei were stained with 1 μg/ml Hoechst 33342. Cellomics ArrayScan VTI HCS Reader was used to analyze the cells as previously described. (Vakkila et

al., 2004)   Briefly, the system scans multiple fields in a well to acquire images from a predefined numbers of cells as determined by the Hoechst channel. The system analyzes fluorescent intensity (mean pixel intensity) of nuclear and cytoplasmic regions (488 nm).

2.3.6 QRT-PCR

Total RNA was extracted and purified from cultured cells with an RNeasy mini-kit (Qiagen, Valencia, CA). RT was performed using the TaqMan® One-Step RT-PCR Kit (ABI systems, Foster City, CA). Expression levels of IL-6, IL-1β, and GAPDH genes were analyzed using SYBR® Green kit (ABI Systems) and ABI PRISM 7700 sequence detection system. Primers sequences were: IL-6, forward 5'-TGAGTGTGAAAGCAGCA AGGA-3', reverse 5'-TCGCCT GATTGAACCCAGATT-3'; IL-1β, forward 5'-CCAGCT TCTGATGAGCAACCA-3', reverse 5'-CAGATGCGCCTG CTTCTAGG-3'; GAPDH, forward 5'-GGCGTGAACCACGAG AAGTAT-3', reverse 5'-CCTCCACGATGCCAAA GTG-3'. Relative expression of the marker genes was calculated from the delta-Ct (threshold cycle) of the targeted gene normalized to the delta-Ct of GAPDH.

2.3.7 Caspase-3 substrate assay

The cells were seeded in 96-well plates, and following treatments 25 μl of lysis buffer was added directly to the plate and incubated for 2 hours at 4 ºC with agitation. To each well was added 12.8 μl of Caspase buffer (312.5 mM HEPES Ph 7.5, 31.25% w/v sucrose, 0.3125% w/v CHAPs), 0.8 μl of DMSO, 4 μl of 100 mM DTT, 6.6 μl dH_2O and 0.8 μl of 10 mM DEVD-pNA substrate (BioVision, Mountain View, CA). The plates were incubated overnight at 37 ºC and plates were read at 405 nm with a Benchmarked Plus microplate reader (Biorad, Hercules, CA).

2.3.8 DNA fragmentation assay

The cells were seeded in 96-well plates and following treatments the cells were subjected to an in situ terminal deoxynucleotidyl transferase (TUNEL) detection kit, TiterTACS (Trevigen, Gaithersburg, MD) according to the manufacture's protocol.

2.3.9 Statistical Analysis

A one-tailed Students t test was used for comparison of two groups. Significance was defined as $P<0.05$

2.4 RESULTS

2.4.1 Inhibition of NF-κB activation using siRNA

We used RNA interference technology to deplete two major components of the NF-κB pathway, p65 and IKK2, from ABAE cells. siRNA targeting of either p65 or IKK2 significantly silenced expression of these genes such that the protein levels were marginally detectable 48 hours following the transfection as assessed by western blotting analysis (Figure 6A).

To investigate the effect of the siRNA treatments on VEGI-induced NF-κB activation we assayed for NF-κB p65 translocation into the nucleus, a characteristic of activated NF-κB. Following the transfections with the respective siRNA, ABAE cells were treated with recombinant VEGI for 30 minutes. The cells were fluorescently labeled for p65 and nuclear levels were quantified using imaging analysis (Figure 6B). VEGI exposure to the scrambled siRNA control group resulted in an approximate 2-fold increase of nuclear NF-κB p65 concentrations as compared to non-stimulated cells. The p65 siRNA sufficiently depleted the p65 protein in the cells and, consequently, only a negligible amount of p65 could be detected in

nuclei following VEGI stimulation. VEGI exposure to the IKK2-silenced cells showed approximately a 40% decrease in nuclear NF-κB concentrations as compared to the VEGI-stimulated scrambled control group.

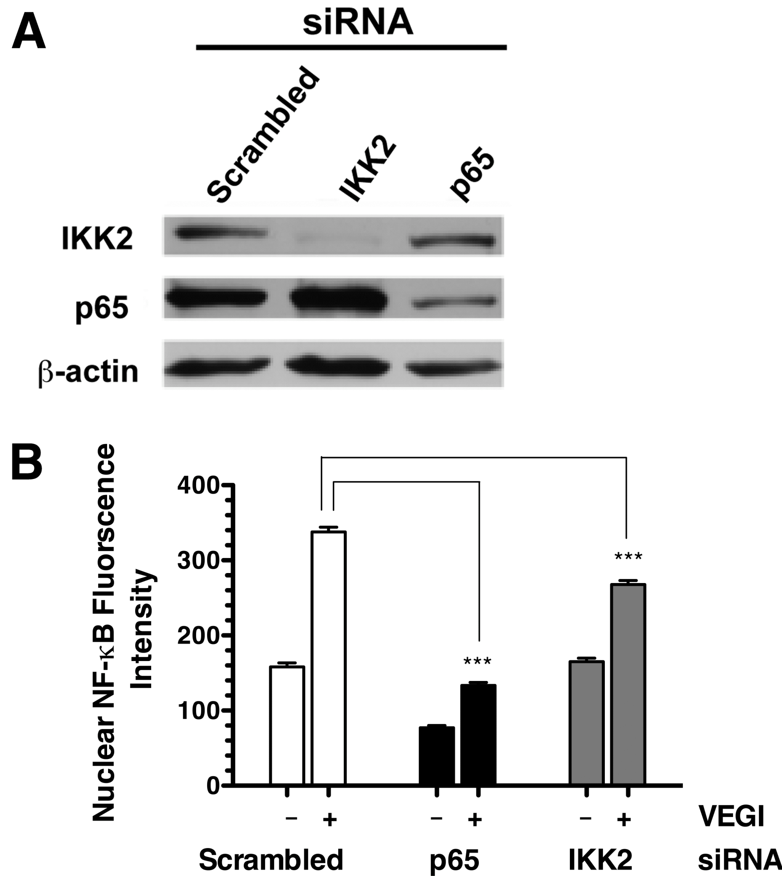

Figure 6. RNAi depletion of NF-κB p65 and IKK2 prevents VEGI-induced NF-κB activation in ABAE cells. ***A,*** **Western Blotting analysis of IKK2 and p65 in ABAE cells treated with either scrambled-, IKK2- or p65-siRNA (100 nM each, 48 h), as indicated. β-Actin was used as a loading control.** ***B,*** **Nuclear levels of activated NF-κB following siRNA (100 nM, 48 h) and VEGI (2 units, 30 min) treatment. Cells were fluorescently labeled for p65 and subjected to automated fluorescent imaging analysis to determine fluorescent intensity of nuclear p65. Values are means ± SEM of duplicate measurements. Data are from a typical experiment out of three independent experiments. At least 3000 cells were analyzed. Asterisks, $P < 0.0001$.**

We then determined the effect of siRNA treatments on the ability of VEGI to upregulate gene expression of the cytokines IL-6 and IL-1β, two well-known NF-κB-responsive genes. Following the transfections with the respective siRNA, ABAE cells were treated with VEGI for 7 hours. The cells were then harvested and RNA was extracted and subjected to qRT-PCR to determine IL-6 and IL-1β expression levels. VEGI exposure to the scrambled control cells led to a 700–fold increase of IL-6 expression levels. Cells silenced of p65 or IKK2 exhibited a 90% and 95% decrease, respectively, in IL-6 production (Figure 7A). Additionally, VEGI exposure to the scrambled control cells led to a 5–fold increase of IL-1β expression levels. Cells silenced of p65 or IKK2 exhibited a 50% and 95% decrease, respectively, in IL-1β production (Figure 7B). Here, we show p65 and IKK2 siRNA treatments inhibited VEGI-stimulated NF-κB nuclear translocation and the ability of VEGI to upregulate expression of NF-κB-target genes therefore demonstrating the siRNA treatments are effective at inhibiting NF-κB activation.

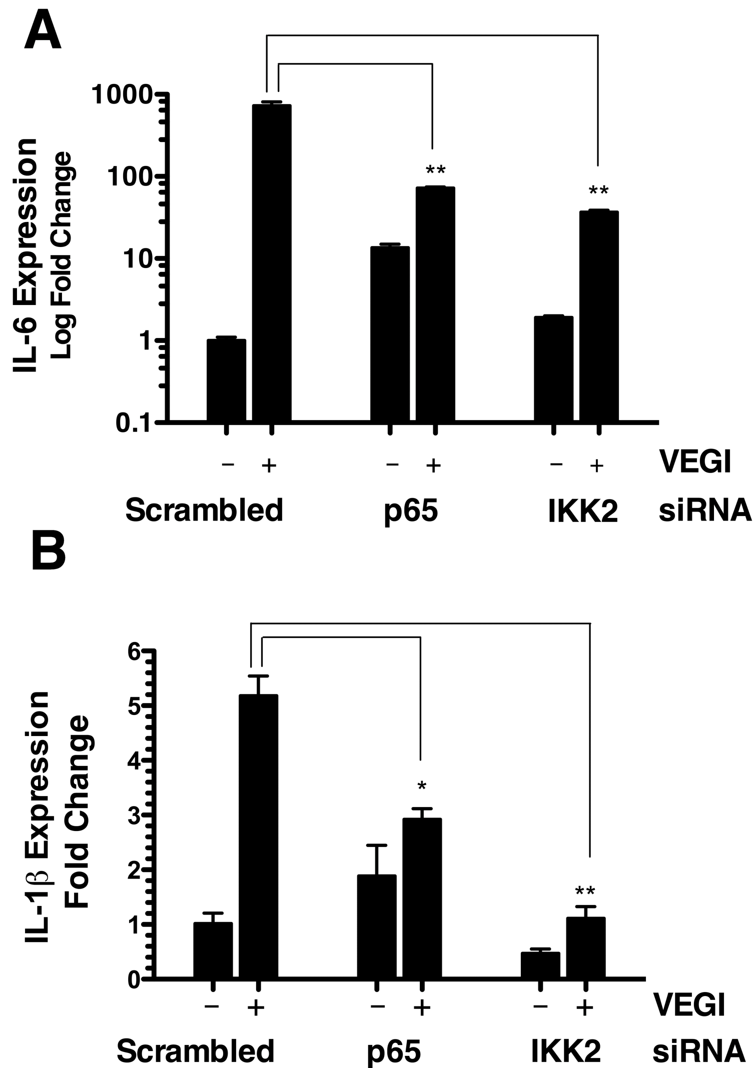

Figure 7. RNAi depletion of NF-κB p65 and IKK2 diminishes the expression of VEGI regulation of NF-κB genes. ***A,*** **Quantitative RT-PCR analysis of IL-6 expression in ABAE cells following treatment with the indicated siRNA (100 nM, 48 h) and VEGI (2 units, 7 h).** ***B*****, Quantitative RT-PCR analysis of IL-1β expression in ABAE cells following treatment with the indicated siRNA (100 nM, 48 h) and VEGI (2 units, 7 h). Values are means ± SD of duplicate measurements. Data are from a typical experiment out of two independent experiments; **P<0.001, *P <0.05.**

2.4.2 Inhibition of NF-κB activity coincides with increased VEGI cytotoxicity

We then determined if NF-κB inhibition effected VEGI regulation of ABAE cell viability. Following the transfections with the respective siRNA, ABAE cells were exposed to various concentrations of VEGI for 48 hrs and then subjected to a calcein viability assay. We determined the half-maximum inhibition value (IC_{50}) on the p65-silenced cells to be about 0.2 units of VEGI activity, a nearly 10-fold decrease from the scramble control treated cells (Figure 8A). The IC_{50} value of VEGI on the IKK2-silenced cells was determined to be about 0.4 units, an approximate 5-fold decrease from the scramble control treated cells (Figure 8B). These results show that endothelial cells when prevented from activating the NF-κB pathway became more sensitive to VEGI cytotoxicity.

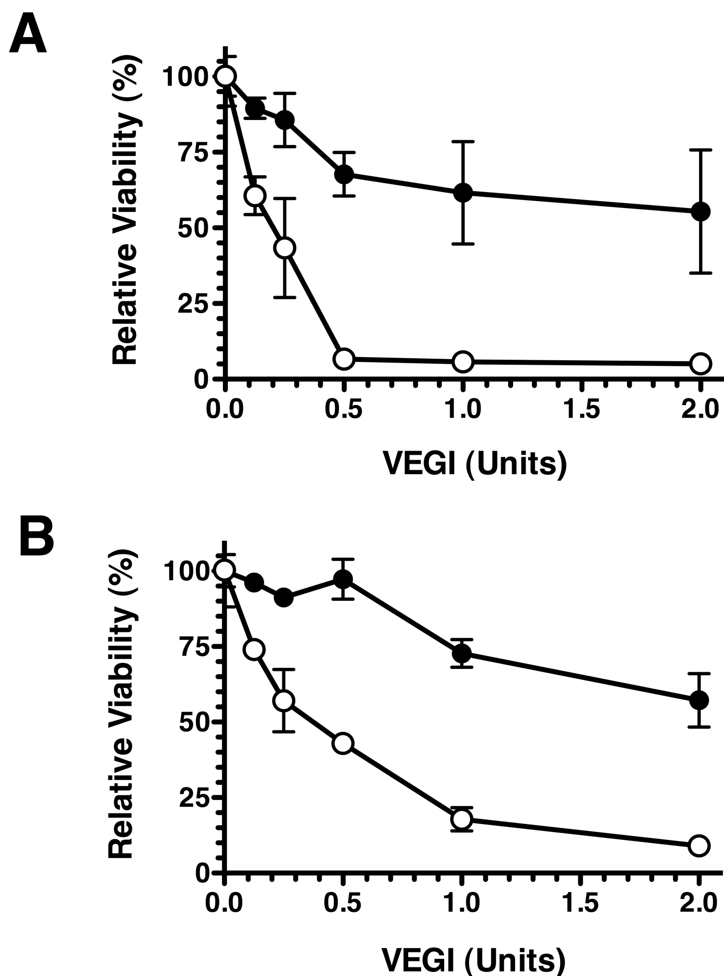

Figure 8. Silencing of the NF-κB pathway potentiates the cytotoxicity of VEGI in ABAE cells. The cells were treated with the respective siRNA (100 nM, 48 h) treated with VEGI at the indicated concentrations (48 h), and then subjected to a calcein viability assay. *A,* Percentage of viable cells treated with either the scrambled siRNA (closed circles) or the p65 siRNA (open circles). *B,* Percentage of viable cells treated with either the scrambled siRNA (closed circles) or the IKK2 siRNA (open circles). Values are means ± SD of duplicate measurements. Data are from a typical experiment of at least two independent experiments.

Since the recombinant VEGI protein was produced in E.coli, we wanted to ensure the observed sensitization was not a result any present endotoxin found in the protein preparation. We repeated the siRNA treatments and exposed the cells to heat-denatured VEGI and found a complete loss of activity in the p65-silenced cells (Figure 9A) as well as the IKK2-silenced cells (Figure 9B). These results demonstrate that the observed effect shown above is associated with the recombinant VEGI.

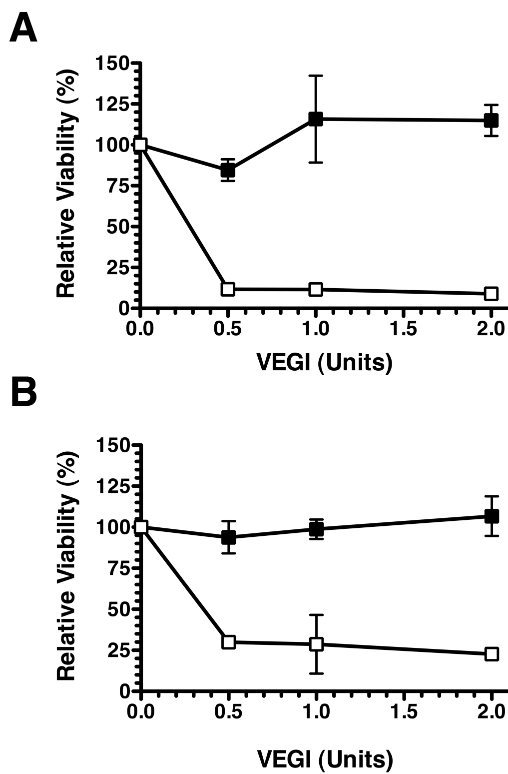

Figure 9. Enhanced Cytotoxicity is specific towards VEGI. ***A,*** **Percentage of viable p65-depleted cells treated with either native VEGI (open squares) or heat-denatured VEGI (closed squares).** ***D,*** **Percentage of viable IKK2-depleted cells treated with either native VEGI (open squares) or heat-denatured VEGI (closed squares). Values are means ± SD of duplicate measurements. Data are from a typical experiment of at least two independent experiments.**

2.4.3 Inhibition of NF-κB activity enhances VEGI-induced apoptosis

Since VEGI cytotoxicity results from induction of apoptosis, we measured caspase-3 activity to determine if there was an increase in the apoptotic potential of VEGI. Following the transfections with the respective siRNA, the cells were treated with various concentrations of VEGI for 7 hours and then subjected to a caspase-3 substrate (DEVD) assay (Figure 10A). VEGI exposure on p65-silenced cells resulted in a rapid increase of caspase-3 activity showing a 80-fold increase at 2 units of VEGI as compared to the scrambled control treated cells. In the IKK2-silenced cells we observed an increase of caspase-3 activity only at 2 units of VEGI. To determine a dose-response effect of VEGI on the IKK-2-silenced cells we repeated the experiment extending the VEGI treatment to 24 hrs (Figure 10B), and observed a marked increase in caspase-3 activity at various VEGI concentrations in the IKK2-silenced cells as compared to the scrambled control treated cells.

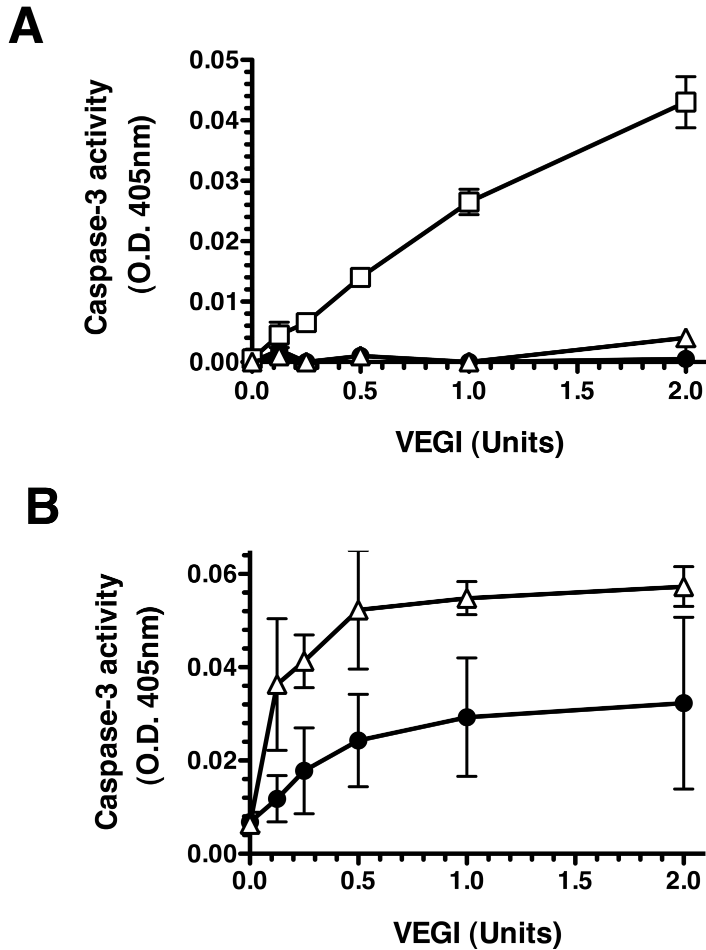

Figure 10. Silencing of the NF-κB pathway facilitates VEGI-induced Caspase-3 activation. ***A,*** **Caspase-3 activity in NF-κB pathway-silenced cells in response to VEGI. ABAE cells were treated with either the scrambled (Circles), p65 (Squares), or IKK2 (Triangles) siRNA (100 nM, 48 h), then exposed to VEGI at indicated concentrations; caspase-3 activity was measured 7 h after VEGI treatment.** ***B,*** **ABAE cells were treated with the scrambled (Circles), or IKK2 (Triangles) siRNA (100 nM, 48 h), then exposed to VEGI at indicated concentrations; caspase-3 activity was measured 24 h after VEGI treatment. Values are means ± SD of duplicate measurements. Data are from a typical experiment out of two independent experiments.**

In addition to caspase-3 activity, we determined the degree of DNA fragmentation to measure apoptosis. Following the transfections with the respective siRNA, ABAE cells were exposed to VEGI for either 7 or 24 hours then subjected to a TUNEL assay. At 7 hours we observed an approximate 3-fold increase in DNA fragmentation in the p65-silenced cells as compared scrambled control treated cells (Figure 11A). At 24 hours, we observed an approximate 2-fold increase in DNA fragmentation in the IKK2-silenced cells as compared to scramble control treated cells (Figure 11B). These results demonstrate inactivation of the NF-κB pathway enhances the potential of VEGI to induce apoptosis in endothelial cells.

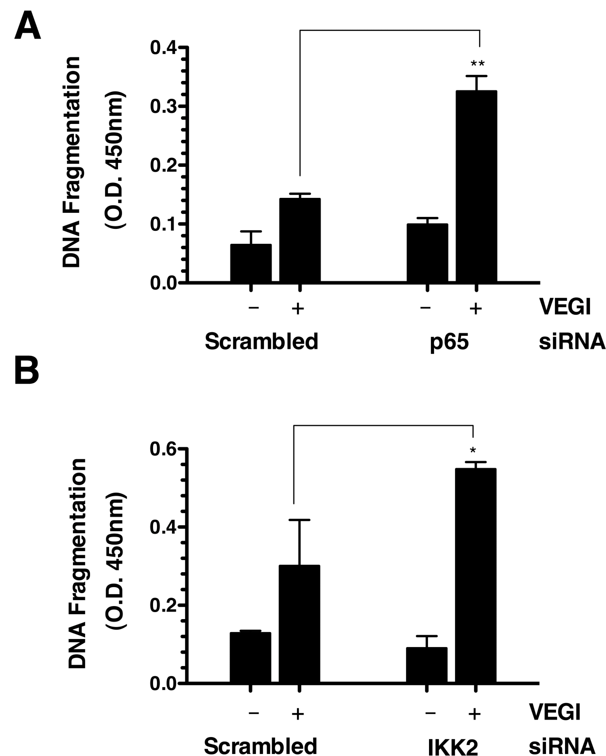

Figure 11. Silencing of the NF-κB pathway facilitates VEGI-induced DNA fragmentation. ***A,*** **Extent of DNA fragmentation (TUNEL assay) in ABAE cells treated with either the scrambled or p65 siRNA (100 nM, 48 h) responding to VEGI (2 units), measured at 7 h.** ***B,*** **Extent of DNA fragmentation in either the scrambled or IKK2 siRNA (100 nM, 48 h) treated cells responding to VEGI (2 units), measured at 24 h. Values are means ± SD of duplicate measurements. Data are from a typical experiment out of two independent experiments; **P<0.001, *P <0.05.**

2.4.4 Inhibition of NF-κB activation with small molecules

We next used two small-molecule inhibitors, curcumin and BMS-345541, to prevent NF-κB activation in ABAE cells. Curcumin and BMS-345541 are able to inhibit IKK1 and IKK2 activity. (Pan et al., 2000; Burke et al., 2003) We first confirmed the ability of these inhibitors to prevent NF-κB nuclear translocation. ABAE cells were incubated with either one of the inhibitors for 2 hours, and then exposed to VEGI for 30 min. Analysis of fluorescent intensity of nuclear NF-κB p65 showed a 3-fold increase in the control cells as compared to the non-stimulated cells. In the presence of curcumin and BMS-345541 there was about a 75% and 45% inhibition, respectively, in NF-κB p65 nuclear concentrations following exposure to VEGI (Figure 12).

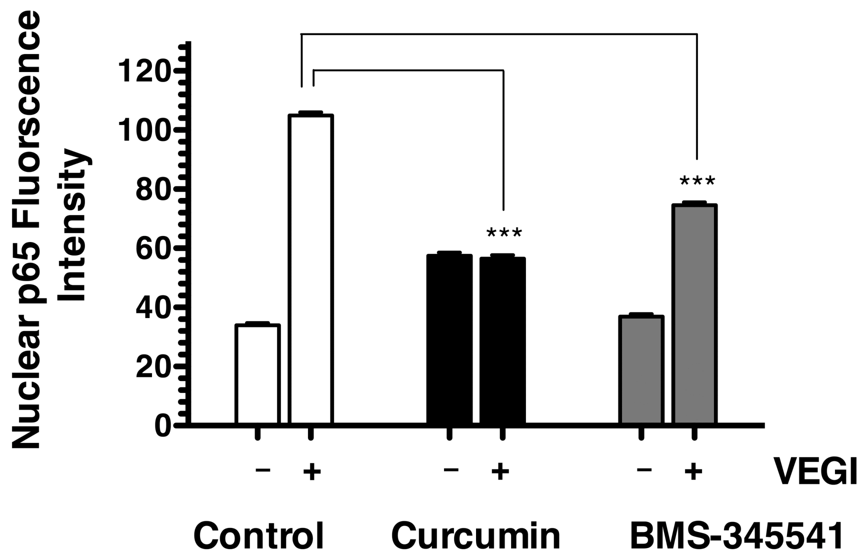

Figure 12. IKK inhibitors prevent VEGI-induced NF-κB nuclear translocation. *A*. Nuclear levels of activated NF-κB. ABAE cells were treated with either curcumin (25 μM) or BMS-345541 (1 μM) for 2 h, and then exposed to VEGI (2 units, 30 min). Cells were fluorescently labeled for p65 and subjected to automated fluorescent imaging analysis to determine fluorescent intensity of nuclear p65. Values are means ± SEM of two measurements, At least 9000 cells were analyzed; Asterisks, P < 0.0001.

We also determined the ability of these compounds to inhibit VEGI-induced gene regulation of IL-6 and IL-1β. ABAE cells were incubated with one of the inhibitors for 2 hours, and then treated with VEGI for 7 hours. QRT-PCR analysis showed the VEGI treatment led to a 2500–fold increase of IL-6 expression levels as compared to the non-stimulated cells. In the presence of curcumin or BMS-345541 there was a 99% and 80%, respectively, inhibition of IL-6 expression levels (Figure 13A). VEGI treatment led to a 40–fold increase in IL-1β expression levels as compared to the non-stimulated cells. In the presence of curcumin and BMS-345541 there was a 90% and 40%, respectively, inhibition of IL-1β expression levels (Figure 13B). These results confirmed the effectiveness of the inhibitors to prevent VEGI-induced NF-κB activation.

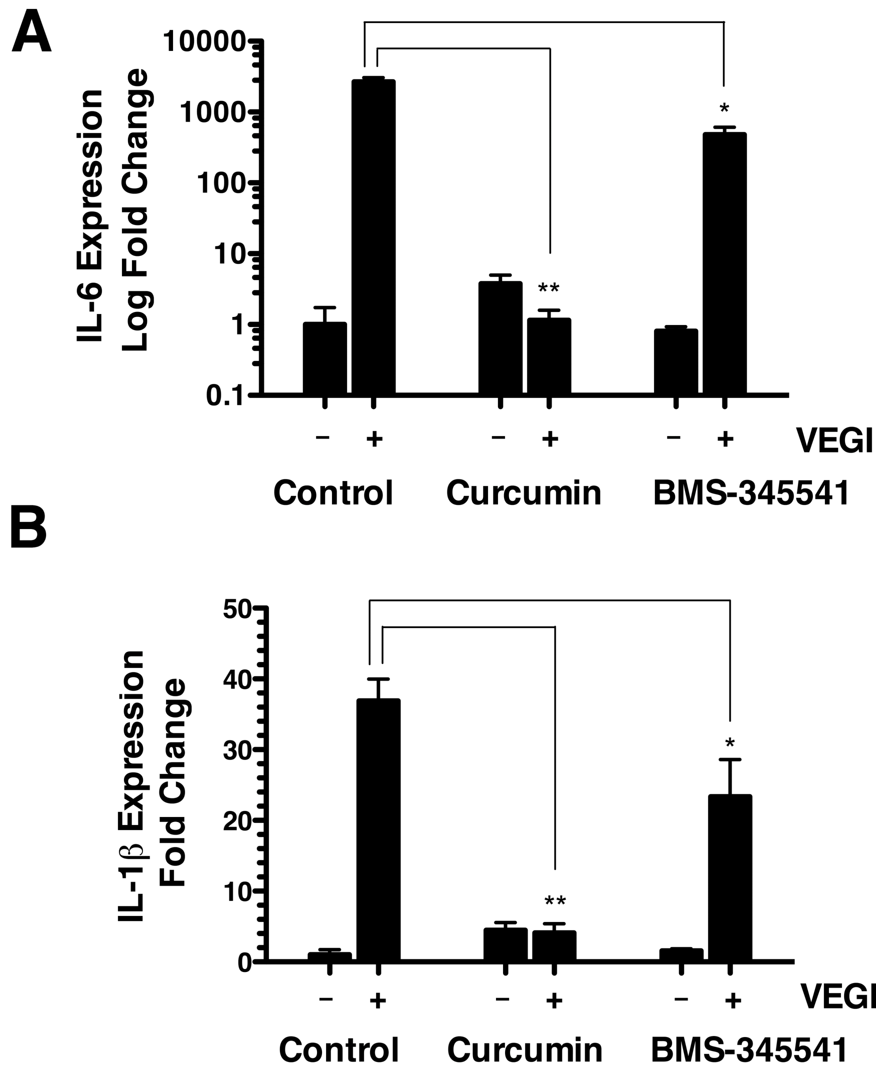

Figure 13. IKK inhibits diminished VEGI regulation of NF-kB responsive genes. ***A,*** **Quantitative RT-PCR analysis of IL-6 expression in ABAE cells treated with curcumin (25 mM) or BMS-345541 (1 mM) for 2 h then exposed to VEGI (2 units, 7 h).** ***B,*** **Quantitative RT-PCR analysis of IL-1β expression of ABAE cells treated with curcumin (25 mM) or BMS-345541 (1 mM) for 2 h then exposed to VEGI (2 units, 7 h). Values are means ± SD of duplicate measurements. Data are from a typical experiment out of two independent experiments; **P<0.001, *P <0.05.**

2.4.5 Small-molecule inhibition of NF-κB activity sensitizes ABAE cells to VEGI cytotoxicity

We next determined the effect of the NF-κB pathway inhibitors on VEGI-mediated ABAE cell death and caspase-3 activation. We determined a 2-fold and 8-fold decrease in the VEGI IC_{50} values in the presence of curcumin and BMS-345541, respectively, as compared to cells cultured without these molecules (Figure 14A). Furthermore, caspase-3 activation upon exposure to various concentrations of VEGI significantly increased when the cells were first incubated with either curcumin or BMS-345541 (Figure 14B). These results further demonstrate that inhibition of the NF-κB pathway sensitizes the cells to VEGI-induced apoptosis, thus corroborating the findings obtained with the siRNA.

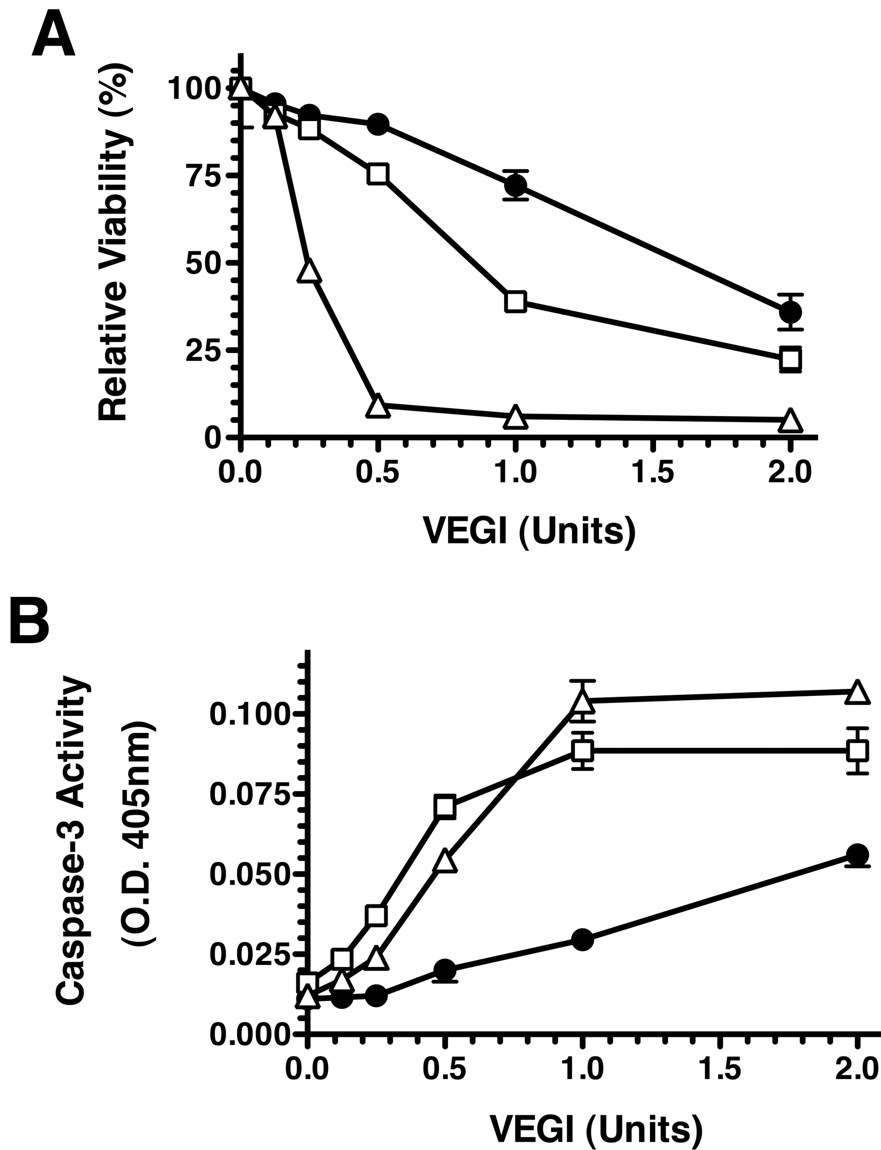

Figure 14. IKK inhibitors potentiate the cytotoxicity of VEGI and apoptosis in ABAE cells. ***A,*** **Percentage of viable cells in the absence of an inhibitor (Circles) or treated with 12.25 mM curcumin (Squares) or 1 nM BMS-345541 (Triangles) for 2 h, treated with VEGI at indicated concentrations for 48 h, and then subjected to calcein viability assay. Values are means ± SD of duplicate measurements. Data are from a typical experiment out of two independent experiments.** ***B,*** **Caspase-3 activity in ABAE cells treated with IKK inhibitors and VEGI. ABAE cells in the absence of an inhibitor (Circles), or treated with 12.25 μM curcumin (Squares) or 1 μM BMS-345541 (Triangles) for 2h, and then exposed to VEGI at indicated concentrations; the activity was measured 9 h after VEGI treatment. Values are means ± SD of duplicate measurements. Data are from a typical experiment out of two independent experiments.**

2.5 CONCLUSIONS

Whether a cell lives or dies depends on a variety of stimuli that mediate either pro-survival or pro-apoptotic signaling pathways or at times both. These pathways in turn modulate a network of proteins, and the balance of these proteins determines whether or not programmed cell death proceeds. (Aggarwal, 2003; Kucharczak et al., 2003) An aberrant balance of these factors may lead to excessive apoptosis or ineffective induction of cell death, both conditions resulting in a variety of disease states. In vascular biology excessive endothelial cell apoptosis weakens vascular integrity and is associated with numerous conditions such as atherosclerosis (Tricot et al., 2000), allograft vasculopathy (Dong et al., 1996), heart failure (Rossig et al., 2000), and diabetic retinopathy (Mizutani et al., 1996). Conversely ineffective apoptosis is associated with excessive angiogenesis as implicated in tumor growth, age-related macular degeneration, and arthritis (Folkman, 2007). Thus, it is of great importance to characterize survival and apoptotic factors and their molecular mechanisms to further our knowledge of the endothelial homeostasis in normal and disease states.

VEGI is an endogenous inhibitor of endothelial cell growth and angiogenesis. The molecular mechanisms involved in this cytokine's inhibition effect remain undefined. In this investigation we determined that inhibition of NF-κB activation facilitated VEGI-mediated endothelial cell death. NF-κB is an integral factor of TNF biology commonly known for its role in modulating inflammation and cytokine production but also determined to be a key regulator in controlling apoptosis among several of the TNF ligands (Aggarwal, 2003). NF-κB involvement in protection from apoptosis was most prominently shown in $p65^{-/-}$ mice, which die at embryonic day E (15) due to excessive apoptosis of hepatocytes (Beg et al., 1995). NF-κB suppression of apoptosis is a result of gene induction and extensive research is directed towards understanding

the anti-apoptotic genes regulated by NF-κB (Karin and Lin, 2002). NF-κB suppression of apoptosis is a result of gene induction and extensive research is directed towards the understanding of anti-apoptotic genes regulated by NF-κB. Notable NF-κB responsive genes include the c-IAP family of proteins known to prevent the proteolytic processing pro-caspases -3, -6, and -7 (Deveraux et al., 1998). The adaptor protein c-FLIP is another well-characterized anti-apoptotic factor that is regulated by NF-κB. c-FLIP interacts with FADD and procaspase-8 and prevents the initiation of caspase pathway (Kreuz et al., 2001). Other notable NF-κB inducible factors include the Bcl-2 family members Bcl-2 and Bcl-XL which are known for maintaining the integrity of the mitochondrial membrane (Lee et al., 1999; Tamatani et al., 1999). Thus, there are number of genes that might be involved in VEGI-induced NF-κB activation and it will be of future interest to determine which factor(s) is important in suppressing apoptosis mediated by VEGI in endothelial cells.

For this investigation we utilized siRNA for a highly specific approach to prevent activation of the canonical NF-κB pathway. A possible disadvantage of silencing either p65 or IKK2 could be the depletion of basal survival factors required for normal cell integrity. An advantage of the small-molecule inhibitors is their brief introduction (2 h) can result in a rapid disabling of the NF-κB pathway. Thus, providing a short time frame between the inhibition of the NF-κB pathway and the exposure to VEGI, minimizing possible adverse cellular effects that could come about in the long-term suppression of the NF-κB pathway. The siRNA and the small-molecule inhibition data therefore provide complimentary results that helped to clarify inducible NF-κB activation.

We were able to establish that endothelial cells became sensitized to VEGI in the presence of NF-κB inhibitors. We showed a decrease in the IC_{50} value of VEGI in the presence of either

curcumin or BMS-345541. The use of NF-κB inhibitors to improve the efficacy of several anticancer agents is under investigation (Nakanishi and Toi, 2005). Another member of the TNF family, TNF-related apoptosis-inducing ligand (TRAIL), is a promising cancer therapeutic and efforts are directed to use this ligand in combination with NF-κB inhibitors to improve this cytokine's therapeutic potential (Romagnoli et al., 2007; Shankar et al., 2008). VEGI is an promising anti-angiogenic therapeutic shown to be effective in inhibiting tumor growth in several murine models including a colon cancer model (Zhai et al., 1999a), a prostate cancer model (Zhai et al., 1999a), a xenograft breast cancer model (Zhai et al., 1999b), and a Lewis lung carcinoma model (Hou et al., 2005). The efficacy of VEGI to inhibit tumor growth is thought to be primarily attributable to the inhibition of tumor angiogenesis. Anti-angiogenic agents such as VEGI are of principal importance in cancer research as well in research of several vascular diseases such as infantile hemangiomas, peptic ulcers, ocular neovascularization (Folkman, 2007). Our studies reveal that the synergy between VEGI and NF-κB inhibitors increases the potency of VEGI to initiate endothelial cell death. Further analysis of in vivo models will be of interest to determine if a similar synergy is observed in reducing blood vessel growth in tumor models and other vascular disease states.

In conclusion, we show that inhibition of VEGI-induced activation of the predominant NF-κB complex results in an increased apoptotic potential of VEGI. It is thus plausible that inducible NF-κB activation suppresses VEGI-induced endothelial cell apoptosis.

2.6 ACKNOWLEDGMENT

We thank Talal El-Hefnawy and Adam Farkas for their technical assistance.

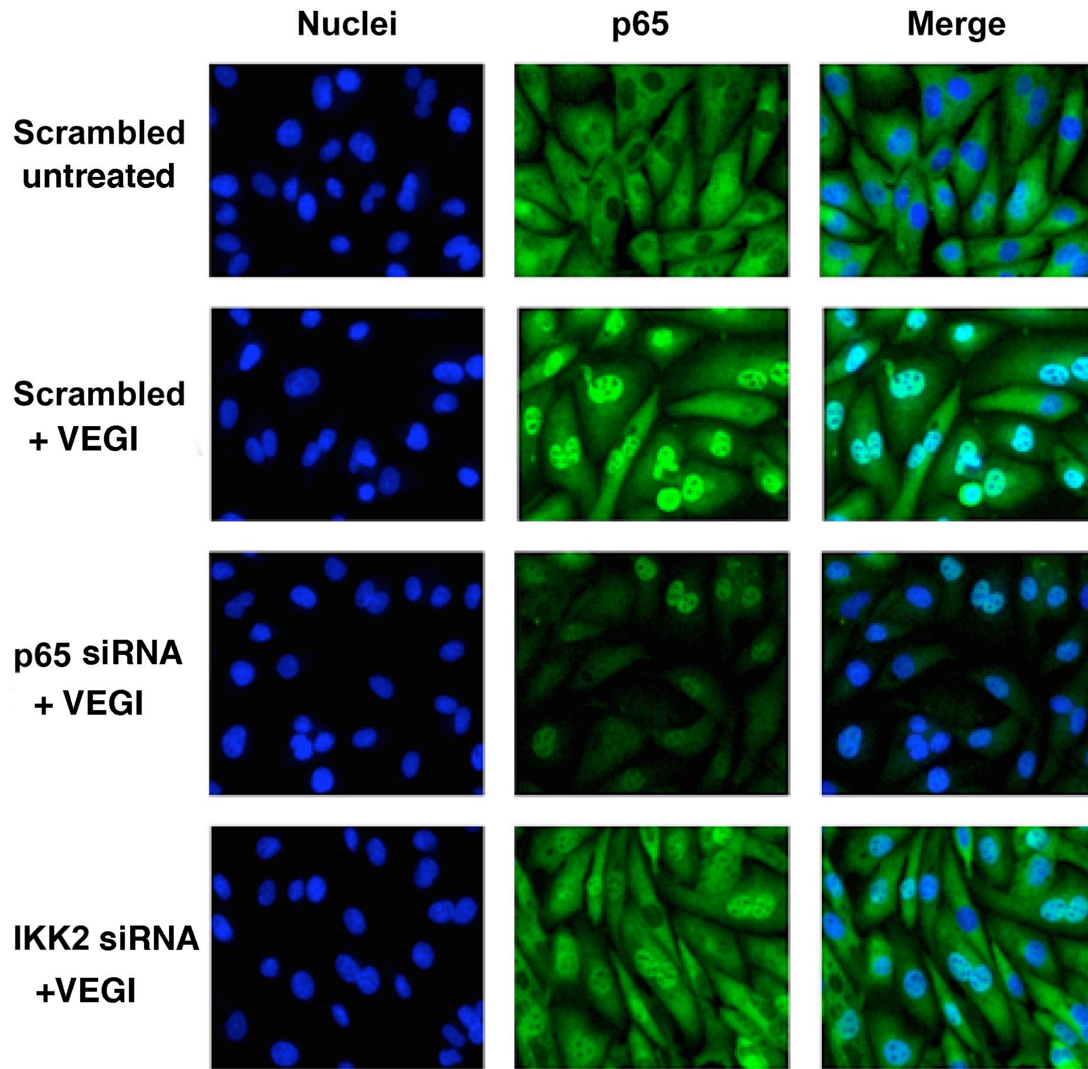

Figure 15. Supplemental figure of the nuclear translocation assay from Figure 6B. Representative images of siRNA treated cells with VEGI (2 units) Hoescht nuclear staining and p65 fluorescent staining.

3.0 DR3-DEPLETED ENDOTHELIAL CELLS ARE PROTECTED FROM THE CYTOTOXICITY OF VEGI, LPS, AND TNFα

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3.1 ABSTRACT

Death receptor-3 (DR3; TNFRSF25) is a receptor for VEGI for T cells; however, it is yet to be determined if DR3 is the receptor responsible for VEGI-mediated endothelial cell death. In addition, it is unknown if different VEGI isoforms utilize the same receptor to mediate their cellular activities. Here investigated if DR3 is the necessary receptor required for VEGI-mediated endothelial cell apoptosis. We used RNA interference (RNAi) to silence expression of the DR3 gene in ABAE cells and determined how this affected the activities of two VEGI isoforms: VEGI-192 and VEGI-251. We found that DR3-depleted endothelial cells exhibited a markedly diminished responsiveness toward the cytotoxic and apoptosis-inducing activity of both VEGI isoforms. Interestingly, we obtained similar results with lipopolysaccharide (LPS) and TNFα as both of these factors were also unable to mediate endothelial cell death in DR3-depleted cells. However, treatment of DR3-depleted cells with the chemotherapeutic, doxorubicin, resulted in a similar decrease in cell viability as compared to the control cells. We also found that VEGI-192 treatment on DR3-depleted cells resulted in an enhanced activation of NF-κB. These data reveal that depletion of DR3 from ABAE cells had a unique impact on the cells. These data suggest DR3 is regulating a critical component(s) in the extrinsic apoptosis-inducing pathways mediated by VEGI, LPS, and TNFα. We hypothesize during normal cell growth DR3 is involved in repressing the NF-κB pathway, such that the siRNA depletion of DR3 from the cells results in an increased amount of activated NF-κB which then leads to an enhanced pro-survival in the cells. We determined a novel role of DR3 in the regulation of the apoptotic pathway however do to these unexpected results we cannot confirm a ligand-receptor relationship between VEGI and DR3

3.2 INTRODUCTION

VEGI is a type II transmembrane protein belonging to the TNF superfamily of ligands (Yue et al., 1999). The human VEGI gene spans about 17 kb and consists of four exons with three VEGI transcripts generated by the use of cryptic splice sites and alternate exons (Chew et al., 2002). The three isoforms results in peptides containing 174aa, 192aa, and 251aa so denoted by their amino acid count. The VEGI-174 isoform contains transmembrane domain suggesting a membrane-bound variant of VEGI. The VEGI-192 does not contain a transmembrane domain nor a secretory domain and it is unclear of the subcellular localization of the protein. The VEGI-251/TL1A isoform encodes a 251aa peptide that contains a secretory signal peptide at the N-terminus, upon cleavage of the signal peptide the remaining 179aa peptide exists as soluble variant of VEGI (Migone et al., 2002). These alternatively spliced isoforms differ in a small stretch of amino acids at the their N-terminus, however all isoforms share the 151 aa stretch of amino acids known as the TNF homology domain (THD), a domain common to other TNF ligands that is known to be responsible for receptor binding. Expression of the VEGI THD domain (1-151aa) alone is able to inhibit endothelial cell growth (Wang et al., 2000).

VEGI is a ligand for two receptors belonging to the TNFR superfamily of receptors: decoy receptor 3 (DcR3) and death receptor 3 (DR3) (Migone et al., 2002). DcR3 is a soluble receptor thought to be a negative regulator of cell signaling (Roth et al., 2001). DR3 is a cell-surface receptor capable of mediating cellular activities. Sequence homology of DR3 reveals that it is closely related to TNFR1 and is characterized by cysteine-rich domains common to other TNFR receptors (Marsters et al., 1996). DR3 belongs to a subset of the TNF receptor family that contains an intracellular death domain capable of activating the caspase pathway. DR3 is enriched in immune cells and ectopic expression of DR3 results in the aggregation and

recruitment TNFR-1 associated death domain (TRADD) protein and Fas-associated domain containing (FADD) protein as downstream effectors of apoptosis (Kitson et al., 1996; Screaton et al., 1997).

It is yet to be determined if DR3 is the leading receptor responsible for mediating the inhibitory activities of VEGI on endothelial cells. Here we sought to determine a functional ligand/receptor relationship between VEGI and DR3 in endothelial cells. We found that two VEGI isoforms could no longer initiate apoptosis in ABAE cells that had been depleted of DR3 by siRNA. Surprisingly, we also found that both LPS and TNFα, which are not known to be the ligands of DR3, were similarly unable to induce apoptosis in DR3-depleted endothelial cells. We also determined that VEGI-induction of NF-κB was not diminished in DR3-silenced cells, but was rather enhanced in DR3-depleted cells. We hypothesize that DR3 is a negative modulator of NF-κB activation and the removal of DR3 is causing an increased propensity for NF-κB activity. It is likely an increase in NF-κB activity would then result in an increased expression of anti-apoptotic genes and fortify the cells from various apoptotic stimuli. We also speculate that since VEGI continues to sufficiently induce NF-κB in DR3-depleted cells that VEGI may be utilizing an unidentitfied receptor in endothelial cells.

3.3 MATERIALS AND METHODS

3.3.1 Cells and reagents

Adult bovine aortic endothelial (ABAE) cells were purchased from Lonza (Walkersville, MD) and maintained in endothelial growth medium (EGM-MV) media. ABAE cells were used in passages 4-10. DR3 (NT) mouse polyclonal antibody was purchased from ProSci (Santa Cruz, CA). TNFα was purchased R&D systems (Minneapolis, MN) LPS was purchased from Sigma-Aldrich (St. Louis, MO). VEGI-192 was prepared as previously described (Tian et al., 2007). VEGI-251 was prepared as previously described (Jin et al., 2007a).

3.3.2 siRNA transfection

Custom designed siRNA were synthesized by Sigma-Proligo (St. Louis, MO). siRNA sequences were designed against the bovine DR3 mRNA: DR3 siRNA-1 5'- CGGGAAAACCACTACG AAA-3', and DR3 siRNA-2 5'-GCCCTAAGTACGGTTACTT-3'. A scrambled siRNA control was purchased from Ambion (Austin, TX). siRNA duplexes were complexes with Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA) diluted and plated in opti-MEM reduced serum medium. 100 nM of siRNA was added with Lipofectamine RNAiMax at a ratio of 40:1 of pMol to lipid mixture. Transfections proceeded overnight at 37 ºC, 5% CO_2 after which the cells were placed back in normal growth media.

3.3.3 Western Blot analysis

Cells were subjected to lysis in a buffer containing 50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 0.5% Sodium Deoxycholate, and a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Total protein lysate was resolved using 12% SDS-

PAGE. The proteins were then transferred onto a Hybond-ECL nitrocellulose membrane, blocked with 5% nonfat dried milk powder in TBST buffer (20 mM Tris-HCl (pH 7.4), 137 mM NaCl, and 0.1% Tween 20) for 1 hour at room temperature, and incubated overnight at 4 ºC with primary antibodies against the target protein. The membrane was then washed and incubated with the appropriate HRP-conjugated secondary antibody, washed with TBST, and developed with the ECL system (Amersham Biosciences, Pittsburgh, PA).

3.3.4 Viability assay

ABAE cells were seeded in black 96-well well plates. Following the treatments, the cells were washed once with PBS. Calcein-AM (Invitrogen Carlsbad, CA) 1 μg/ml in PBS was then added to the cells and incubated for 30 minutes at room temperature. The cells were washed with PBS and fluorescent intensity, 485nm excitation: 520 nm emission was measured on a Tecan Safire fluorescence plate reader (Tecan, San Jose, CA)

3.3.5 Caspase-3 substrate assay

ABAE cells were seeded in 96-well plates and following treatments 25 μl of lysis buffer was added directly to the plate and incubated for 2 hours at 4 ºC on a shaker. Plates were then centrifuged and cleared lysates were transferred to a new 96-well plate. For each well containing 25 μl cleared lysates was added 12.8 μl of Caspase buffer (312.5 mM HEPES Ph 7.5, 31.25% w/v sucrose, 0.3125% w/v CHAPs), 0.8 μl of DMSO, 4 μl of 100 mM DTT, 6.6 μl dH_2O and 0.8 μl of 10 mM DEVD-pNA substrate (BioVision, Mountain View, CA). Plates were incubated overnight at 37 ºC and plates and then read at 405 nm with a Benchmarked Plus microplate reader (Biorad, Hercules, CA).

3.3.6 DNA fragmentation assay

ABAE cells were seeded in 96-well plates and following treatments the cells were subjected to the apoptosis detection kit TiterTACS™, Trevigen (Gaithersburg, MD) following the manufacture's protocol. TiterTACS™ detects DNA double strand breaks using the in situ terminal deoxynucleotidyl transferase (TUNEL) assay followed by colorimetric analysis. Absorbance was measured at 450 nm with a Benchmarked Plus microplate reader.

3.3.7 Nuclear translocation assay

Translocation of NF-κB from cytoplasm to nucleus was analyzed using an automated fluorescence imaging system. ABAE cells were cultured in a black 96-well plate and following treatments the cells were fixed with 4% paraformaldehyde for 10 min. The cells were then permeabilized with PBS containing 1% Triton X-100 for 5 min. NF-κB was labeled by adding mouse anti-p65 antibody and Alexa Fluor 488 goat anti-mouse antibody (Invitrogen, Carlsbad, CA). Nuclei were stained with 1 mg/ml Hoechst 33342. Cellomics ArrayScan VTI HCS Reader analyzes fluorescent intensity (mean pixel intensity) of nuclear and cytoplasmic regions according to the target channel (488 nm) using a defined algorithm.

3.3.8 QRT-PCR

Total RNA was extracted and purified from cultured ABAE cells by an RNeasy mini-kit (Qiagen, Valencia, CA). RT was performed in 100 µL reaction volumes with random hexamer priming using the TaqMan® One-Step RT-PCR Kit (ABI systems, Foster City, CA). The expression levels of the genes for IL-6, IL-8, and GAPDH were amplified using SYBR® Green

kit (ABI Systems) and quantitative real time polymerase chain reaction was performed on ABI PRISM 7700 sequence detection system. Primers sequences were as follows; IL-6 Forward 5'-TGA GTG TGA AAG CAG CAA GGA-3' reverse 5'-TCG CCT GAT TGA ACC CAG ATT-3', IL-8 Forward 5'-GAAGAGAGCTGAGAAGCAAGA -3' Reverse 5'-ACCCACACAGAACA TGAGGC -3', GAPDH Forward 5'-GGC GTG AAC CAC GAG AAG TAT-3' Reverse 5'-CCT CCA CGA TGC CAA AG TG-3'. Relative expression of the marker genes was calculated from the delta-Ct (threshold cycle) of the targeted gene normalized to the delta-Ct of GAPDH.

3.3.9 Statistical Analysis

A one-tailed Students t test was used for comparison of two groups. Significance was defined as $P<0.05$

3.4. RESULTS

3.4.1 siRNA inhibition of DR3 expression

In order to deplete DR3 from ABAE cells we used RNA interference technology. We designed two siRNA that targeted different sites of the bovine DR3 mRNA. Western blot analysis showed that the siRNA treatments of ABAE cells with DR3 siRNA-1 and siRNA-2 were effective at silencing DR3 gene expression (Figure 16A). Densitometry analysis showed that cells transfected with DR3 siRNA-1 and siRNA-2 exhibited a 65% and 85% decrease, respectively, of DR3 protein levels as compared to the scrambled control cells (Figure 16B).

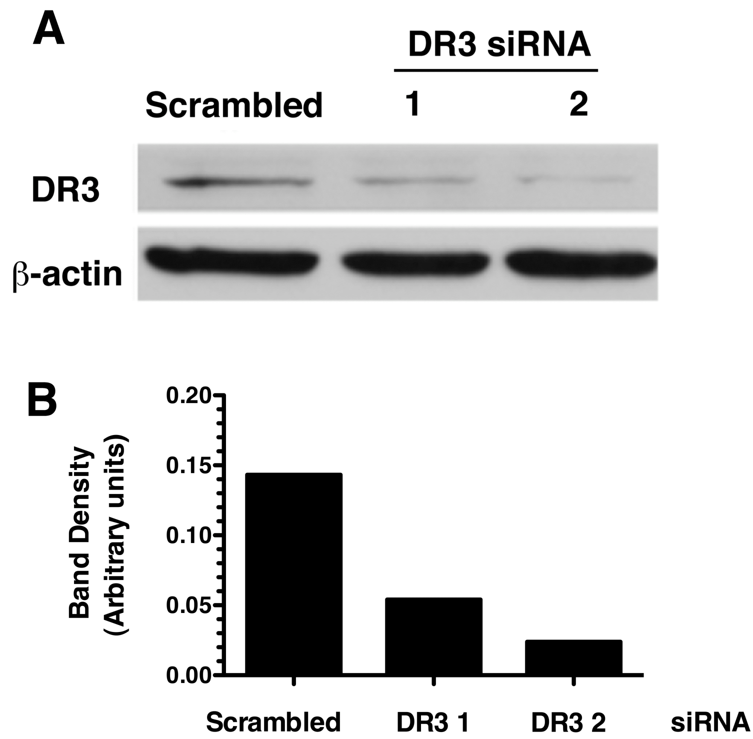


Figure 16. siRNA depletion of DR3 from ABAE Cells. ABAE cells were transfected with DR3 siRNA (100nm) targeting two different regions along the bovine DR3 mRNA. *A,* Western blot analysis of DR3 72 h following the transfection. β-actin was used as loading control. *B,* Densitometric analysis of the blot.

3.4.2 DR3-depleted ABAE cells are protected from the cytotoxcity exhibited by the VEGI isoforms

We next determined how DR3-silencing in ABAE cells affected the cytotoxicity of VEGI-192 and VEGI-251. Forty-eight hours following the transfection with the respective siRNA, ABAE cells were exposed to either recombinant VEGI-192 or VEGI-251 for 48 hours. The cells were then subjected to a calcein viability assay. Figure 17A, VEGI-192 exposure to the scramble siRNA treated cells exhibited a dose-dependent decrease in cell viability, achieving an approximately 70% decrease in cell viability with the highest dose of VEGI (2 units). VEGI-192 exposure to ABAE cells transfected with DR3 siRNA-1 achieved an approximate 45% reduction in cell viability with 0.5 VEGI units and there was only a minimal decrease in viability with further VEGI concentrations. VEGI-192 exposure to the ABAE cells transfected with DR3 siRNA-2 showed a 25% reduction with 0.25 VEGI units and no further decrease in viability was observed through the highest dose of VEGI (2 units). We repeated experiment using the VEGI-251 isoform (Figure 17B). VEGI-251 exposure to the scrambled siRNA treated cells exhibited a dose-response and achieved a 50% decrease in cell viability with 1 unit of VEGI. VEGI-251 exposure to the ABAE cells treated with DR3 siRNA-2 only achieved a 15% decrease in cell viability through the highest concentration of VEGI (2 units). These results indicate that ABAE cells depleted of DR3 are less responsive to the cytotoxicity of both VEGI isoforms.

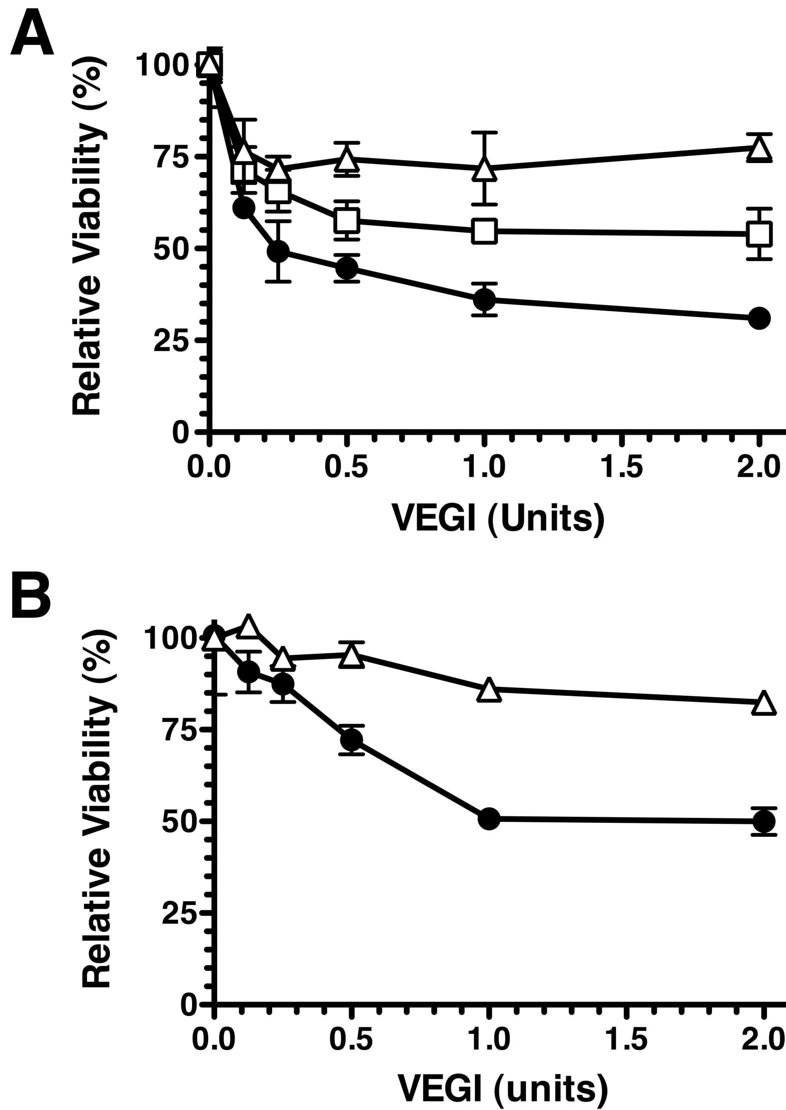


Figure 17. Reduced VEGI-cytotoxicity in DR3-depleted cells. Viability of DR3-silenced ABAE cells following treatment with the VEGI isoforms. *A,* Cells were transfected with scrambled siRNA (closed circles), DR3 siRNA-1 (open squares) or DR3 siRNA-2 (open triangles) then treated with VEGI-192 at indicted concentrations for 48 hours and subjected to a calcein assay. *B,* Viability of ABAE cells treated with scrambled siRNA (closed circles) or DR3 siRNA-2 (open triangles) then treated with VEGI-251 for 48 hr and subjected to a calcein assay. Values are means ± SD of duplicate measurements. Data are from a typical experiment of at least two independent experiments.

3.4.3 DR3 depletion from ABAE cells attenuates apoptosis initiated by the VEGI isoforms

VEGI reduces ABAE cell viability by inducing apoptosis we therefore determined the apoptotic potential of VEGI-192 and VEGI-251 in DR3-depleted cells. We first measured activation of caspase-3, a hallmark of apoptotic induction. ABAE cells were transfected with the respective siRNA for 72 h and then exposed to various concentrations of VEGI-192 for 9 h. Whole cell lysates were extracted and subjected to a caspase-3 substrate (DEVD) assay (Figure 18A). Treatment of VEGI-192 on cells depleted of DR3 using siRNA-1 or siRNA-2 showed a marked reduction of activated caspase-3 as compared to the scrambled control treated cells resulting in a 40% and 60% inhibition, respectively, of activated caspase-3 at 2 units of VEGI. We next treated DR3-depleted cells with the VEGI-251 isoform and performed a caspase-3 substrate assay (Figure 18B). Exposure to VEGI-251 on the control siRNA treated cells exhibited a dose-response having an approximately 3-fold increase of caspase-3 activation at the 2 units of VEGI. Treatment of VEGI-251 on DR3-depleted cells using siRNA-2 showed a minimal effect on caspase-3 activation with increasing VEGI concentrations.

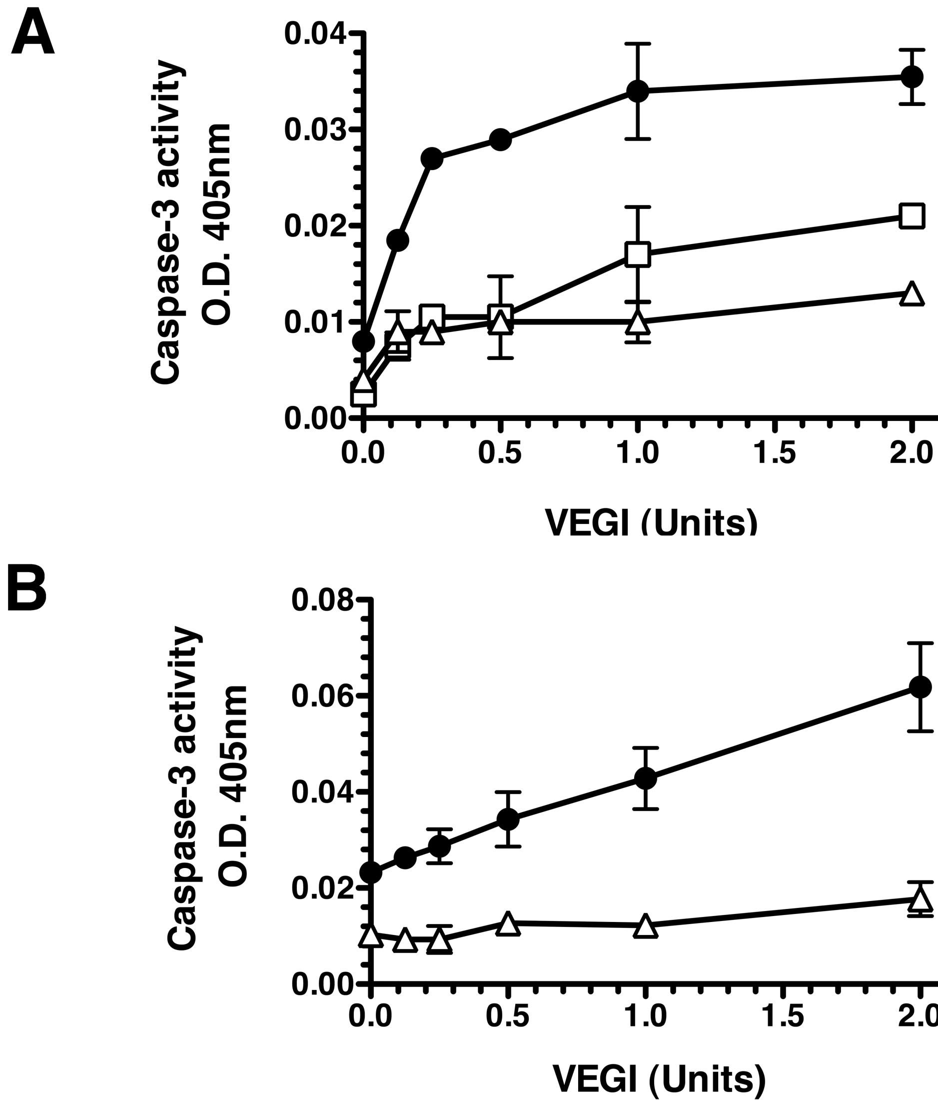


Figure 18. DR3-depletion from ABAE cells attenuates VEGI-induced caspase-3 activation. ***A,*** **ABAE cells were transfected with scrambled siRNA (closed circles) or DR3 siRNA-1 (open squares) or DR3 siRNA-2 (open triangles) then treated with VEGI-192 at indicated concentrations for 9 h and subjected to a caspase-3 substrate assay.** ***B,*** **ABAE cells transfected with scrambled siRNA (closed circles) or DR3 siRNA-2 (open triangles) then treated with VEGI-251 at indicated concentrations for 9 h and subjected to a caspase-3 activation assay. Values are means ± SD of duplicate measurements. Data are from a typical experiment of at least two independent experiments.**

In addition to the caspase-3 assay we measured DNA fragmentation for a second measurement of apoptosis. ABAE cells were transfected with the scrambled siRNA or DR3 siRNA-2 then treated with VEGI-192 and subjected to a TUNEL assay (Figure 19A). Treatment of VEGI-192 showed a 90% reduction of the amount of DNA fragmentation as compared to the scrambled control cells. We also performed a TUNEL assay with VEGI-251 treatment on DR3-depleted cells (Figure 19B). Treatment of VEGI-251 on DR3-depleted cells showed minimal changes in DNA fragmentation as compared to the scrambled control cells. The caspase-3 and TUNEL assays showed that both VEGI isoforms are unable to facilitate apoptosis in DR3-depleted cells.

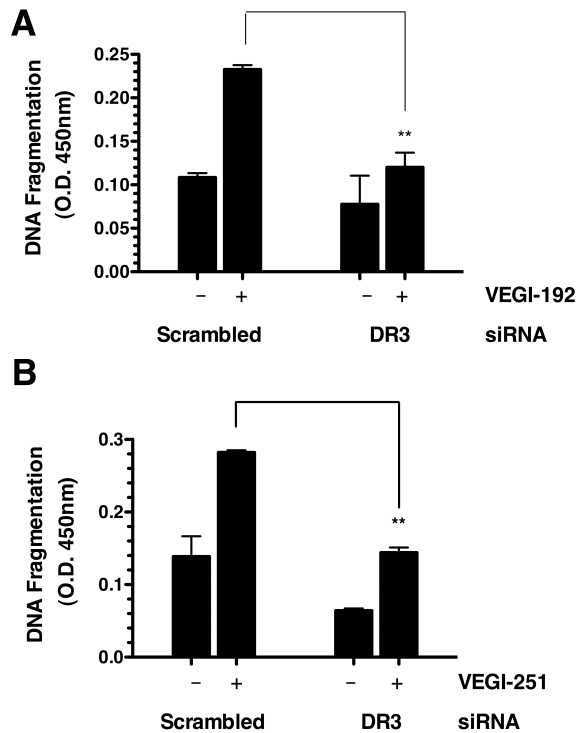


Figure 19. DR3-depletion from ABAE cells attenuates VEGI-induced DNA fragmentation. ***A and B*****, ABAE cells transfected with scrambled or DR3 siRNA-2 then treated with the indicated VEGI isoform for 9 h and subjected to a TUNEL assay. Values are means ± SD of duplicate measurements; **P<0.001. Data are from a typical experiment of at least two independent experiments.**

3.4.4 DR3 depletion of ABAE cells attenuates the cytotoxicity mediated by LPS and TNFα but not doxorubicin

We next determined if the loss of VEGI activity on DR3-depleted cells described above was specific. As a negative control to determine specificity we chose LPS, a component of the outer membrane of Gram-negative bacteria. LPS, acting through toll-like receptor 4, induces apoptosis and initiates cellular activity in endothelial cells similar to VEGI (Choi et al., 1998). Following treatments with the respective siRNA, the cells were treated with various concentrations of LPS and subjected to a calcein viability assay (Figure 20A). LPS treatment on the scrambled controls cells led to about a 50% decrease in endothelial cell viability. LPS treatment on the DR3-depleted cells only led to about a 20% decrease in endothelial cell viability and no further decrease in cell viability was shown with increasing concentrations of LPS. We next determined if LPS could mediate apoptosis in DR3-depleted endothelial cells. Cells were treated with the respective siRNA, then treated with LPS for 9 h and analyzed for caspase-3 activity (Figure 20B). LPS treatment on the scrambled siRNA-treated control cells led to a 3-fold increase in caspase-3 activation. LPS treatment on the DR3-depleted cells resulted in a 75% reduction of activated caspase-3. These results indicate that DR3-depleted endothelial cells are protected from LPS-induced apoptosis.

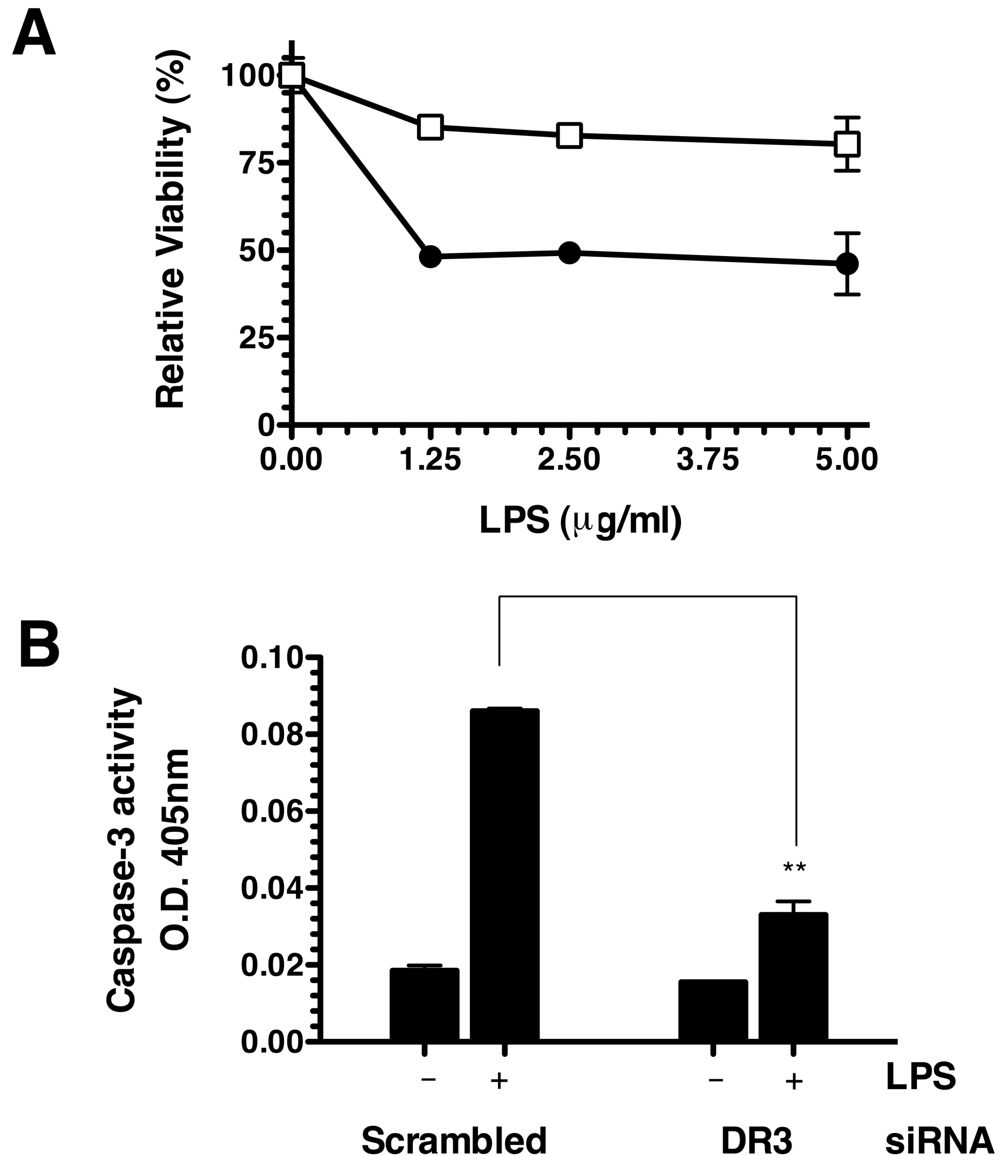


Figure 20. DR3-depletion from ABAE cells attenuates LPS mediated endothelial cell death. ***A,*** **Viability of ABAE cells exposed to LPS. ABAE cells were transfected with scrambled siRNA (closed circles) or DR3 siRNA-2 (open squares) then treated with LPS at indicated concentrations for 48 h and subjected to a calcein assay.** ***B,*** **Caspase activation following LPS exposure to DR3-depleted cells. ABAE cells were transfected with scrambled or DR3 siRNA-2 then treated with LPS (1μg/ml) for 9 h and subjected to a caspase-3 substrate assay. Values are means ± SD of duplicate measurements; **P<0.001. Data are from a typical experiment of at least two independent experiments.**

To further investigate if other apoptotic stimuli are able to mediate cell death in DR3-depleted cells we investigated TNFα and doxorubicin activity on ABAE cells. TNFα is able to induce apoptosis in endothelial cells through TNFR-1 and possibly shares very similar signaling components with VEGI-induced pathways (Hsu et al., 1996). Doxorubicin is a chemotherapeutic that intercalates in DNA to interrupt DNA synthesis, resulting in apoptosis (Oyarzo et al., 2006). Cells were transfected with the respective siRNA, treated with various concentrations of either TNFα or doxorubicin, and then subjected to a calcein assay. Figure 21A, TNFα exposure to the scramble control siRNA-treated cells resulted in a 70% reduction in viability. TNFα treatment exposure to the DR3-depleted cells only achieved a 25% reduction in cell viability with the highest concentration used. In sharp contrast, doxorubicin treatment of both the scrambled control siRNA-treated cells and the DR3-depleted cells led to a similar decrease in viability (Figure 21B). These results suggest the DR3-depleted endothelial cells are resistant to certain stimuli that are known to activate the extrinsic apoptotic pathway, but not those that stimulate the intrinsic apoptotic pathway.

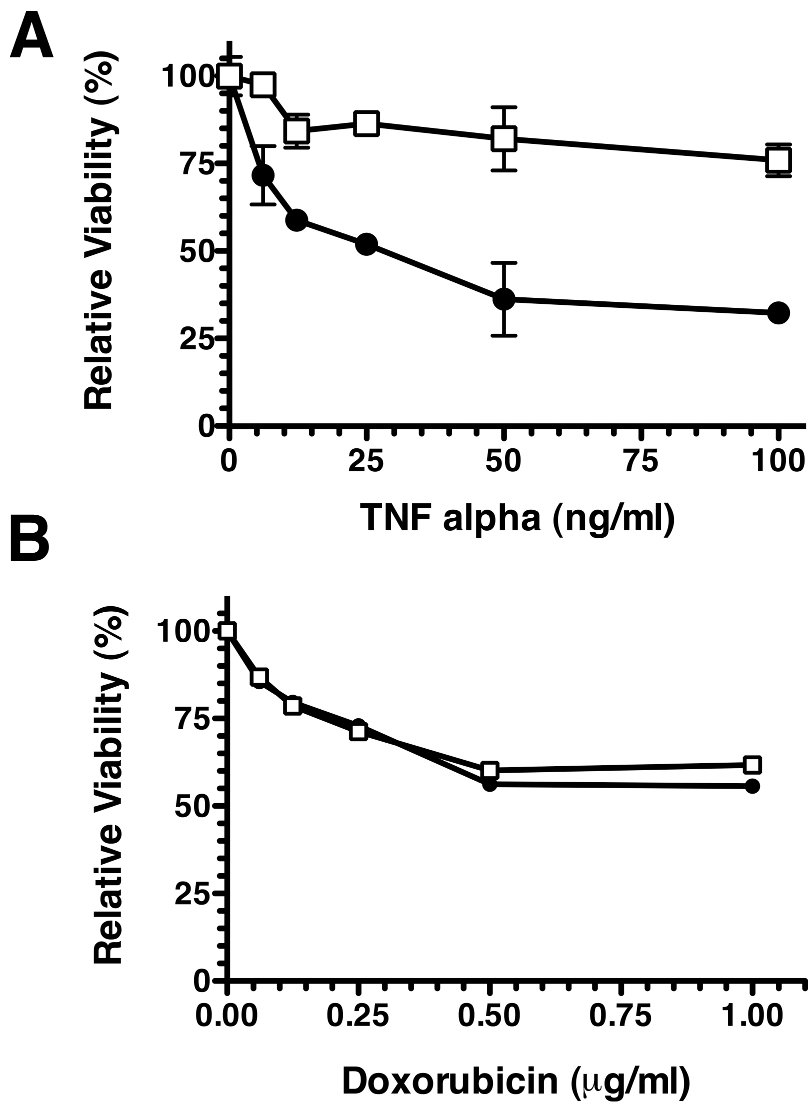


Figure 21. DR3-depletion from ABAE cells attenuates TNF but not doxorubicin mediated ABAE cell death. ***A and B*****, ABAE cells transfected with scrambled (closed circles) and DR3 siRNA-2 (open squares) treated with indicated stimuli at various concentrations. Values are means ± SD of duplicate measurements.**

3.4.5 DR3-depletion increases VEGI-192 stimulated NF-κB activation

To investigate if DR3 has a role in the activation of VEGI cellular activities other than apoptosis we next investigated activation of NF-κB. ABAE cells were treated with the respective siRNA then treated with VEGI-192 and subjected to a NF-κB p65 nuclear translocation assay (Figure 22A). VEGI exposure to the scrambled control siRNA-treated cells exhibited a dose-response showing a 2-fold increase of nuclear NF-κB concentrations with 2 units of VEGI as compared to the untreated cells. VEGI exposure to the DR3-depleted cells exhibited a similar dose-response with approximately a 3-fold increase in nuclear NF-κB concentrations with 2 units of VEGI as compared to the untreated cells. When comparing the cells treated with scrambled or DR3 siRNA in the absence of VEGI there was a slight increase in nuclear NF-κB levels in the DR3 siRNA treated cells. We next quantified the cytoplasmic levels of NF-κB p65 from the same experiment to determine if there was a difference of p65 levels in resting cells of the two siRNA-treated groups (Figure 22B). We determined approximately a 25% increase in the cytoplasmic levels of p65 in the DR3-depleted cells as compared to the scrambled control group. These results indicate that VEGI is able to sufficiently activate NF-κB when cells are depleted of DR3. Additionally, DR3-depletion from ABAE cells results in a slightly higher concentration of NF-κB p65 concentrations in the nucleus and in the cytoplasm.

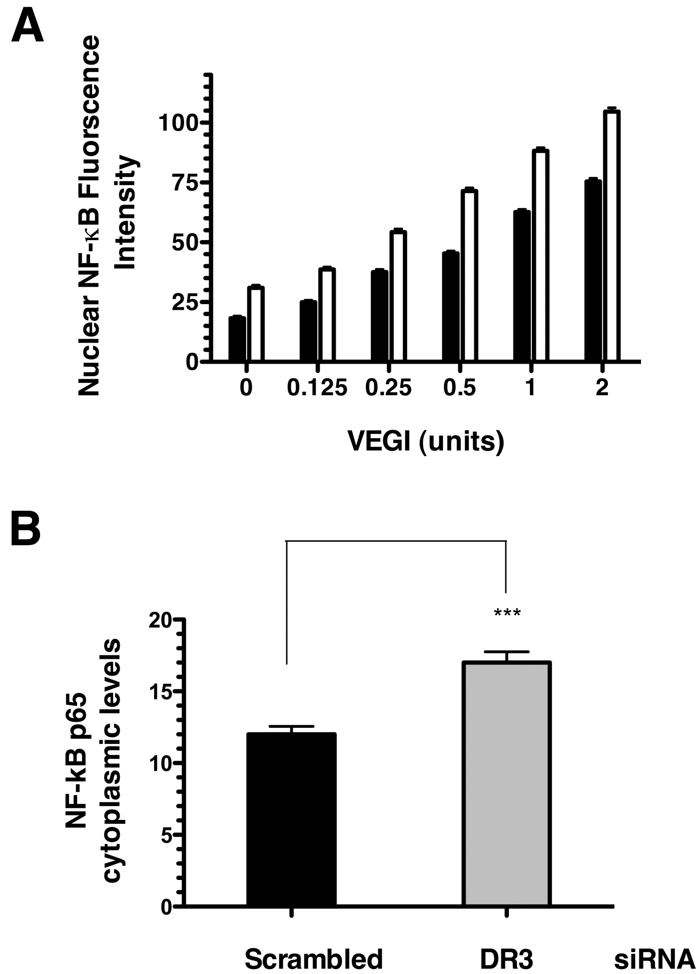
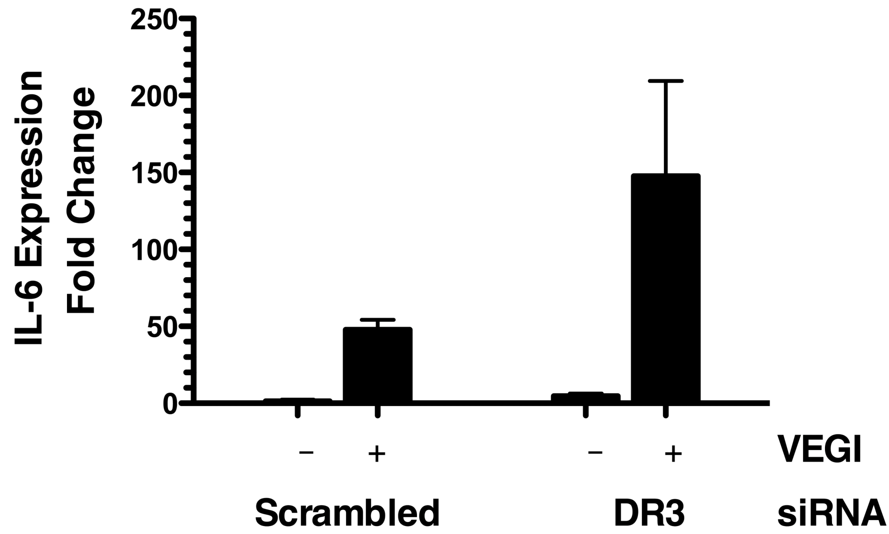


Figure 22. Enhanced NF-κB activity in DR3-depleted cells. ***A,*** **Nuclear levels of activated NF-κB following siRNA and VEGI (2 units, 30 min) treatment. Cells were fluorescently labeled for p65 and subjected to automated fluorescent imaging analysis to determine fluorescent intensity of nuclear p65.** ***B,*** **Cytoplasmic analysis of NF-κB p65 levels of cells transfected with scrambled siRNA or DR3 siRNA-2. Values are means ± SEM of duplicate measurements. At least 3000 cells were analyzed. Data are from a typical experiment of at least two independent experiments.**

To further investigate enhanced activation of NF-κB in DR3 depleted cells we determined the expression levels of the NF-κB-target genes, IL-6 and IL-8 following exposure to VEGI. Cells were transfected with the respective siRNA, then exposed to VEGI-192 for 7 hours and subjected to qRT-PCR analysis of IL- and IL-8. Figure 23A, VEGI exposure to the scrambled control siRNA-treated cells led to a 50-fold increase in IL-6 expression levels. VEGI exposure to the DR3-depleted cells led to approximately a 150-fold increase in IL-6 expression levels . Figure 23B, VEGI-192 exposure to the control siRNA treated cells led to approximately a 200-fold increase in IL-8 expression levels. VEGI-192 exposure to the DR3-depelted cells lead to approximately a 600-fold increase in IL-8 expression levels. These results further establish enhanced NF-κB activity in response to VEGI.



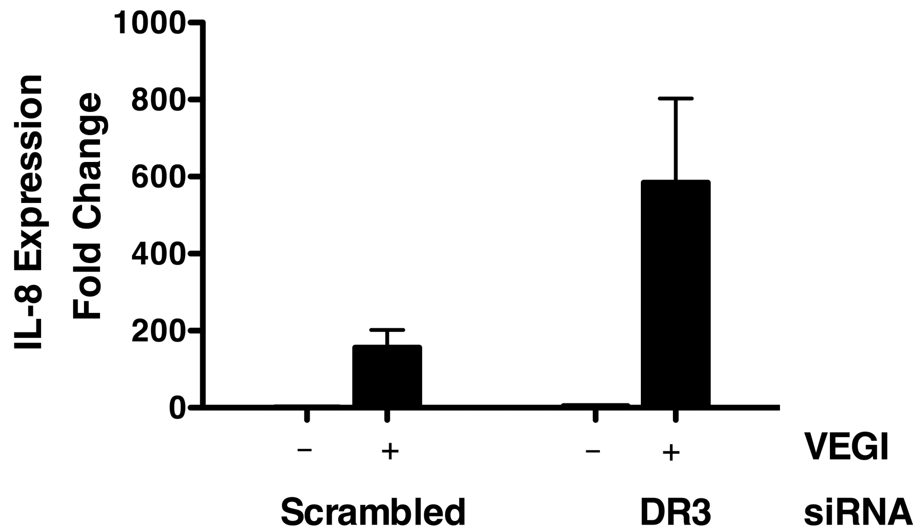


Figure 23. Increased expression NF-κB regulate genes in DR3-depleted cells. ***A,*** **Quantitative RT-PCR analysis of IL-6 expression in ABAE cells following treatment with the indicated siRNA and VEGI (2 units, 7 h).** ***B,*** **Quantitative RT-PCR analysis of IL-8 expression in ABAE cells following treatment with the indicated siRNA and VEGI (2 units, 7 h).**

3.5 CONCLUSIONS

Here we investigated if DR3 is the receptor responsible for VEGI-induced endothelial cell death. Interaction of VEGI-251 with DR3 was convincingly shown by flow cytometry analysis, co-immunoprecipitation, and Biacore analysis (Migone et al., 2002). However, the VEGI preparation used in the Migone et al. investigation did not exhibit any inhibitory actions towards endothelial cells and hence no direct correlation was made between the VEGI-251/DR3 interaction and endothelial cell bioactivity. Migone et al. suggested that the VEGI-174, but not VEGI-251, can inhibit endothelial cell growth while. Thereby raising the notion that there may be an endothelial-specific receptor for VEGI-174. Another inconsistency surrounding VEGI and DR3 interaction in endothelial cells relates to the DR3 expression pattern in human tissue. VEGI has a prominent role in regulating the vasculature however DR3 is mainly enriched immune cells. Suggesting there may a more functionally related receptor expressed in endothelial cells that accounts for the role of VEGI in vascular biology. Although, it is known that human umbilical cord endothelial cells express DR3 (Chinnaiyan et al., 1996) and here we report DR3 expression in ABAE cells, the presence of a receptor is not conclusive to determine a functional significance between a ligand and receptor. Furthermore, the TNF families of ligands are known to bind to multiple receptors for cell signaling. For instance, TNFα binds to TNFR-1 and TNFR-2 and the TRAIL ligand binds to DR4 and DR5. Therefore, it is a characteristic of the TNF family ligands to bind multiple receptors.

Recent studies provide some evidence for the notions that VEGI isoforms may interact to different receptors. Analysis of the x-ray crystal structure of VEGI-251 predicts a very different AA' loop in comparison to the VEGI-174 isoform as a result in differences in the N-terminus

sequences (Jin et al., 2007a). The AA' loop is important for receptor binding in the TNF family member, TRAIL. Therefore it is suggestive that the AA' loop among different isoforms can result in alterations in the receptor binding domain and possibly affect the specificity for a receptor. Another recent study provides evidence to the notion that VEGI binds to multiple-receptors. Al-Lamki et al found VEGI can mediate apoptosis, induce NF-κB activation, and regulate TNFR expression in isolated kidney cultures (Al-Lamki et al., 2008). In $DR3^{-/-}$ knockout mice VEGI was no longer able to induce apoptosis or activate NF-κB however VEGI was still able to regulate TNFR expression. Thus, apoptosis and NF-κB activation can be attributed to signaling via DR3 yet VEGI continued to regulate TNFR expression in the absence of DR3 suggesting that VEGI is utilizing a different receptor for some cellular activities.

In order to investigate the receptor that mediates endothelial cell death initiated by VEGI we first investigated if DR3 is the responsible receptor. We determined that in cells depleted of DR3, two VEGI isoforms could not mediate endothelial cell apoptosis. Initially we understood these data to represent our hypothesis that DR3 is in fact the receptor utilized by VEGI to initiate endothelial cell death. However, when DR3-depleted endothelial cells were treated with either LPS or TNFα these apoptotic stimuli also could not induce apoptosis. Direct binding studies with TNFα and DR3 report that TNFα is not a ligand for DR3 (Bossen et al., 2006). LPS, known to bind to toll-like receptor 4, does not contain a TNF homology domain and therefore there is no precedence for LPS to bind to members of the TNF receptor family (Fenton and Golenbock, 1998). So it is unlikely that TNFα and LPS are in endothelial cells in a very similar fashion (Frey and Finlay, 1998; Messmer et al., 1999). Thus, a possible reason for the observed loss of action from LPS and TNFα on DR3-depleted from endothelial cells is because the apoptotic pathway is nonfunctional.

There are at least two possibilities that DR3-depletion may have rendered the apoptotic pathway nonfunctional. The first is constitutive activation of DR3 could be regulating an essential factor(s) in the apoptotic pathway. Thus, removal of DR3 from the cells would removal this factor resulting in a gap in the apoptotic pathway. A second possibility for the nonfunctional apoptotic pathway may come about if DR3 is involved in downregulating certain pro-survival factors. Thus, following the removal of DR3 these factors would become upregulated and lead to enhance suppression of the apoptotic pathway.

A response to the first possibility, that DR3 is involved in regulating an essential factor, we observed no protective effect in DR3-depleted cells from doxorubicin. This suggests that cells depleted of DR3 contain the necessary molecules to complete the apoptotic pathway initiated by this stimuli. Doxorubicin induces apoptosis via the intrinsic apoptotic pathway (Oyarzo et al., 2006). So there will some differences in the apoptotic molecules involved in the apoptotic pathway mediated by doxorubicin as compared to the other tested stimuli, which induce apoptosis via the extrinsic apoptotic pathway. There are some overlapping molecules used both pathways such as caspase-3, so the ability of doxorubicin to initiate cell death in the DR3-depleted cells suggest that removal of DR3 is not affecting certain downstream effector apoptotic molecules. However, this does leave the possibility that DR3 is able to regulate an apoptotic molecule that is involved in the extrinsic pathway. For example the adaptor protein FADD is a molecule used by all LPS, TNFα, and VEGI but is not used in the intrinsic pathway (Choi et al., 1998; Baud and Karin, 2001; Wen et al., 2003).

A response to the second possibility, that DR3 is downregulating a pro-survival factor, we show that there is enhanced NF-κB activation following VEGI exposure to DR3-depleted ABAE cells. In Chapter 2 we determined that activation of NF-κB is suppresses the apoptotic pathway.

It follows that if there were an increase in NF-κB activation then there would be an increased suppression of the apoptotic pathway. Although the data we present here involves VEGI-induced NF-κB activity, it should be noted that both LPS and TNFα can also induce NF-κB in endothelial cells and it is possible that there are increased levels of NF-κB activation following the treatment with these stimuli. Our preliminary data suggests that increased NF-κB activity in DR3-depleted cells could at least play a partial role in protecting these cells from several apoptotic stimuli and possible be the only mechanism involved.

One question arises if the mild to moderate increase in NF-κB activity in DR3-depleted cell would be sufficient enough to exhibit the observed protective effect. It is possible the moderate increases in NF-κB activation could result in an amplified suppression effect on the apoptotic pathway. We show that VEGI regulation of IL-6 and IL-8 genes revealed a 2-fold further increase in expression levels in DR3-depleted cells as compared to control cells. It is quite possible that other NF-κB-target genes are similarly enhanced. NF-κB is capable of upregulating several anti-apoptotic factors and it is possible that these factors work simultaneously to shut down the apoptotic pathway. Further investigations would be of interest to determine if the enhanced NF-κB activity in DR3-depleted cells is the mechanism resulting in the resistance from several apoptotic stimuli.

These findings give preliminary evidence in a novel role of DR3 to control the apoptotic potential of several stimuli. However, the question still remains whether or not DR3 is the major endothelial-cell receptor for VEGI. Due to the unexpected effect of depleting DR3 from the cells it is difficult to ascertain a functional relationship between VEGI and DR3 as a ligand receptor pair. One pertinent observation is the fold-induction of NF-κB following VEGI exposure to

DR3-depleted cells. If DR3 is the sole receptor in endothelial cells we would expect depletion of DR3 to exhaust the ability of VEGI to activate NF-κB. Since VEGI continues maintain a significant effect on fold-induction of NF-κB suggesting that there is an alternate receptor mediating VEGI-induced NF-κB activation. Future investigations to determine unknown VEGI receptors should be a focus. Protein/protein interactions may be the most useful studies to help determine unknown receptors, after which further investigations of the functional significance of each receptor can be done.

In conclusion we were unable to establish a functional relationship between VEGI and DR3 on the cell-surface of endothelial cells. However, we did unravel two important findings. First, that DR3 is involved in controlling the extrinsic apoptotic pathway under normal conditions, possibly through deregulating the NF-κB pathway. Second, we provide preliminary evidence of VEGI having multiple-receptors in endothelial cells.

4.0 DISCUSSION

The emphasis of this thesis was to investigate the molecular mechanisms of VEGI activity on endothelial cells. We focused on the NF-κB pathway and the identification of the receptor used in VEGI-mediated endothelial cell death in order to gain a better understanding of VEGI signaling. The receptor study interestingly led us back to an investigation of NF-κB. Thus, emphasizing the intimate relationship between NF-κB and members of the TNF superfamily. Due to it role in multiple biological processes and its association with several human diseases multiple NF-κB is one of the most extensively studied molecules.

4.1 VEGI SPECIFICITY TOWARDS ENDOTHELIAL CELL DEATH

4.1.1 VEGI-stimulated NF-κB activation in Endothelial cells and TF-1 cells

One of the more prominent roles of NF-κB is its regulation of apoptosis. In Chapter 2 we established such a role of NF-κB in VEGI-mediated signaling. When we inhibited NF-κB pathway using either siRNA or small-molecule inhibitors there was an increase in VEGI-induced endothelial cell death. These results suggest that NF-κB during normal activation by VEGI exhibits an anti-apoptotic role in endothelial cells. It should be noted that this anti-apoptotic role is not sufficient to prevent VEGI-mediated endothelial cell death. In a previous report describing VEGI signaling in the erythroleukemic cell line, TF-1, NF-κB was determined to suppress the apoptosis (Wen et al., 2003). VEGI-induced NF-κB activation in TF-1 cells is able to prevent cell death. Figure 24 shows cell models summarizing our study of VEGI signaling in endothelial cells and the previous report of VEGI signaling in TF-1 cells. VEGI is known to stimulate

multiple cell-types including T cells, macrophages, dendritic cells. However, only in endothelial cells is cell death the result following VEGI stimulation. It is possible that NF-κB activation is a determining factor in the specificity of VEGI's cytotoxicity towards different cell-types. NF-κB may act as a guard on the apoptotic pathway and only in endothelial cells will the VEGI response be allowed to complete the apoptotic pathway and result in cell death.

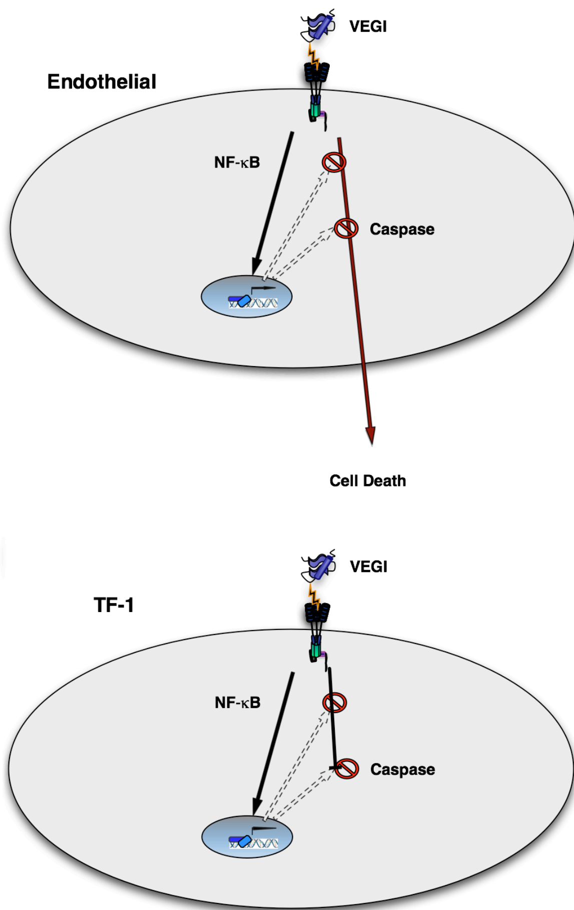


Figure 24. VEGI signaling in endothelial cells versus TF-1. As determined in Chapter 2, VEGI signaling leads to activation of NF-κB and the Caspase pathway. NF-κB activation suppresses apoptosis however cell death still pursues. In a previous report VEGI stimulation of TF-1 cells also leads to NF-κB and caspase activation however the NF-κB response is able block the apoptotic pathway and prevent cell death.

4.1.2 Mechanisms to control NF-κB activity

We state that NF-κB activity may be used to control VEGI specificity. It is unclear how VEGI can utilize NF-κB for only a partial affect on the apoptotic pathway in one cell-type and the complete suppression of the pathway in another cell-type. Several mechanisms are reported to help understand the control of NF-κB activity.

Each member of the NF-κB family binds to slightly different consensus sequences found on gene promoters. For instance, the p50 member binds to GGGACTTTCC while the p52 member prefers to bind to the sequence GGGATTCCCC (Fujita et al., 1992; Duckett et al., 1993). Different compositions of the NF-κB heterocomplex will therefore result in alterations in promoter binding leading to a unique gene expression profile (Perkins, 1997). The vascular cell adhesion molecule-1 (VCAM-1) is regulated by specific NF-κB subunits (Shu et al., 1993). Shu et al determined that p65 alone or in a dimer with p50 can stimulate VCAM-1 expression. In contrast, binding of the p52 subunit to the VCAM-1 promoter inhibited VCAM-1 expression and opposed p65 regulation of VCAM-1.

Simultaneous signaling transduction of NF-κB and other transcription factors are able to regulate NF-κB activity (Perkins, 1997). A promoter containing multiple consensus elements of various transcription factors may give rise to a competition for DNA binding. Alternatively, the presence of DNA binding proteins may be necessary to allow for subsequent binding of transcription factors, as reported in IFNβ gene expression regulated by virus infection. NF-κB activation initiated by virus infection can upregulate IFNβ expression levels however TNFα−induced NF-κB activation does not effect IFNβ expression (Thanos and Maniatis, 1995). The IFNβ enhancer contains four elements available for various transcription factors. Only in the

presence of the enhancer proteins PRD, HMG I, and ATF2/c-Jun occupying the IFNβ enhancer can NF-κB bind to that promoter and initiate transcription. The enhancer proteins, referred to as the enhancesome, are able to bend DNA in such a way to allow NF-κB to bind. TNFα can activate NF-κB but does not regulate the proteins of the enhancesome therefore NF-κB does not effect IFNβ expression. Another example of heterologous signaling pathways affecting NF-κB gene regulation is shown in a study of IL-8 expression by DR3 and TNFR1 (Su et al., 2006). Although both receptors are able to upregulate IL-8 transcripts they require a different set of signaling pathways. The axis TAK1/ASK1-MKK4/MKK7-JNK2 is responsible for DR3 expression of IL-8 whereas the axis ASK1-MKK4-JNK1/JNK2/p38MAPK is required for TNFR-1 expression of IL-8.

NF-κB translocation to the nucleus is another way to regulate NF-κB activity (Perkins, 1997). The IκB family of proteins, responsible for sequestering NF-κB in the nucleus, are known to be activated and degraded in response to different stimuli. For example IκBα can be targeted and degraded by TNFα, IL-1, LPS, and phorbol ester. However, LPS and IL-1 can only target IκBβ via an unknown mechanism (Thompson et al., 1995). Additionally, each IκB member can modulate NF-κB differently. IκBβ results in a longer sustained activation of NF-κB members as compared to that of IκBα. Additionally, IκBβ competes with IκBα to bind to the NF-κB members (Perkins, 1997).

The differences of NF-κB suppression on the apoptotic pathway following VEGI stimulation of endothelial cells and TF-1 cells may be attributed to some the mechanisms stated above. For instance it is possible that each cell-type may contain a different distribution of factors of the NF-κB pathway, which can lead to a unique gene expression or possibly a

shorter/longer activation NF-κB. If NF-κB is in fact not the primary mechanism determined to be involved in cell-type specificity for VEGI-mediated cell death we still would expect NF-κB to play an essential role. For instance, there may be an endothelial-specific receptor for VEGI that mediates apoptosis. However, it would still be necessary for NF-κB activation to have a tapered role on the apoptotic pathway and allow cell death to pursue.

4.2 NF-κB INHIBITION LOOP DURING DR3 SIGNALING

In chapter 3 we show that DR3-depletion by siRNA can result in the enhanced NF-κB inducible activity. It is possible that DR3 during normal conditions is involved in deregulating the NF-κB pathway. The ability of DR3 to activate the NF-κB pathway as well as repress the NF-κB pathway would create a situation in which there is an inhibitory loop of NF-κB activation during DR3 signaling (Figure 25). DR3 and other death receptors are normally thought of as having dual signaling arms with NF-κB representing the pro-survival arm and the caspase pathway representing pro-death arm. The pro-survival arm is able to regulate and suppress the pro-death arm as discussed above. If DR3 activation resulted in an inhibitory loop to prevent NF-κB activation then pro-death would become unchecked and there would be increase in the apoptotic potential once DR3 is activated. If this inhibitory loop is specific for DR3 in endothelial cells and if it is determined that DR3 is the receptor for mediating VEGI-induced cell death, this may account for VEGI specificity towards endothelial cell death.

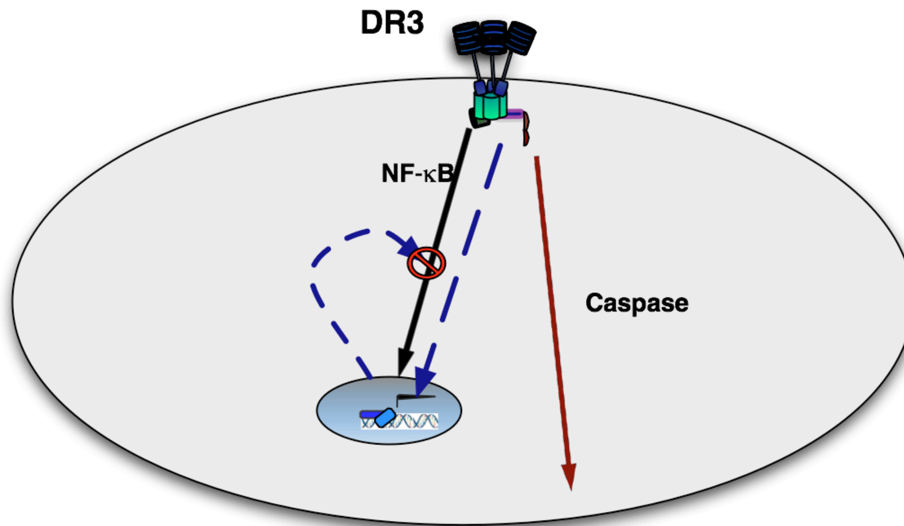


Figure 25. Potential NF-κB inhibitory loop in DR3 signaling. DR3 is able to repress the NF-κB pathway by an unknown mechanisms (Dashed Blue Arrows). DR3 is also able to activate the NF-κB pathway (Black Arrow), resulting in a potential inhibitory loop on NF-κB activation following DR3 stimulation. This would suppress the pro-survival pathway and increase the apoptotic potential of DR3.

4.2.1 Mechanisms that repress the NF-κB pathway

The mechanism involved in DR3 repression of the NF-κB pathway is unknown. However, it is presumed that DR3 is able to upregulate or downregulate factors via gene regulation, which in turn opposes activation of the NF-κB pathway. DR3 may directly regulate the expression of factors in the NF-κB pathway. We determined there was a 25% increase in p65 levels in ABAE cells following the depletion of DR3. This suggests that DR3 is involved in removing p65 from the cells during normal conditions. Several studies reveal other mechanisms used to sequester NF-κB activity (Kucharczak et al., 2003; Scheidereit, 2006). Caspase-3 inhibits the NF-κB pathway be cleaving and deactivating proteins of the NF-κB pathway. In particular p65, p50, and c-REL are known substrates for caspase-3 (Ravi et al., 1998). Thus, it is possible that DR3 activation of caspase-3 can cleave and disable several NF-κB members in this fashion. Another

possible route to repress NF-kB activity is via the modulation of the phosphatases PP2A and PP2Cβ which are known to deactivate IKK (DiDonato et al., 1997). Deubiquitnaiton of UB^{lys63} conjugated to the adaptor proteins TRAF or NEMO is another potent way to sequester NF-κB activity. The Lys63-specific ubiquitin C-terminal deubiquitinase CYLD is able to bind IKKγ and TRAF and selectively degrades UB^{lys63} chains from IKKγ, TRAF2, TRAF6 or TRAF7, which disables these factors and thereby represses NF-κB activity (Brummelkamp et al., 2003; Kovalenko et al., 2003). These are possible directions that can be investigated in order to determine how DR3 is able to sequester NF-κB activity. A first approach to understanding DR3 activity on NF-κB is to measure proteins levels of the canonical pathway, IKK1, IKK2, IKKγ, IκBα, p65 and p50.

4.3 MULTIPLE RECEPTOR HYPOTHESIS FOR VEGI

We were unable to determine if DR3 is utilized by VEGI for mediating cell death. The pathways activated by VEGI however do resemble the typical activation of a death receptor. In figure 24 we depict a cell model for VEGI signaling derived from a single receptor however it is possible that these pathways are derived from two or more receptors. Studies with TNFα interaction with TNFR-1 and TNFR-2 reveal how dual receptors can activate distinct and overlapping pathways. TNFR-1 is a typical death receptor capable of activating both pro-survival and apoptosis pathways. TNFR-2 does not contain a death domain and intracellular signaling is restricted to the activation of NF-κB and MAPK pathways. Additionally, TNFα activation of TNFR-1 and TNFR-2 result in opposing cellular responses in endothelial cells (Luo et al., 2006). In isolated murine endothelial cells TNFR-2 was shown to be responsible for the activation of NF-κB regulated genes, endothelial cell survival, and migration. In contrast TNFR-1 caused the

inhibition of migration and resulted in apoptosis.

So it is possible that pro-survival signaling can arise from one receptor and pro-death can arise from a second receptor. In Chapter 3 we show that DR3-depleted cells continues to respond to VEGI-induced NF-κB activation. This suggests a second receptor for NF-κB activation. Since, DR3 is a death-domain containing receptor it would be expected to be involved the induction of apoptosis, that is, no other higher affinity death receptors can be activated by VEGI.

A recent study gives a clue to an unknown receptor for VEGI in endothelial cells. An investigation of VEGI in chicken revealed VEGI mRNA transcripts in multiple tissues and organs (Takimoto et al., 2005). It was further determined that a recombinant VEGI preparation of the chicken homologue exhibited cytotoxicity to the mouse fibroblast L929 cells as wells as chicken fibroblasts. Interestingly, the presence of TNFα is not detected in chicken and the authors hypothesized that VEGI may function as a substitute for TNFα in chickens. In fact the same group determined that the chicken homologues of VEGI and TNFR-2 can interact and mediate cell signaling (Takimoto et al., 2008). Thus, corroborating the hypothesis that VEGI is the alternate for TNFα in chicken. Although there is an evolutionary distinction between chicken and human or bovine (the cell-typed used for our studies) these findings provide further evidence of VEGI using multiple receptors for cell signaling. Although it has been determined that human VEGI does not bind to human TNFR-2 as studied by a flow-cytometry based assay (Bossen et al., 2006). There are ligand/receptor interactions that require certain post-translational events (Gutierrez et al., 2004) and a certain post-translational modification may be required for VEGI/TNFR-2 interaction that was duplicated in the previous reported flow cytometry assay. A functional assay such a siRNA depletion of TNFR-2 may be useful approach to determine if

VEGI can utilize TNFR-2 for cell signaling, in particular NF-κB activation.

4.4 DR3 REGULATION OF APOPTOSIS

We show that once DR3 is depleted from endothelial cells the cells become protected from several apoptotic ligands. These results suggest that DR3 may play a crucial role in the apoptotic pathway. It is unclear how DR3 is able to modulate the apoptotic pathway. In Figure 26 we propose two possible mechanisms of modulating the apoptotic pathway. One possibility is that DR3 is regulating a factor required for the apoptotic pathway, such that depletion of DR3 results in a gap in the pathway. Another possibility relates to the observation that DR3 in repressing the NF-κB pathway such that removal of DR3 increases NF-κB activity. Increased NF-κB activity would increase production of anti-apoptotic proteins and be more effective at sequestering the apoptotic pathway and preventing cell death.

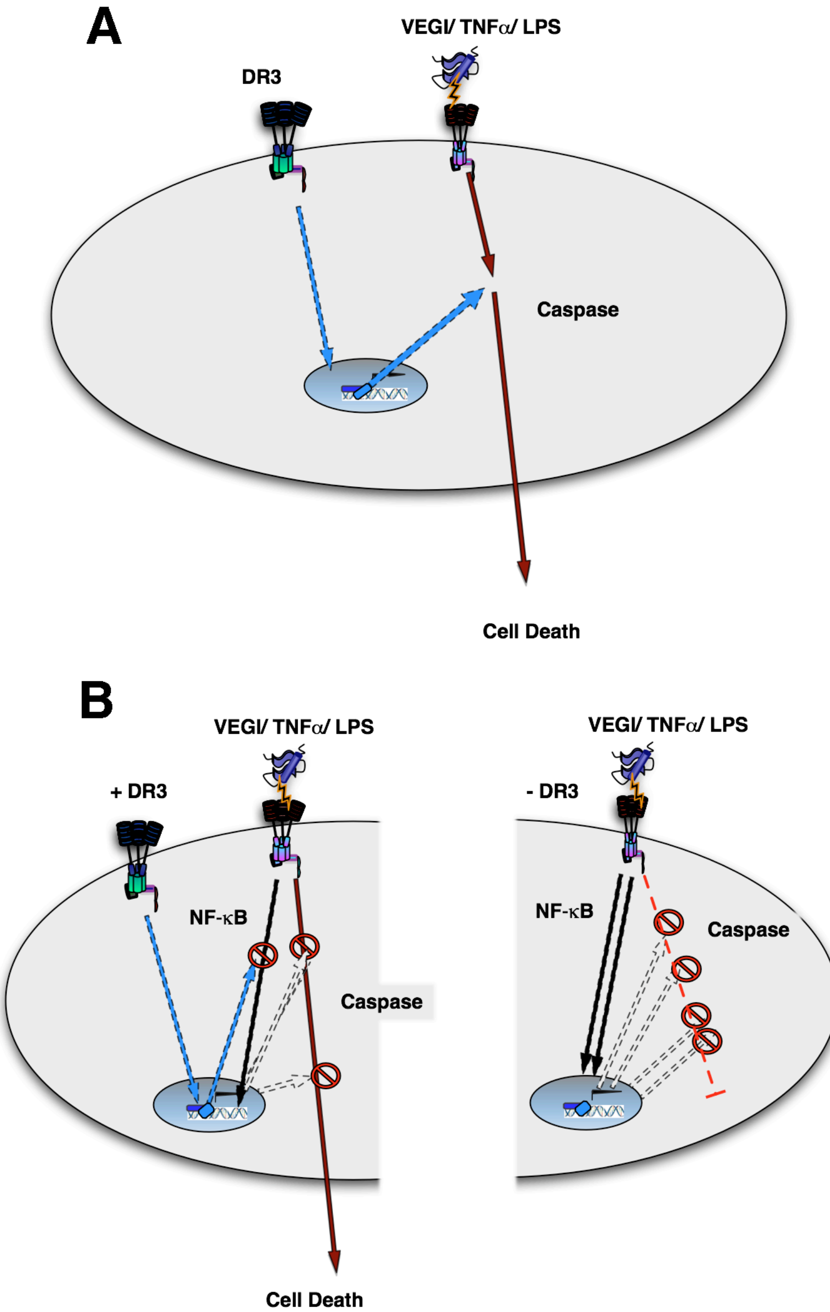


Figure 26. Two potential mechanisms that DR3 may regulate the apoptotic pathway. (A) DR3 may be involved in regulating a necessary factor utilized by the extrinsic apoptotic pathway. (B) Left, DR3 is able to repress the NF-κB pathway which would result in the diminished expression of anti-apoptotic factors. Right, when DR3 is depleted NF-κB activity becomes uninhibited leading to increased expression of anti-apoptotic proteins and enhanced survival.

Regardless of the mechanism that DR3 is utilizing to regulate the apoptotic pathway our results suggest that DR3 is constitutively active under normal conditions. However, it is unclear what is activating DR3 during normal growth conditions. One such possibility is a serum factor, possibly VEGI, may be involved in stimulating DR3. So in the presence of normal growth media that factor is continuously stimulating DR3. A second possibility is that E-Selectin may be activating DR3 during cell contacts. Colon cancer cells can communicate with endothelial cells via an interaction between DR3 and E-Selectin (Gout et al., 2006). This study investigated the cross-interaction with these two cell-surface proteins derived from different cell-types. Since, E-Selectin and DR3 are likely to be co-expressed in endothelial cells it is possible that these two receptors can interact during endothelial cell-cell contacts and possibly result in the constitutive activation of DR3.

4.5 INFLAMMATION AND ENDOTHELIAL CELL ACTIVATION

We determined an anti-apoptotic role in NF-κB activation during VEGI stimulation of endothelial cells. Not directly studied here but an important role of NF-κB activation in endothelial cells is its role in inflammation. Microvascular cells at the site of inflammation are known to directly regulate the inflammation process as well as communicate with inflammatory cells. During acute inflammation the quiescent endothelium undergoes a process of "endothelial cell activation" that initiates endothelial cells to help combat the inflammatory stimulus (Pober and Cotran, 1990). Endothelial cell activation in the absence of gene expression is known as type I activation, and endothelial cell activation that is dependent on gene expression is referred to as type II activation (Pober and Cotran, 1990). Both type I and type II are marked by an increased in local blood flow, a localized leakage of plasma-protein-rich fluid, a localized recruitment and

activation of circulating leukocytes, and pain caused by mediators released by leuokocytes on C-type sensory nerve fibres. (Pober and Sessa, 2007). Type I activation of endothelial cells is typically activated by G-protein-coupled receptor that leads to release of Ca^{2+} ions from the endoplasmic reticulum and the production of prostaglandin I2, a potent vasodilator (Egan and FitzGerald, 2006). Type I activation also involves inactivation MLC phosphatase, the phosphatase responsible for contracting actin filaments and involved in forming cell junctions. Inactivation of MLC leads to the opening of endothelial cell junctions thereby promoting vascular leakage of plasma proteins and neutrophil extravasation (Lorant et al., 1991; Teixeira et al., 1993).

Type I activation lasts for about 10-20 minutes and spontaneously resolves due to receptor desensitization. Type II activation is a more lasting inflammatory response and persists as long as chemokines are still being produced and until the inflammatory stimulus is removed. The protypic members of type II activation are TNFα and IL-1 and their activation of the transcription factors NF-κB and AP-1 (Pober and Sessa, 2007). Type II activation can lead to induction of inflammatory molecules such as IL-8 production, which is responsible for neutrophil recruitment and upregulation of E-selectin. Type II activation can also lead to induction of the chemokine COX2, leading to increased blood flow similar to Type I activation (Smith et al., 2005). TNFα stimulation also involves actin reorganization and the opening intercellular junctions resulting in vascular leakage (Petrache et al., 2003). NF-κB negative feedback loops are involved in bringing type II activation to an end (Winsauer and de Martin, 2007).

Since VEGI is an endothelial autocrine factor with similar signaling activities to TNFα we may expect VEGI to be involved in Type II activation of endothelial cells during acute

inflammation. We determined that VEGI can upregulate the chemokines IL-1β, IL-6 and IL-8 via NF-κB thus exhibiting the potential of VEGI to direct endothelial cells to produce important cytokines involved in the inflammatory process. Further investigation would be of interest to determine if VEGI-induced NF-κB activation is playing a role in type II endothelial cell activation.

4.6 NF-κB AS A TARGET FOR CANCER THERAPY

NF-κB is gaining attention as an important factor in cancer biology because of its involvement in tumor progression and therefore a potential target for anti-cancer drug development. Constitutive NF-κB activity is found in breast, prostate, colorectal, and ovarian cancers and certain forms of leukemia and lymphoma (Karin et al., 2002). The multifunction activities of NF-κB may attribute to tumor progression as several steps. The classical steps thought to be involved in cancer progression require the pre-neoplastic cell to become self-sufficient in growth signals, avoid both growth inhibitors and apoptosis, attain the ability to develop a limitless proliferation potential, increase mobility and metastasis potential, and modulate its nutrient supply by regulating angiogenesis (Hanahan and Weinberg, 2000). NF-κB can affect all of these processes by acting on the premalignant cell itself or by acting on inflammatory cells that are able to modulate the microenvironment (Karin and Greten, 2005). One of the most prominent roles of NF-κB in tumor progression is the ability to regulate anti-apoptotic factors such as BCL-2 a known oncogene (Danial and Korsmeyer, 2004). In certain leukemias and lymphomas inhibiting NF-κB activation triggered apoptosis suggesting that the growth of these cells are dependent of NF-κB (Furman et al., 2000). NF-κB activation can also lead to the recruitment of several cells to the tumor microenvironment including macrophages, dendritic cells, neutrophils, mast cells, T cells and B cells (Greten et al., 2004, Pikarsky et al., 2004). These cells can produce

cytokines utilized by tumors for growth, production of angiogenic factors, and the production of proteases to degrade the extracellular matrix and support the growth of the tumor vasculature. NF-κB regulated genes IL-6 and IL-8 are known to be growth factors for premalignant cells and are involved in the stimulation of angiogenesis (Sparmann and Bar-Sagi, 2004).

Therefore there is close relationship between NF-κB activation, inflammation, and cancer progression. We propose that NF-κB inhibitors can be used in combination with VEGI as approach to cancer therapy. This approach would create a multi-targeting effect on the tumor. First NF-κB inhibition can work directly on the tumor to prevent activation of anti-apoptotic genes and proliferation. Additionally, NF-κB inhibition can prevent recruitment of inflammatory molecules that may be involved in the tumor progression. Also VEGI combined with NF-κB inhibitors would create a potent apoptotic effect on the tumor vasculature.

It should be noted that it is possible that prolonged and substantial inhibition of NF-κB may not be practical when implemented as therapeutics because NF-κB is required for many fundamental processes (Nakanishi and Toi, 2005). Thus, a more intermittent treatment of NF-κB inhibitors may be required. An ideal approach to inhibiting the NF-κB pathway would be to target certain factors in the pathway that are only required for inducible NF-κB involved in the induction of anti-apoptotic genes and does not interfere with normal cellular functions. We discussed above several possible routes of stimulating and controlling NF-κB responsive genes. Elucidating the details of the VEGI-induced NF-κB pathway may provide for an ideal target.

4.7 VEGI AND THE ANGIOGENIC BALANCE

The role of VEGI as an anti-angiogenic factor is well established and shown in several *in vitro* and *in vivo* models. As a regulator of endothelial cell apoptosis it is possible that VEGI is

involved in the angiogenic balance and may oppose the actions of the pro-angiogenic factors such as VEGF and bFGF. One of the emerging pictures from our studies is the multiple level of balancing that may arise in the angiogenic balance and vascular homeostasis. We established balancing role of NF-κB and apoptosis initiated by VEGI. So VEGI may oppose pro-angiogenic factors but also activates pro-survival genes. These multiple levels of balance would contribute to the highly orchestrated process and fine-tuning of the angiogenic balance and vascular homeostasis.

4.8 ENDOGENOUS INHIBITORS TO NORMALIZE THE VASCULATURE

Endogenous inhibitors are thought to provide a promising approach to anti-angiogenic therapies because of their natural ability to inhibit endothelial cell growth. However, endogenous inhibitors role in normal physiology is thought to restore and maintain an angiogenic balance. Thus, endogenous inhibitors may contain checks so as not to overexert their inhibitory effect and avoid vascular damage. We determined VEGI utilizes NF-κB in order to control it's apoptotic potential. It is possible that VEGI and other endogenous inhibitors may not serve as ideal drugs that can completely deplete the tumor vasculature because of their partial survival activity.

Initially the use of anti-angiogenic drugs in clinical trials, only produced modest results and did not yield long-term survival benefits (Mayer, 2004). When the anti-angiogenic drug, bevacizumab, was used in combination with chemotherapy there was an unprecedented 5-month increase in colorectal cancer patients (Hurwitz et al., 2004). This treatment combination suggests that chemotherapy is able to inhibit growth of the tumor cells and bevacizumab is able to prevent angiogenesis. However, this dual treatment is thought to be a paradox as anti-angiogenic therapy would diminish oxygen delivery, needed by the chemotherapeutics in order to be effective (Jain,

2005b). To account for this paradox it was hypothesized by Rakesh Jain that the bevacizumab may "normalize" the tumor vasculature providing a more efficient delivery of drugs and oxygen to the tumor.

The tumor vasculature is highly tortuous and is structurally and functionally abnormal (Tian et al., 2002; Jain, 2005a). These abnormalities in the tumor vasculature lead to a tumor microenvironment that is characterized by a high interstitial hypertension, hypoxia, and acidosis (Padera et al., 2004). The tumor is able to survive under these conditions however these conditions create a barrier for drug delivery (Jain, 2005b). Chemotherapy and radiation therapy are oxygen-dependent and in hypoxic conditions will reduce the efficacy of these therapies. Also, hypoxia and low pH compromise the efficacy of the immune cells that infiltrate the tumor (Jain, 2005b). Another barrier in the drug delivery attributed to the tumor vasculature are the large holes found in the vasculature leading to vasculature leakiness. This would result in an accumulation of the drug in certain parts of the tumor microenvironment and not all of the tumor regions would be accessible to the anti-cancer agents (Hobbs et al., 1998; Jain, 1999). Due to these barriers in the tumor vasculature Jain suggested if the tumor vasculature is normalized to be a more functional and structural conduit this will in turn lead to improved drug delivery to the tumor. Additionally, the normalized vasculature may prevent malignant cells from entering the circulation and lower the metastatic potential of a primary tumor.

A slight variation of the normalization hypothesis to improve drug therapy is to stabilize the vasculature to maintain cancer in a stable disease. Overexpression of the Angiopoetin-1 results in the inhibition of tumor growth. Angiopoetin-1 increased pericyte coverage of the vasculature allowing for more functional blood vessels. It is hypothesized that this would revert the tumor state back to dormant state and prevents erratic angiogenesis that is required for tumor

progression.

Endogenous inhibitors of angiogenesis play a pivotal role in controlling the angiogenic balance but they become downregulated during tumor progression. Re-introduction of endogenous inhibitors in the tumor microenvironment may help to restore the angiogenic balance and maintain vascular homeostasis and direct the blood vessels to a more functional and quiescent state. Thus it may be possible that endogenous inhibitors such as VEGI can improve drug delivery to the tumor or maintain cancer as a stable disease.

4.9 FINAL WORD

We focused on characterizing the molecular mechanisms of VEGI. We determined that NF-κB is a controlling factor in VEGI-mediated endothelial cell death and provide some potential new approaches to cancer therapy with the use of VEGI and NF-κB inhibitors. We also investigated if DR3 is the receptor responsible for VEGI-mediated endothelial cell death. We were unable to make a ligand/receptor correlation between DR3 and VEGI. However, we uncovered a novel role of DR3 and its needed presence for the induction of cell death by several apoptotic ligands, thereby establishing a unique role of DR3 in endothelial cell biology. These studies resulted in a clearer understanding of both VEGI and DR3 and it is the hope that these studies will make an impact cancer therapy.

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