PERLECAN DOMAIN V INDUCES VEGF SECRETION IN BRAIN ENDOTHELIAL CELLS THROUGH $\alpha_5\beta_1$ INTEGRIN DEPENDENT MECHANISM

A NOVEL INSIGHT IN BRAIN TISSUE RECOVERY FOLLOWING ISCHEMIA

A Dissertation

by

DOUGLAS NELSON CLARKE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2010

Major Subject: Biochemistry

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Approved by:

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ABSTRACT

Perlecan Domain V Induces VEGF Secretion in Brain Endothelial Cells Through $\alpha_5\beta_1$ Integrin Dependent Mechanism

a Novel Insight in Brain Tissue Recovery Following Ischemia.

(December 2010)

Douglas Nelson Clarke, B.S., Texas State University

Co-Chairs of Advisory Committee: Dr. Gregory J. Bix
Dr. Sumana Datta

Stroke is the leading cause of long term disability and the third leading cause of death in the United States. Perlecan plays a significant role in brain development by sequestering and delivering growth factors to developing neuronal precursor cells in a neurovascular niche. Previous results demonstrated that perlecan proteolysis results in the cleavage of perlecan's most C-terminal domain five (DV) in the post-ischemic brain. As post-stroke angiogenesis is an important step in post-stroke brain repair, I focused on the mechanism of DV's role in brain angiogenesis in vitro.

I first demonstrated that DV significantly increased brain endothelial (BE) cell migration, proliferation and tube-like formation suggesting DV is a pro-angiogenic factor for BE cells. I next investigated VEGF secretion from BE cells in the presence of DV. DV significantly increased VEGF secretion into the cell media, which was both dose and time dependent. Using quantitative real-time PCR, DV induced a maximal nine-fold

increase in VEGF expression, compared to control, indicating DV is an upstream regulator of VEGF transcription. DV treated cells show an increase in phosphorylation of ERK-(1/2) that could be blocked by the pharmacological inhibitor U0126. This inhibitor could also block DV's effect on VEGF mRNA expression and secretion indicating ERK is involved with DV's effect on VEGF regulation. Optical sensor binding assays confirmed that DV binds to the $\alpha_5\beta_1$ integrin with a K_d of 160nM, and cells treated with DV showed a visual representation of integrin $\alpha_5\beta_1$-DV colocalization. Furthermore, shRNA-mediated knockdown of integrin α_5 blocked DV's effect on VEGF mRNA expression, indicating integrin α_5 is involved with DV's regulation of VEGF expression.

In conclusion, these results demonstrate that DV has an unexpected pro-angiogenic effect in brain angiogenesis. This occurs via a previously unreported interaction between DV and the $\alpha_5\beta_1$ integrin, resulting in the activation of the ERK, eIF4A and HIF1α signaling pathway and an ultimate increase in VEGF mRNA expression and VEGF secretion. As DV is generated post-stroke, these results suggest a novel mechanism by which brain tissue recovery following ischemia is influenced by processed fragments from the extracellular matrix.

DEDICATION

This dissertation is dedicated to my mother and father,

Christine Winter and William Robert Clarke.

Thank you for your love and support throughout the years.

How else could I become what I've become?

All your plans and hopes and even fears

Now come together in what I have done.

Know that I am grateful for your love.

Your hard work is mirrored now in mine.

On you all my accomplishments must shine.

Underneath my pride, your spirits move.

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I would like to thank my sister, Kathryn Marie Clarke, for being my best friend, my motivator and my number one fan. I would like to thank my brother Daniel Clarke, here's "looking to the forward". I would like to thank my grandmothers, Gretel Winter and Viola Clarke; I'll always keep my nose to the grind stone. I would like to thank the Leavelle family for their thoughts and supportive prayers. I would like to thank my roommates Joel William Gray and Mathew Thomas Kuhn. From the beginning of

graduate school till the end they were always there willing to stay up late, talk science and provide a shoulder to lean on, in particular Chopper.

Finally and most importantly, I would like to thank Nichole Whitney Leavelle. Who would have ever guessed a corny post-it flip book would have gotten us to come this far. Here's to the first of many accomplishments we will endure together. I love you.

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INTRODUCTION

Stroke, also referred to as a "brain attack", is the leading cause of long term disability and the third leading cause of death in the United States. Two types of stroke exist. Ischemic stroke is where there is loss of blood flow to a brain region due to a blood clot occluding a cerebral artery, and hemorrhagic stroke, where there is an excessive amount of blood released in the cranium due to the rupture of a blood vessel. Eighty seven percent of patients who are affected by neurovascular stroke suffer from ischemic stroke while the remaining population suffers from hemorrhagic stroke. Regardless of the type of stroke suffered, the brain attempts post-stroke repair. However, while incremental advances have been made in acute stroke treatment, understanding of the mechanisms underlying brain self-repair after stroke remains poor. Therefore, the problem of brain repair and stroke rehabilitation is an emerging research priority (Grotta et al., 2008), with the underlying goals of identifying and improving brain reparative process.

In order to identify and foster brain reparative processes for better patient outcomes, the neurovascular unit must be appreciated. The neurovascular unit consists of several cell types within the brain including endothelial cells, astrocytes, pericytes and neurons, all of which are closely knit together by the extracellular matrix (ECM).

This dissertation follows *Journal of Cell Biology.*

The ECM, in turn, plays many important roles in cell biology by regulating cell morphology, controlling cell fate, providing scaffolding, and regulating cell-to-cell interactions (Greenberg and Kunlin, 2005).

Within the last two decades, research has focused on investigating the cryptic fragments that are released from the extracellular matrix when exposed to active proteases such as matrix metalloproteinases (MMPs) and cathepsins. During ischemia, there is an increased release of these proteases within the stroke ischemic core (central region of irreversible neuronal injury) and penumbra (area surrounding the ischemic core) that leads to proteolysis of the ECM. Processing of the ECM can lead to the generation of ECM fragments with potential effects on the surrounding neurovascular unit. Frequently, these fragments have been shown to be negative regulators of new blood vessel development from pre-existing vasculature (angiogenesis) among other neurovascular effects.

Among several components of the ECM, perlecan, a heparan sulphate proteoglycan, has been shown to be a source for such a cryptic fragment, its C-most terminal domain, domain five (DV). Previous research has characterized this 82kDa fragment as a negative regulator of angiogenesis outside of the brain via interaction with the $\alpha_2\beta_1$ integrin and inhibiting vascular endothelial growth factor (VEGF) activity (Nystrom et al., 2009).

Although the anti-angiogenic capacities of these cryptic fragments have been exploited for therapies used in pathological diseases such as cancer, little research has

been performed investigating the roles of these cryptic fragments on the neurovascular unit following ischemic stroke. There is a basic understanding following ischemia that ECM proteolysis is induced, but underlying questions still remain unanswered: are cryptic ECM fragments generated after ischemic stroke? What happens if/when these cryptic fragments are released? Do they affect the surrounding vasculature and how?

In this dissertation, I focus on the mechanism by which perlecan's DV fragment unexpectedly *induces* brain angiogenesis in vitro. My research provides novel insight on the regulatory effects mediated by the ECM, particularly perlecan's DV fragment, and a plausible mechanism describing brain tissue recovery following neurovascular stroke.

Stroke

It is estimated stroke will cost $73 billion in direct and indirect costs for health care during the year of 2010. Currently, more than six million people, greater than or equal to 20 years and older, in the United States have suffered from a stroke. More women than men will suffer from a stroke for two reasons: women tend to live longer than men and suffer from an imbalance of hormones incurred following menopause. The most common high-risk factors for both sexes in stroke are hypertension, smoking, diabetes mellitus and depression (Lloyd-Jones, 2010).

Currently the only FDA approved drug for acute ischemic stroke treatment is tissue plasminogen activator (tPA). tPA is a clot busting agent suitable for patients who suffer from ischemic stroke or heart attack. The major down side to tPA is its limited

therapeutic window of three to four and a half hours following the onset of stroke symptoms. Unfortunately, many stroke patients do not recognize their stroke symptoms as such until it is too late for tPA administration, or worse are initially misdiagnosed in the emergency room putting them outside the window of opportunity for tPA (Lloyd-Jones, 2010).

Two types of stroke exist: ischemic and hemorrhagic. An ischemic stroke can generally be defined as an event in which blood supply to the brain has been blocked, typically by a thrombus, leading to the depletion of sufficient oxygen and nutrients needed for cell survival. Patients who suffer from hemorrhagic stroke can either suffer from an intracerebral or subarachnoid hemorrhagic stroke. An intracerebral hemorrhage occures when there is bleeding within the brain, while a subarachnoid hemorrhage occurs when there is bleeding in the subarachnoid space. Eighty seven percent of patients who are affected by stroke suffer from ischemia, ten percent suffer from intracerebral hemorrhagic and the remaining three percent suffer from subarachnoid hemorrhage This dissertation will focus on the more common ischemic stroke.

Immediately following an ischemic insult, an ischemic cascade takes place that can ultimately lead to apoptosis. The initial processes following ischemia are energy failure, loss of ion homeostasis, depolarization, and water influx (Siesjo, 2008). Apoptosis following ischemia is initiated by internal and external events. The “intrinsic pathway” occurs when there is a disruption in oxygen levels that leads to the disruption

of the mitochondria and subsequent release of caspases, following ATP-dependent ion transport failure. Once this happens, there is an influx of cytosolic calcium ions within the cell which leads to cell stress and the release of glutamate. The release of excess glutamate, the presumed prime suspect in ischemic core damage, stimulates N-methyl-D-aspartate (NMDA) or D, L-α-amino-3-hydroxy-5-methyl-isoxazolpropionic acid (AMPA) receptors causing neighboring cells to uptake more intracellular calcium ultimately leading to cell death. Sadly, pharmacological studies blocking NMDA and AMPA receptors have not been entirely successful, leaving this model of ischemic damage open to further investigation.

Dying cells can have negative paracrine effects on neighboring cells by releasing apoptotic factors such as toxins, cytokines, and proteases, thus activating the "extrinsic pathway" (Broughton, 2009). These apoptotic factors then interact with pro-apoptotic cell surface receptors to induce caspase signaling cascades and ultimately the direct induction of apoptosis. The newly released apoptotic factors and resulting inflammatory responses cause the ischemic core to continuously grow while also putting the ischemic penumbra at risk for necrosis.

The area of severe ischemia, where irreversible neuronal injury occurs, is defined as the ischemic core. The area surrounding the ischemic core, the ischemic penumbra, is also at risk for cell death yet viable several hours following ischemic injury, (Ginsberg, 1997) but not indefinitely (**Figure 1.1**). Following middle cerebral artery occlusion (MCAO in the mouse, the ischemic core has a cerebral blood flow at or below 20% of normal and is unable to reverse injury because of potassium steady-state elevations which cause anoxic depolarization (AD). AD is a sudden and profound depolarization of neurons and glia in cortical and subcortical gray matter (Jarvis, 2001), and ionic dyshomeostasis, which refers to a disruption of intercellular calcium levels. In contrast, the ischemic penumbra has a cerebral blood flow approximately 20% to 40% higher than normal. It is electrically silent but above ionic dyshomeostasis (Ginsberg, 1997) leaving the cells in a quiescent but viable stage. Therefore, targeting the viable cells within the ischemic penumbra with neuroprotective therapies may potentially lead to the rescue of neuronal injury, cell death and promotion of brain repair.

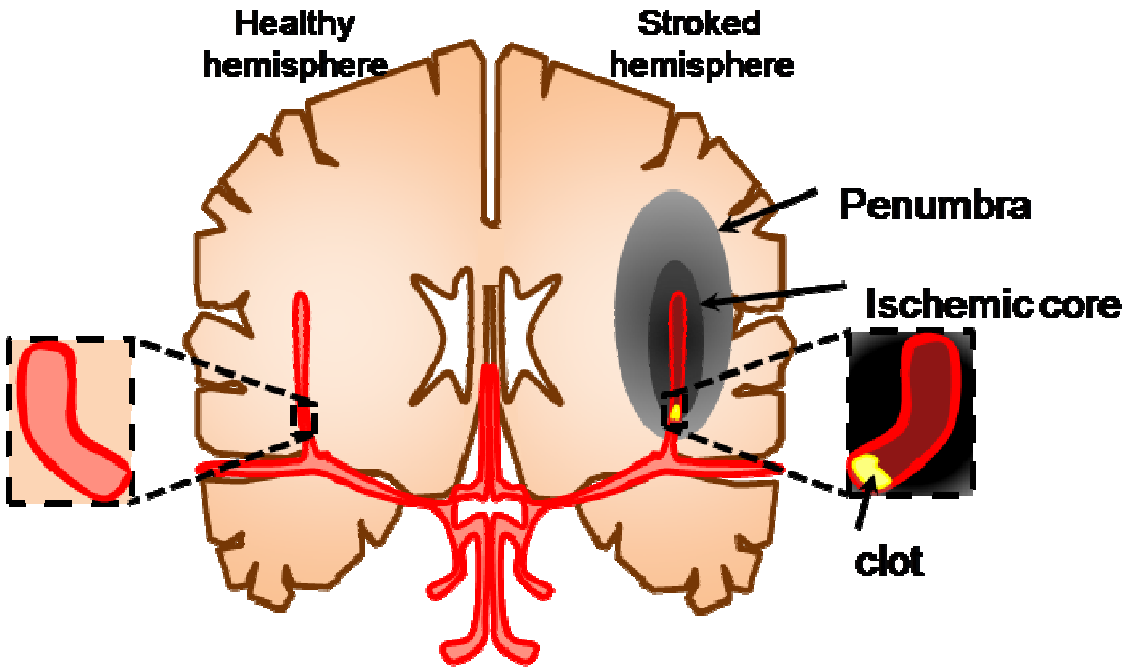

Figure 1.1. Ischemic stroke schematic. This figure illustrates the consequence of a cerebral blood vessel clot occlusion, i.e. an ischemic stroke. The core is defined as the area surrounding the blood vessel distal to the clot where irreversible neuronal injury occurs. The ischemic penumbra/peri-infarct brain that surrounds the core is also at risk for cell death yet is still viable several hours following ischemic injury, thus allowing for neuroprotective therapies to rescue neuronal injury and cell death. Both areas are shaded in various colors of gray.

Neurogenesis and angiogenesis are vital in brain repair not only to replace neurons that have rapidly degenerated post stroke, some as soon as one day following stroke (Hayashi et al., 2003), but also to help in reperfusion of blood supply and nutrients that are vital for cell survival. Yet the regulation of neurogenesis and angiogenesis following ischemia are not well understood. This lack of understanding leads to an underlying disconnect between the bench and bedside for current stroke therapies and a critical need for identifying and improving brain reparative processes.

Brain self-repair

Brain recovery following ischemia involves the formation of new blood vessels (angiogenesis) and the re-population of neurons (neurogenesis). Indeed, following ischemia embryonic/developmental molecular signals are reactivated to regulate neurogenic and angiogenic processes for brain repair. Both of these processes occur in close proximity, i.e. in a neurovascular niche, (Ohab et al., 2006) (Krupinski et al., 1993) (Krupinski et al., 1994b) which affords mutually supportive growth factor-mediated neuron-endothelial cell cross-talk (Jones et al., 2001), (Stumm et al., 2002), (Guo et al., 2008). In the neurovascular niche, diffusible growth factors help afford cross-talk between the closely associated brain endothelial cells and the neuronal precursor cells in a fashion that is consistent with the developmental association of neurogenesis and vasculogenesis (Wurmser et al., 2004).

In order for proper neurogenesis to occur following ischemia, neural stems cells must proliferate, differentiate and migrate to the ischemic penumbra where they

mature to neurons and integrate into the parenchymal tissue (Guo et al., 2008). For post-stroke neurogenesis in mice and rats, neuronal progenitor cell proliferation is significantly enhanced in the subventricular zone (SVZ) and the hippocampal dentate gyrus (DG). This occurs as early as two to three days post-stroke and correlates with a specific expression pattern of cytokines, chemokines and vascular growth factor signaling (Guo et al., 2008). Cell proliferation continues but peaks after one to two weeks and returns to control levels by three to four weeks (Jin et al., 2001).

Once generated, these newly formed neuronal progenitor cells continue on for approximately two to three weeks in the DG but only for a week in the SVZ (Dempsey et al., 2003). Instead, the SVZ neuronal progenitor cells largely migrate as far as the striatal stroke penumbral area and once there, differentiate into mature striatal neurons and astrocytes (Parent et al., 2002). Unfortunately, relatively few neural progenitors migrate into stroked tissue and those few that complete the trip usually fail to become mature neurons for unknown reasons (Jin et al., 2003).

For angiogenesis in mice, endothelial cell proliferation can occur as early as 12 to 24 hours post middle-cerebral artery occlusion in the ischemic middle cerebral artery territory (Hayashi et al., 2003). These newly generated endothelial cells then migrate towards the ischemic penumbra in response to a number of endothelial cell chemoattractants such as vascular endothelial cell growth factor (VEGF) and platelet derived growth factor (PDGF). The endothelial cells then form new blood vessels in

peri-infarct cortex after three to seven days, (Hayashi et al., 2003) with angiogenesis continuing for at least 21 days after.

In addition to providing nutritive blood flow to the surrounding tissues, previously and newly formed angiogenic blood vessels are beneficial following ischemia because they serve many roles in brain repair. As noted earlier, neuronal migration is a key step towards proper brain repair. In order to help neuronal migration, newly formed angiogenic blood vessels can serve as physical scaffolds or "railroad tracks" for new neurons to migrate toward the ischemic core, even in the absence of blood flow (Ohab et al., 2006). Ohab et al. (Ohab et al., 2006), have demonstrated the importance of close physical associations between neuroblasts and endothelial cells in order to help neuroblast survive and get to areas, such as the ischemic penumbra, of vascular remolding following stroke.

The newly produced vasculature also helps promote neurogenesis and neuroblast migration by the secretion of growth factors that influence the biological activity of neuroblasts. Angiopoietin 1 (Ang1) and stromal-derived factor 1 (SDF1) (Hohenstein et al., 2005), (van Weel et al., 2007) are endothelial secreted factors that act on the neuroblast Tie2 and CXCR4 receptors, respectively (Jones et al., 2001), (Stumm et al., 2002). Erythropoietin (EPO) also increases the number of immature neurons in the peri-infarct tissue (Zhang et al., 2005). Neuroprotection, neuronal migration, and neural stem cell renewal are also afforded by vascular production of brain-derived neurotrophic factor (BDNF) binding to neuronal TrkB receptors (Guo et

al., 2008), (Snapyan et al., 2009), (Li et al., 2006). However, this may not be the case in the adult mouse and rat subventricular zone (Galvao et al., 2008). A recently defined example of neurovascular cross-talk occurs in brain endothelial cell-neural stem cell co-cultures. Neural stem cell Nitric Oxide (NO) reportedly induces brain endothelial cell release of BDNF and VEGF, which in turn induce endothelial cell angiogenesis via VEGFR2 and TrkB receptors. The release of BDNF and VEGF neuronal stem cell renewal effects (Li et al., 2006).

In addition to VEGF, BDNF and EPO, several reports describe other important growth factors and their roles in neurovascular remodeling. For example, the expression of basic fibroblast growth factor 2 (FGF-2) has been shown to increase the migratory capacity and proliferation of neural progenitor cells (NPC) following transplantation into the neonatal ischemic cortex (Dayer et al., 2007). Furthermore, Teramoto et al (Teramoto et al., 2003) have demonstrated that epidermal growth factor (EGF) can be used to promote brain self-repair by increasing endogenous neuronal replacement following ischemia. Finally, Insulin-like growth factor I (IGF-1) has also shown promise in brain ischemic injury. Current evidence suggests that IGF-1 is neuroprotective, has the ability to cross the blood brain barrier, and when over

expressed following acute ischemic injury can improve motor performance in mice (Zhu et al., 2008). Collectively, niche neurovascular coupling appears to represent an important means of post-stroke brain repair that could be therapeutically exploited (Arai et al., 2009).

Angiogenesis and the extracellular matrix

Blood vessels are composed of two interacting cell types, endothelial cells and perivascular cells. Endothelial cells line the inside of the vessel wall, while perivascular cells envelope the surface of the vascular tube (Bergers, 2005). Angiogenesis is the formation of new blood vessels from pre-existing blood vessels, including the remodeling of adult endothelial cells to arteries, veins and capillaries (Hayashi et al., 2003), (Serini et al., 2006). Angiogenesis occurs in multiple steps: detachment of pericytes from the vascular tube, breakdown of the extracellular matrix (ECM), proliferation and migration of new endothelial cells, and finally tube morphogenesis (**Figure 1.2**). The onset of angiogenesis occurs as a response to a stimulus such as a growth factor or cytokine, or following an insult such as a wound. Following pro-angiogenic stimuli, pericytes detach, allowing previously inhibited endothelial cell proteases to begin breaking down the ECM (Saunders et al., 2006). Once the ECM is broken down, endothelial cells begin to proliferate and migrate out towards new angiogenic stimuli. Following migration, endothelial cells form new tubes and release attractants for pericytes to once again come back and re-stabilize the vascular tube (**Figure 1.2**).

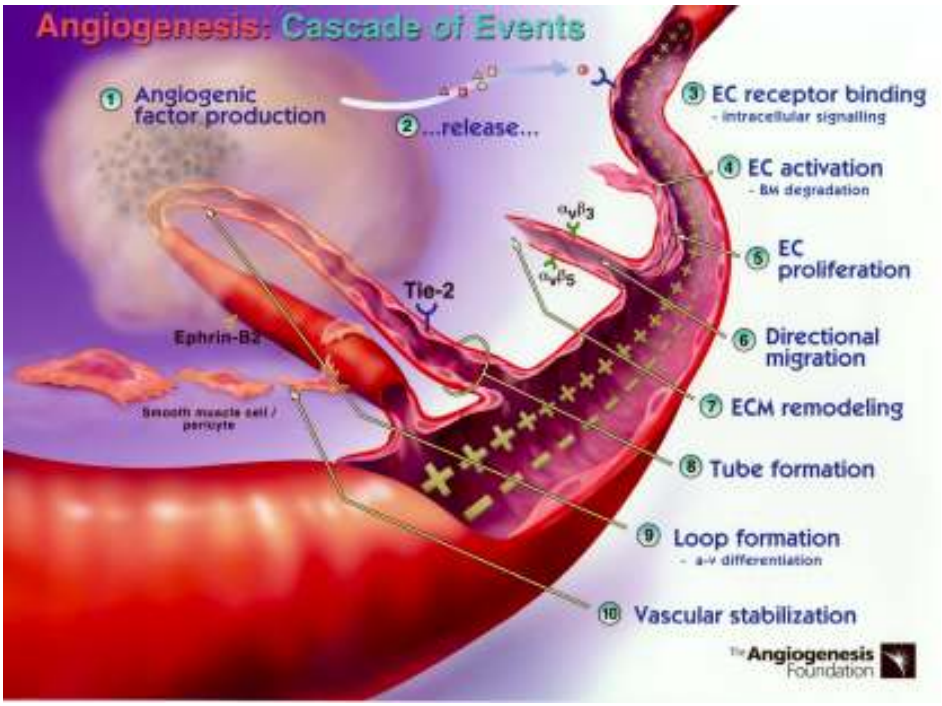

Figure 1.2. The angiogenic process. Once a pro-angiogenic stimulus has been sensed by endothelial cells, endothelial cells are activated and release pericytes for vascular tube destabilization. Following vascular tube destabilization, endothelial cells begin to proliferate and release proteases responsible for extracellular matrix degradation. The endothelial cells migrate out towards angiogenic stimuli and begin to remodel forming new tube vasculature. Once tube formation is complete, vascular stabilization is achieved once again by the recruitment of pericytes. Figure modified from The Angiogenesis Foundation, 2009.

During embryonic development, the vascular system develops by vasculogenesis, the de novo production of blood vessels. Vascular development in the brain is different among species. For example, capillary sprouts migrating into the neuroectoderm from the perineural plexus begin at embryonic day 4.5 in the chick versus embryonic day 11.5 in the rat (Plate, 1999). For the rodent, vascularization in the brain lasts up to 2 weeks following birth, and then is dramatically down regulated to keep up with organ size (Plate, 1999). Once the primitive vascular system develops, angiogenesis initiates, and produces most of the blood vessels in the embryo, including those formed in the brain (Plate, 1999).

Physiological angiogenesis is conditional in adult organisms, occurring only in processes such as the female menstruation cycle and pregnancy. But pathological angiogenesis, a process by which mature, adult blood vessels give rise to new blood vessels, is initiated in disorders such as cancer, wound healing, diabetic retinopathy and stroke. In mice, pathological angiogenesis can occur as soon as 12 hours post-middle cerebral artery occlusion (MCAO), and up to 21 days post-stroke in the ischemic penumbra (Hayashi et al., 2003). Once ischemia has taken place several events happen simultaneously. There are abrupt alterations in the ECM, changes in cell-surface integrin expression on several cell types such as neurons, astrocytes, and endothelial cells, and an increase in vascular permeability (del Zoppo and Milner, 2006). All of these events are required for active angiogenesis to take place in the adult.

The extracellular matrix

The basement membrane is composed of several ECM proteins critical for important biological processes. It is formed by glycoprotein and proteoglycan protomers that assemble and form supramolecular infrastructures (Yurchenco and Schittny, 1990). The ECM has important roles in the control of vascular patterning, morphogenesis, neovessel stabilization, and the formation of organs (Bix et al., 2006), (Serini et al., 2006). One important process the ECM serves is as a substrate and scaffolding for migrating cells during development and the onset of pathogenesis in such cases as wound healing, tissue regeneration and repair (Abrahamson, 1986). A second function of the ECM is to form a protective barrier and common substrate for various cell types to keep them closely knit together. The ECM also is capable of regulating cell fate by activating cell surface receptors and triggering subsequent intercellular signaling cascades. The cerebral extracellular space constitutes roughly 20% of the brain's volume and consists of the proteins laminin-1, entactin/nidogen, Type IV collagen, fibronectin, and perlecan (Fukuda et al., 2004) (Kohling, 2006). Changes in the brain ECM after ischemia are poorly understood, but are potentially important because they can affect multiple cell types.

The glycoprotein laminin-1 was first purified from engelbreth-holm-swarm (EHS) mouse tumor sarcoma cells (Orkin, 1977) (Paulsson et al., 1987). Laminin-1 structure is composed of three short-arm and one long arm glycoproteins linked together in a

fashion that resembles a crucifix. Alterations in laminin-1 during development can lead to several disorders such as blistering skin disease, epidermolysis bullosa, junctionalis and muscular dystrophy (Kohling, 2006). Laminin-1 contains the RGD amino acid sequence binding motif and other accessible interactive domains allowing it to easily bind integrins, heparan and other ECM components such as Type IV collagen and entactin/nidogen.

Entactin/nidogen was first described as two separate entities when purified by Carlin (1981), but later were recognized to be the same macromolecule (Abrahamson, 1986). The structure of nidogen is representative of a dumbbell, with large domains connected to one another by a linker and a rod. The domains of nidogens range from 38 kDa to 85 kDa, the C-terminus domain being the largest. Two isoforms of nidogen exist, nidogen-1 and nidogen-2. Knockout of both nidogen-1 and nidogen-2 is embryonic lethal, yet knockout of just nidogen-1 or nidogen-2 is not, suggesting compensation and redundancy of function between the two isoforms. However, neurological defects such as limb weakness and seizure-like behavior occur when nidogen-1 alone is knocked out, indicating its importance in neurological development (Bader, 2005).

During brain development, fibronectin and its receptor, $\alpha_5\beta_1$ integrin, are highly expressed to promote a pro-angiogenic environment (Davis and Senger, 2008). Fibronectin's positive effect on cell survival and proliferation is mediated through the $\alpha_5\beta_1$ integrin (Milner and Campbell, 2002). Reports demonstrate that vasculogenesis

(the de novo production of blood vessels) and angiogenesis depend primarily on ibronectin and the $\alpha_5\beta_1$ integrin (Serini et al., 2006) suggesting this integrin and ligand promote angiogenesis. Following development, maturation of the CNS no longer requires a pro-angiogenic environment but instead requires a quiescent environment. Therefore, there is a decrease in fibronectin and $\alpha_5\beta_1$ integrin expression and an increase in $\alpha_6\beta_1$ integrin and its ligand laminin type-1 which promote more of a quiescent environment (Milner et al., 2006). More recently, Milner et al. (2008) have demonstrated a pro-angiogenic "switch" following ischemia in which the vasculature reverts to a developmental, pro-angiogenic environment by increasing fibronectin and $\alpha_5\beta_1$ integrin expression.

Perlecan, (**Figure 1.3**), is involved with cell growth, proliferation, and brain development, (Bix and Iozzo, 2005). This >400kDa heparan sulfate proteoglycan is divided into five distinct structural domains (DI-DV), and is encoded by a single gene, which is conserved among humans, *Drosophila*, *C. elegans* and mice Knox and Whitelock, 2006; Hassell et al., 2003; Bix and Iozzo, 2005; Noonan et al., 1991). Non-lethal mutations in perlecan result in the Silverman-Hand maker type of dyssegmental dysplasia and Schwartz-Jampel syndrome (Arikawa-Hirasawa et al., 2001) that are characterized by lethal dwarfism and short stature, respectively.

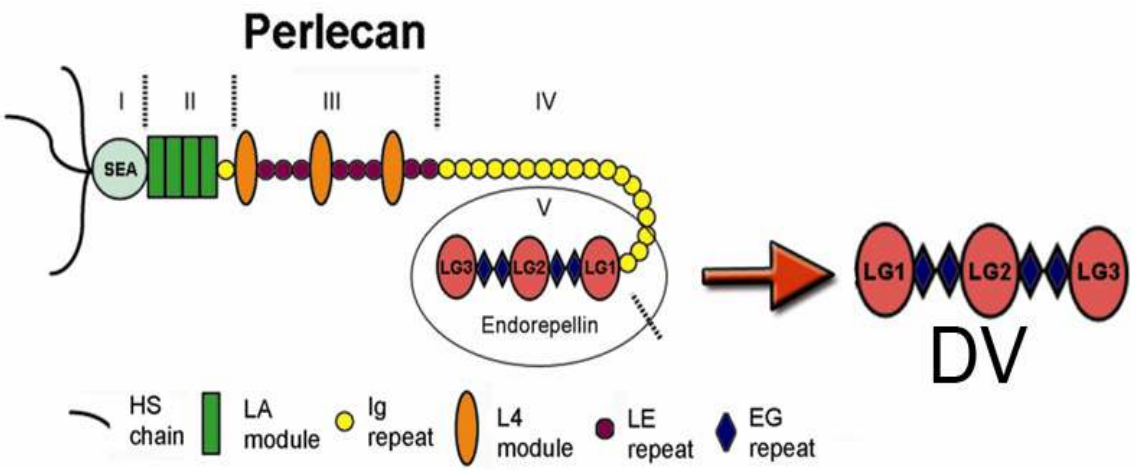

Figure 1.3. Schematic of perlecan and DV. Perlecan is divided into five domains, DI-DV, DI located at the N-terminus and DV the C-terminus. DV is made up of 3 Laminin Globular repeats that are separated by two epidermal growth factor repeats. Figure modified from Bix and Iozzo, 2005.

Perlecan-null mutations lead to malformations in cardiac outflow track, intra-pericardial hemorrhage, severe cephalic and cartilage abnormalities and ultimately death in mice, *C. elegans* and humans (Mongiat et al., 2003),(Bix et al., 2004), (Bix and Iozzo, 2005), (Noonan et al., 1991). In mice, perlecan mutations cause developmental defects of the heart, brain, kidney and skeletal muscles (Farach-Carson, 2007). The perlecan locus in *C. elegans* is referred to as UNC-52; mutations in UNC-52 are linked to body wall muscle defects and abnormal formation of integrin complexes (Rogalski et al., 1995). The perlecan homologue in *Drosophila*, t*rol*- (terribly reduced optic lobes), has a phenotype of small eyes and brains because of quiescent neuroblasts failing to initiate proliferation (Farach-Carson, 2007).

Interestingly, perlecan can be synthesized and secreted by neurons, astrocytes, and endothelial cells (Shee et al., 1998), the latter induced by $VEGF_{165}$ (Kaji et al., 2006). This suggests that multiple cell types are responsible for replenishing perlecan into the vascular basement membrane. Evidence indicates perlecan is the most protease sensitive and rapidly processed ECM protein following ischemia when compared to collagen or laminin after middle cerebral artery occlusion (MCAO), a commonly used animal model of ischemic stroke for rodents and non-human primates (Fukuda et al., 2004). Perlecan proteolysis by the stroke-generated cysteine protease cathepsin L occurs within two hours of MCAO in non-human primates and persists for at least seven days (Fukuda et al., 2004). The sustained processing of perlecan for days after stroke is consistent with studies demonstrating an increase in perlecan production in

neurons and astrocytes after brain injury (Shee et al., 1998). The developmental, nonstroke neurovascular niche located in the subventricular zone, (Palmer et al., 2000) (Merecier et al., 2002) contains perlecan and its absence in mice results in severely impaired neurogenesis because of decreased capture of neurogenic factors (Kerever et al., 2007). As mentioned previously, one product of perlecan proteolysis is the domain V (DV) fragment, which has been shown to inhibit angiogenesis in human umbilical vein endothelial cells (HUVEC) in vitro and in vivo. Proteolysis of perlecan by cathepsin L leads to the cleavage of DV from perlecan (Cailhier et al., 2008), suggesting that following ischemia, DV could be released from perlecan by cathepsin L.

Proteolytic processing of the extracellular matrix

During a stroke, dying and infiltrating inflammatory cells release matrix metalloproteinases (MMPs) and cathepsins, which disturb the blood brain barrier and proteolytically process the ECM (Fukuda et al., 2004). The major players involved with ECM processing following brain ischemia are tissue plasminogen activator (tPA), the MMP family and the cathepsin family. The expression, activity, and roles of these proteases are actively being investigated to better define their importance following brain injury. MMPs, cathepsin-L and tPA expression are up regulated following stoke. Overproduction of MMPs can result in cell death and inflammation. Inhibitors of the MMPs have been shown to reduce edema and infarction size (Durukan and Tatlisumak, 2007).

The initial processing and degradation of ECM is largely thought of as a negative consequence of acute stroke in that it increases blood-brain barrier permeability, but an additional consequence of matrix proteolysis is the generation of bioactive matrix fragments (Tian et al., 2007). Indeed, many matrix components are known to harbor bioactive matrix fragments in their C-terminal regions that can inhibit angiogenesis outside of the central nervous system (Mundel and Kalluri, 2007), (Bix and Iozzo, 2005), but their capability of affecting angiogenesis remains uncharacterized in the brain. Alterations in ECM can start as soon as two hours post-cerebral ischemia in the non-human primate (Milner et al., 2008b). Roughly 60% of cerebral ECM proteins are lost in the ischemic core within 24-hours post MCAO (del Zoppo and Milner, 2006). These results suggest the probability of generating bioactive matrix fragments following ischemia is highly likely.

Currently, the generation and role of biologically active ECM fragments in ischemic stroke is poorly understood. Most of these fragments have been isolated from the ECM of tumor microenvironment and have been shown to inhibit angiogenesis. At least nine ECM derived inhibitors of angiogenesis have been reported (Lo, 2007). Proteolysis of fibronectin can produce a fragment called anastellin (C-terminal) that has antimetastic activity (Yi and Ruoslahti, 2001), while collagen type IV proteolysis generates three anti-angiogenic fragments: arresten, canstatin and tumstatin, depending on the alpha chain composition (Mundel and Kalluri, 2007). Endostatin, the C-terminus of the heparan sulfate proteoglycan collagen type XVIII, is also anti-

angiogenic (Lo, 2007). Importantly, these angiogenesis inhibitors have been characterized primarily, but not exclusively (Ohab et al., 2006), outside of the central nervous system.

Arresten, derived from the C-terminus of the type IV collagen alpha1 chain, inhibits migration, tube formation of stimulated endothelial cells, and the positive proliferative effect of basic fibroblast growth factor (bFGF) stimulated endothelial cells (Mundel and Kalluri, 2007). The mechanism by which arresten induces its anti-angiogenic effect is likely because of interaction with the $\alpha_1\beta_1$ integrin and subsequent blockade of MAPK signaling (Colorado et al., 2000), (Sudhakar et al., 2005). The interaction and blockade has been shown to inhibit hypoxia inducible factor-1α (HIF-1α), an upstream factor that stimulates transcription of VEGF (Durukan and Tatlisumak, 2007). A mutation in arresten has been linked to patients suffering from intracerebral hemorrhaging (Vahedi et al., 2009). This link suggests arresten is present in the brain. Currently there is no evidence the collagen type IV alpha2 or alpha3, parent molecules of canstatin or tumstatin, respectively, are present in the brain.

Endostatin, the C-terminal fragment of collagen type XXVIII, was the first anti-angiogenic peptide used in clinical research performed by Judah Folkman in the late 1990's (1996-1997;92:65-82). Endostatin has been shown by Tian et al. to be rapidly upregulated following ischemic stroke with unknown consequence (Tian et al., 2007). The increase is maximal two hours post-stroke and gradually fades by 48 hours post-stroke, the last recorded time point. More recently, we have demonstrated by post-

stroke day seven in rats, endostatin is undetectable (Lee et al, submitted manuscript) suggesting that endostatin is created rapidly but transiently after stroke. Importantly, the release of endostatin following ischemia could have positive results. Endostatin has been documented to play a role in stabilizing cell-to-cell and cell-matrix adhesions. This could potentially play an important role in stabilizing the blood brain barrier (BBB) and decrease its permeability following ischemia (Durukan and Tatlisumak, 2007).

Perlecan Domain V: a cryptic regulator of endothelial angiogenesis

Perlecan's C-terminal 703 amino acids comprise DV, also known as endorepellin, which is further sub-divided into three laminin-like globular repeats separated by two epidermal growth factor (EGF) repeats (**Figure 1.3**) (Noonan et al., 1991), (Murdoch et al., 1992), (Kallunki and Tryggvason, 1992). Laminin-1 and EGF, two proteins that are involved with regulating cell growth, proliferation, and differentiation, contain structural homology with DV (Hamann, 1995), (Senger, 2002). Each EGF repeat contains 40 amino acids, with six conserved cysteines and conserved glycines for proper folding (Murdoch et al., 1992), (Kallunki and Tryggvason, 1992). DV has multiple binding partners, including endostatin, α-dystroglycan, progranulin and nidogen (Mongiat et al., 2003), (Bix and Iozzo, 2008), (Woodall et al., 2008). These proteins also regulate endothelial cell angiogenesis and in some cases wound healing (Bix and Iozzo, 2008).

Within the past eight years, extensive research on perlecan's DV fragment has characterized its effect on human umbilical vein endothelial cells (HUVECs) and solid tumor endothelial cells. DV has been demonstrated to inhibit angiogenesis in vitro by blocking HUVEC migration, tube formation, and in vivo by inhibiting blood formation in matrigel plug assays, CAM assays and decreasing tumor angiogenesis in rodents (Mongiat et al., 2003), (Bix et al., 2006). DV blocks angiogenesis in HUVECs by autocrine signaling. Specifically, DV binds to the I-domain (i.e., the ligand binding domain) of $\alpha_2\beta_1$ integrin, causing an increase in cAMP levels, protein kinase A (PKA) and focal adhesion kinase (FAK) activation, and ultimately disassembly of actin stress fibers (**Figure 1.4**). More recently, DV's mechanism of action for inhibiting angiogenesis has been further dissected (Nystrom et al., 2009). The work performed by Nystrom et al. demonstrates that DV causes an increase in tyrosine phosphatase SHP-1 activity, which leads to the dephosphorylation of growth factor receptor VEGFR2, the main receptor for vascular endothelial growth factor (VEGF).

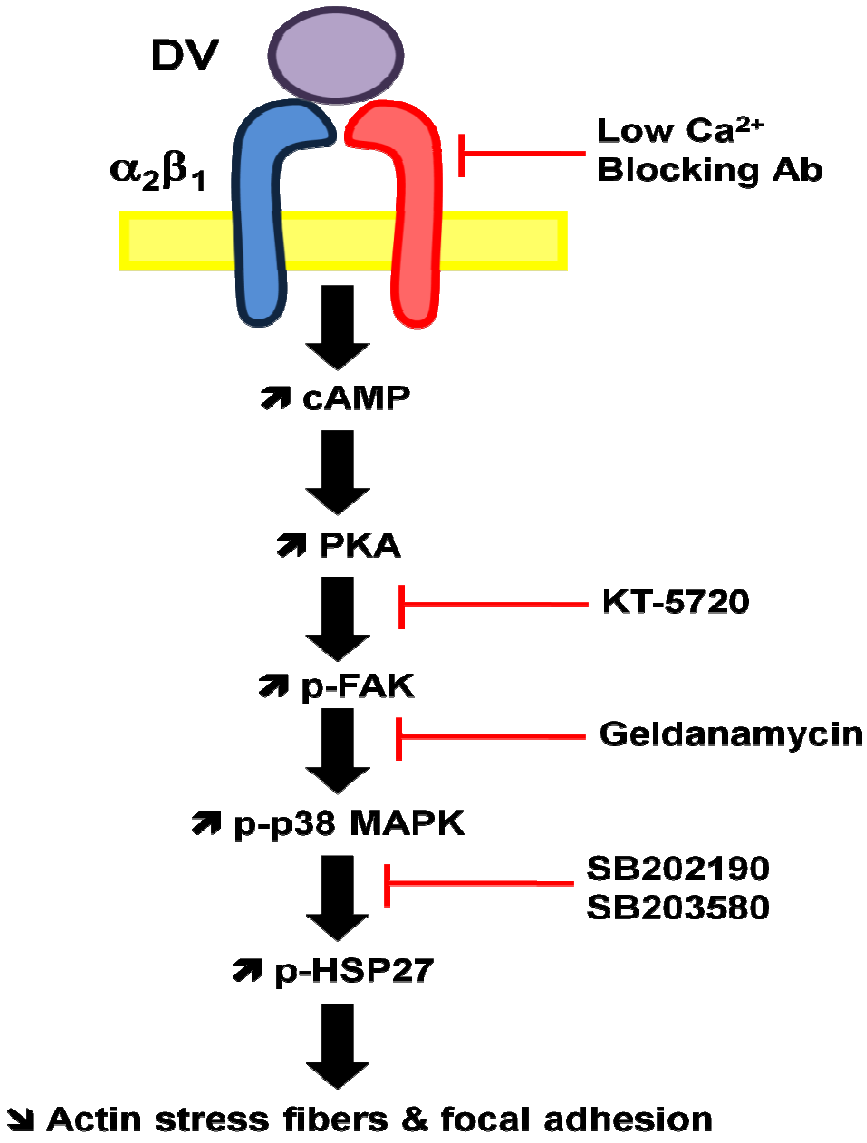

Figure 1.4 DV/ER mechanism for disassembly of actin stress fibers and focal adhesions on HUVEC. Model depicting the pathway by which DV interaction with the α2β1 integrin causes the activation of PKA, P-FAK, P-p38MAPK and P-Hsp27. Activation of this pathway leads to the disassembly of actin stress fibers and focal adhesions.

Domain V and integrins: a different function for brain repair and possible binding partners

One way in which cells interact with the ECM is via cell surface receptors known as integrins. Integrins are a major class of cell-surface receptors consisting of transmembrane, noncovalently linked αβ heterodimers (del Zoppo and Milner, 2006), (Milner and Campbell, 2002), (Mark and Davis, 2002). They are important for cell adhesion, contractility, movement, and growth by physically linking the ECM with the cell cytoskeleton (del Zoppo and Milner, 2006), (Shi and Sottile, 2008), (Wang and Milner, 2006b).With respect to angiogenesis, the interaction between the ECM and integrins helps regulate the migration and proliferation of endothelial cells by altering the presentation or localization of integrins on the cell surface. Endothelial cell migration is key for post-stroke repair, as the newly produced networks of endothelial cells become scaffolds directing the way for the newly migrating neurons in the ischemic penumbra and ultimately the site of injury within the ischemic core.

During migration, integrins assist endothelial cells by first anchoring them to the ECM thereby allowing the cell to polarize and then push off towards any present attractant. ECM ligands binding to integrins influence proliferation by activating intercellular cascades that are responsible for inhibiting or promoting proliferation.

Currently 8 β and 14 α integrin subunits have been identified (Hynes, 2008). Both subunits contain a hydrophobic transmembrane segment, a cytoplasmic domain containing 50 or less amino acids and an extracellular domain that is greater than 75 kDa for the β subunit and greater than 100 kDa for the α subunit (Hynes, 2008).

The first crystal structure of an integrin was the $\alpha_V\beta_3$, solved by Xiong et al. in 2001 (Xiong, 2001). Since then, it is believed that the integrin has different conformations: the bent conformation in which the integrin is resting and has low affinity for ligand, and the extended conformation, which is active and has high affinity for ligand. Activation of integrins was later referred to as the "switch blade model" (**Figure 1.5**).

Bent conformation

non-ligand-occupied, extended conformation with closed headpiece

ligand-occupied, extended conformation with open headpiece

Figure 1.5. Switchblade model for integrin activation. Electron microscopy image averages of αVβ3 conformational states. Integrins are believed to be in a resting, low affinity binding state for ligand. After a stimulus has taken place, the integrins are activated and are able to bind to ligand with high affinity. From Takagi et al 2002.

There are three classes of integrin α subunits: α^{IIb} (which includes α_5), α^M (which includes α_2) and the third subunit, α^4. These subunits contain a transmembrane domain and divalent cation repeats. Divalent cation repeats have been demonstrated to play a role in integrin activation. For example, the $\alpha_5\beta_1$ integrin is activated by Mg^{2+} and Mn^{2+}, while Ca^{2+} will inhibit activation (Mould, 1998). The α^M subunit is distinguished from the other α subunits because it contains an I-domain, a 200 amino acid insertion in its N-terminus, which binds to collagens (Tuckwell et al., 1995) (**Figure 1.6**).

There are three integrin receptors involved with angiogenesis in the brain that are re-expressed following ischemia and could be potential binding partners for DV: α5β1, α6β1 and αVβ3. As mentioned previously, developmental angiogenesis in the brain is mediated by α5β1, which is the receptor for fibronectin. Once the brain begins to mature, there is a switch in expression from the α5β1 integrin to the α6β1 integrin, the receptor for laminin, in order to maintain a quiescent environment (Milner and Campbell, 2002). Following an ischemic event, the brain goes back to a pro-angiogenic environment re-expressing fibronectin and the α5β1 integrin.

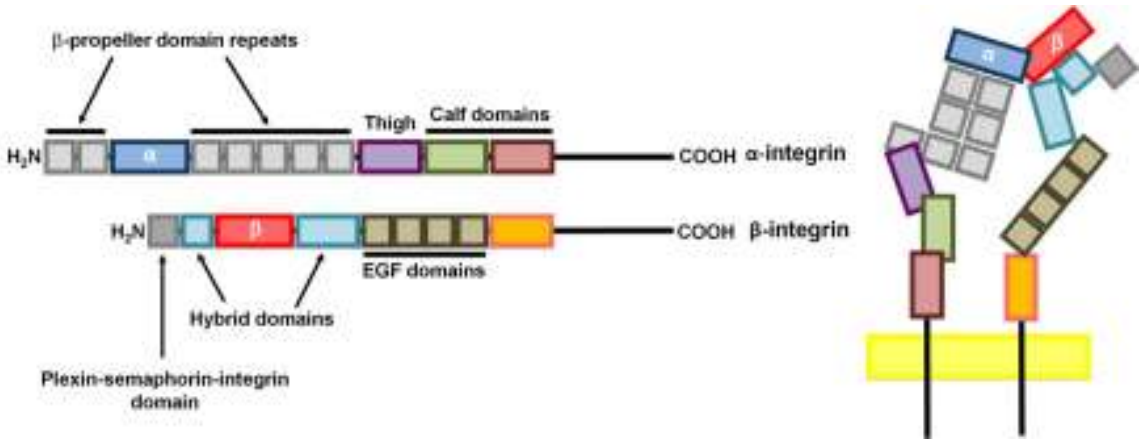

Figure 1.6. Domains of αβ integrin subunits. The α β-propeller domain repeats are shaded in grey. Thigh and calf domains are represented in purple, green and light red. The β plexin-semaphorin-integrin domain is represented in dark grey, hybrid domains are represented in light blue and EGF domains are shown in black.

Directly following ischemic stroke, β_1 expression in endothelial cells and astrocytes in the ischemic core is lost (del Zoppo and Milner, 2006), (Milner et al., 2008b). Transcription of β_1 is increased in tissues surrounding the ischemic core and the ischemic penumbra (del Zoppo and Milner, 2006). Integrin expression following ischemia can be recapitulated in vitro using the oxygen-glucose deprivation (OGD) model. OGD is a model used to recapitulate ischemia in vitro by limiting the availability of oxygen and glucose for cell survival. Endothelial cells that have undergone OGD increase the expression of $\alpha_5\beta_1$ (Milner et al., 2008b) confirming that this model is consistent with in vivo studies.

Hypoxia of the CNS in mice leads to high levels of $\alpha_5\beta_1$ integrin and fibronectin from 0 to 14 days post-hypoxia, with its strongest level of expression at day 4 (Milner et al., 2008a). The $\alpha_V\beta_3$ integrin is normally expressed on cerebral endothelial cells. Ischemia in mice and in non-human primates can stimulate early expression of $\alpha V\beta_3$ integrin within 1 hour (del Zoppo and Milner, 2006), (Lee, 2009). The $\alpha V\beta_3$ integrin is not expressed on quiescent endothelial cells, but it is induced during angiogenesis where it can promote their proliferation and migration (del Zoppo and Milner, 2006), (Lee, 2009).

DV has previously been shown to inhibit angiogenesis in HUVECs and solid tumor endothelial cells via interaction with the $\alpha_2\beta_1$ integrin. Results demonstrate that anti-angiogenic effects of DV in HUVECs do not occur in the absence of $\alpha_2\beta_1$ integrin (Woodall et al., 2008). In human and mouse microvessel brain endothelial cells there is no staining of α_2 integrin (Rakic), (Wang and Milner, 2006b), (McGeer et al., 1990) and α_2 null mice have no reported CNS abnormalities (Chen et al., 2002). Collectively, these results indicate the current receptor for DV is not present in the brain and suggests DV may play an opposite role in the brain because anti-angiogenic effects of DV do not occur in the absence of $\alpha_2\beta_1$ integrin. However, DV may still play a role in angiogenesis following ischemia in the brain because DV has also been shown to block endothelial cell adhesion to fibronectin (Mongiat et al., 2003), suggesting that DV could potentially bind to the $\alpha_5\beta_1$ integrin. As mentioned previously, following ischemia there is an integrin "switch" to promote a pro-angiogenic environment that includes the re-expression of the $\alpha_5\beta_1$ integrin. These data suggest a new receptor for DV that is present in the brain following ischemia.

Following MCAO in male baboons (*Papio Anubis/cynocephalus*), perlecan has been demonstrated to be the vascular matrix that is most sensitive component to proteolysis (Fukuda et al., 2004). Until now, no research has been performed investigating what fragments of perlecan are upregulated following ischemia. Research in our laboratory performed by Dr. Lee confirmed perlecan's DV fragment is significantly upregulated in the stroked hemisphere of mice and rats (**Figure1.7**). Compared to contralateral hemispheres on post-surgery days 1, 3, 5, and 7, and corresponding contralateral hemisphere DV levels, DV in the stroked hemisphere was elevated at post-stroke day 1 (*p=0.0001), followed by slight diminishment at post-stroke day 3 (**p=0.0007) and then further reduction to a plateau at post-stroke days 5 and 7 (#p=0.007, ##p=0.005). These results suggest that following ischemia, perlecan's DV fragment can be generated from full length perlecan and thereby increase the amount of free DV around the cerebral vasculature.

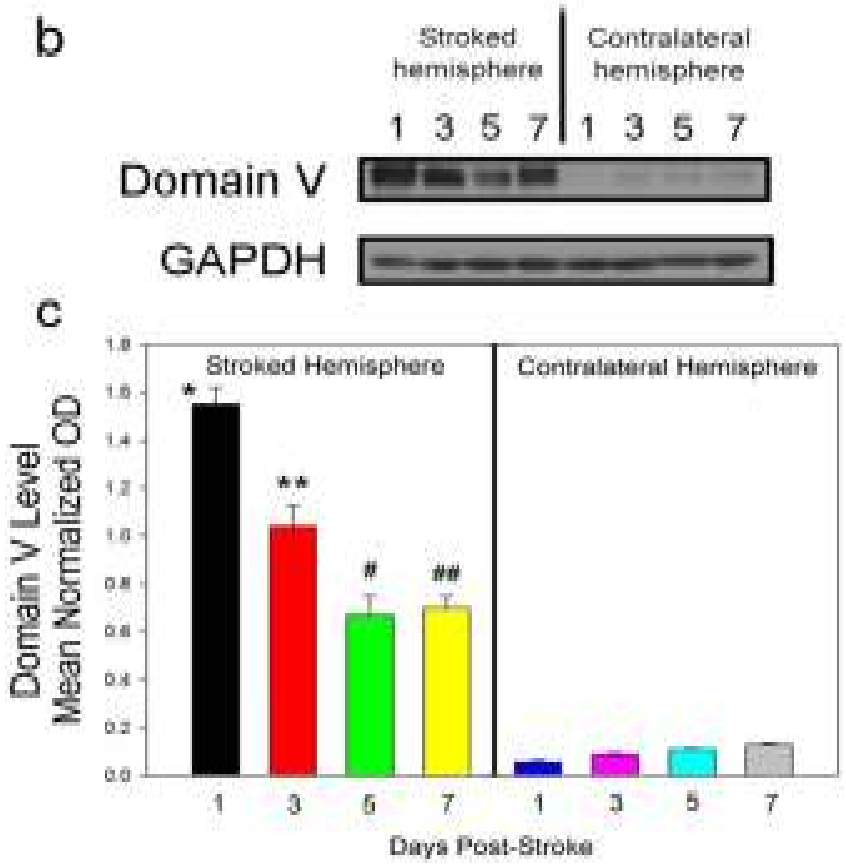

Figure 1.7. Perlecan Domain V (DV) is transiently upregulated after stroke. (b) representative anti-DV western blot analysis with GAPDH loading control of the brains of rats, performed on post-stroke days 1, 3, 5, 7 on separated stroked and contralateral (non-stroked) cerebral hemispheres (brain from same animal on each post-stroke day). (c) Optical density analysis of DV western blot band intensities, mean (± standard deviation) values as normalized to corresponding GAPDH optical densities from n=5 animals per each post-stroke date shown. DV was significantly elevated in the stroked cerebral hemisphere at all days measured (*p=0.0001, **p=0.0007, #p=0.007, ##p=0.005 as compared to corresponding contralateral hemisphere levels).

Vascular endothelial growth factor

As described earlier, angiogenesis requires a multitude of steps in order for proper blood vessel development to occur. Within the vascular basement membrane, several growth factors exist which help support the surrounding vasculature during angiogenesis. Vascular endothelial growth factor (VEGF) was first identified for its vascular permeability function. Later it was recognized to be one of the most potent endothelial cell specific growth factors during angiogenesis (Ferrara, 2004). VEGF was first purified and characterized as a permeability factor in 1983, where it was originally referred to as "tumor vascular permeability factor" (VPF). It wasn't until years later that a different group unknowingly isolated the same protein but characterized it as having endothelial cell specific capabilities, naming it vascular endothelial growth factor, (VEGF) (Senger, 1983) (Senger, 1990). It was not until Connolly et al. sequenced VPF that it was confirmed that VEGF and VPF are the same molecule (Connolly, 1989).

The VEGF molecule is a glycoprotein that forms a homodimer with a molecular weight of 46,000 daltons corresponding to the $VEGF_{165}$ isoform (Ferrara, 1989). The VEGF gene is approximately 14 kilobases in size and consists of seven introns and eight exons (Houck, 1991) (Tischer, 1991) located on chromosome 6p21.3 (Vincenti, 1996).

Splicing of the VEGF gene generates six isoforms correlating to polypeptides that are 121, 145, 165, 183, 189, and 206 amino acids long. The functionality of these isoforms is dependent upon where the splicing occurs. For example, isoforms 189 and 206 are cell associated and act as permeability factors because they contain heparin binding domains and are more basic in charge. VEGF 121 and 165 are both secreted, but 121 is weakly acidic and does not contain exons six and seven, which contain heparin binding domains, and it is therefore freely diffusible (Neufeld, 1996; Park, 1993) .

Each member of the VEGF family utilizes three receptor tyrosine kinases, VEGFR-1/Flt-1, VEGFR-2/Flk-1, and Flt-4 in order to induce their angiogenic signals (**Figure 1.8**). VEGFR-1 and VEGFR-2 are linked to angiogenic responses while VEGFR-3 is also linked to lymphangiogenesis. Under physiological conditions, VEGF promotes endothelial cell proliferation and migration. During development this is detrimental as the vasculature matures. Following development, postnatal angiogenesis only occurs conditionally, during wound healing and the ovulatory cycle.

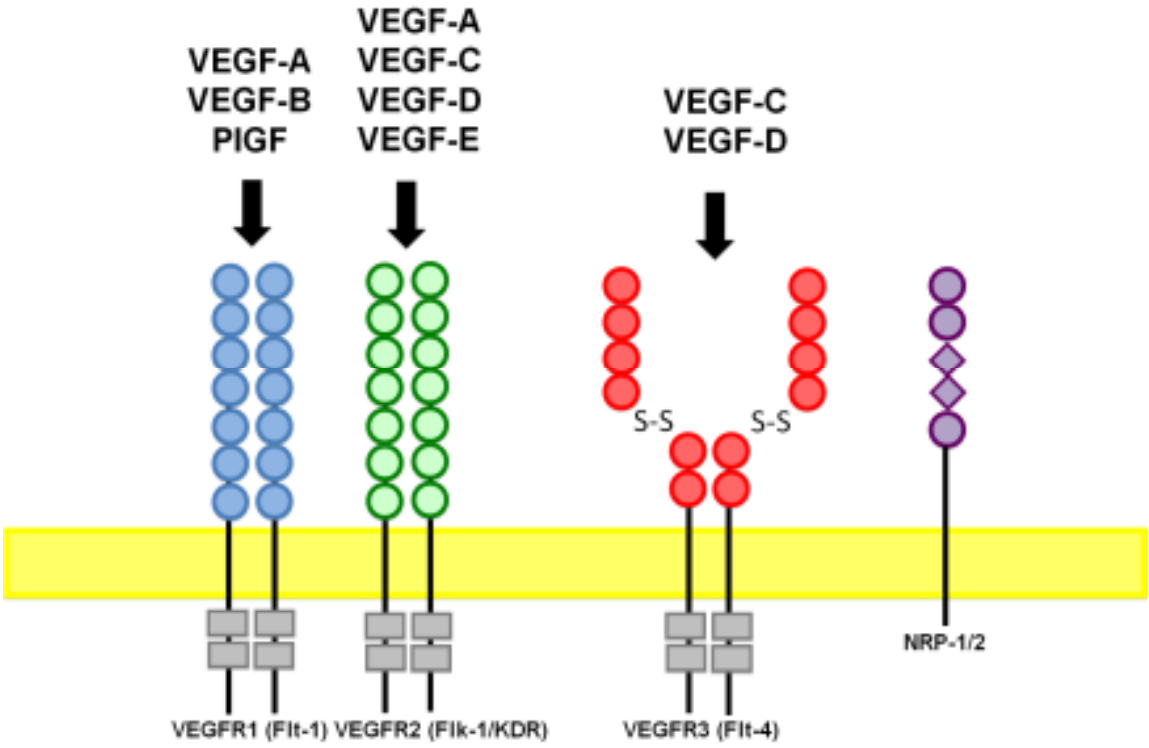

Figure 1.8. Schematic representation of VEGF receptors. VEGF-R1 binds PIGF, VEGF-B and VEGF-A. VEGF-R2 binds to VEGF-A, VEGF-C, VEGF-D, and VEGF-E. VEGF-R3 binds VEGF-C and VEGF-D.

Several growth factors have been isolated and characterized as having effects on angiogenesis. Of these, VEGF stood out via its secretion capabilities and in knock out studies where absence of VEGF proved to be embryonic lethal. Since then, VEGF activity has been identified in several angiogenic in vitro models such as endothelial cell invasion into collagen gels, proliferation, induction of capillary-like structures and endothelial cell sprouting (Zachary, 2001). Other studies demonstrated VEGF capabilities in vivo using the chick chorioallantoic membrane (CAM) assay and rabbit cornea assay (Zachary, 2001). These studies prove that VEGF is capable of strongly promoting angiogenesis in vivo as well.

Expression of VEGF is tightly regulated, yet several factors can influence initiation or inhibition of VEGF expression at the mRNA and protein levels. VEGF expression is up-regulated by activation of several receptors including tyrosine kinase, IGF and the FGF receptors (Tuder, 1995). Activation of these receptors can lead to the subsequent phosphorylation of extracellular-signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3-kinase) / AKT signal transduction pathways, which in turn up-regulate VEGF expression (Berra et al., 2000), (Berra, 2000).

p42/p44 MAP kinase cascade and hypoxia inducible factor 1-α (HIF-1α) are both up-stream regulators of VEGF expression. Under normoxic conditions, mitogen-activated protein kinases (MAPK) are activated through a series of three serine/threonine specific kinase enzymes, MAPKKK, MAPKK, and MAPK. Extracellular signal-regulated kinases (ERK) p44/p42 kinase isoforms are implicated in several cellular

functions including control of protein synthesis, cell growth and cell survival (Berra, 2000). Currently, there are two commercially available inhibitors selective for p44/p42 kinases, 2´-amino-3´-methoxyflavone (PD98059) and 1, 4-diamino-2, 3-dicyano-1, 4-bis [2-aminophenylthio] butadiene (U0126), both of which bind to and inhibit ERK1/2 function.

VEGF is also under tight regulation by oxygen homeostasis. Regulation of oxygen homeostasis involves hypoxia-inducible factor 1 (HIF-1). HIF-1 is a herterodimeric protein consisting of a constitutively expressed HIF-1β subunit and an oxygen regulated HIF1-α subunit. Under normoxic conditions, HIF-1α is hydroxylated on proline residues 402 and/or 564 by prolyl hydroxylase domain (PHD) proteins. Following prolyl hydroxylation, von Hippel-Lindau protein (VHL) binds to the hydroxylated HIF-1α facilitating ubiquitination of HIF-1α (Semenza, 2008). Under hypoxic conditions, HIF-1α is no longer hydroxylated which prevents it from being ubiquitinated by VHL. This allows HIF-1α to form a heterodimer with HIF-1β and subsequently bind to the hypoxia response element (HRE) located -975/-968 on the VEGF promoter (Forsythe et al., 1996). Interestingly enough, work performed by the Pouyssegur group demonstrated that HIF-1 is a target for MAPK phosphorylation, and such an event also allows the induction of VEGF expression under normoxic conditions (Berra, 2000) (**Figure 1.9**).

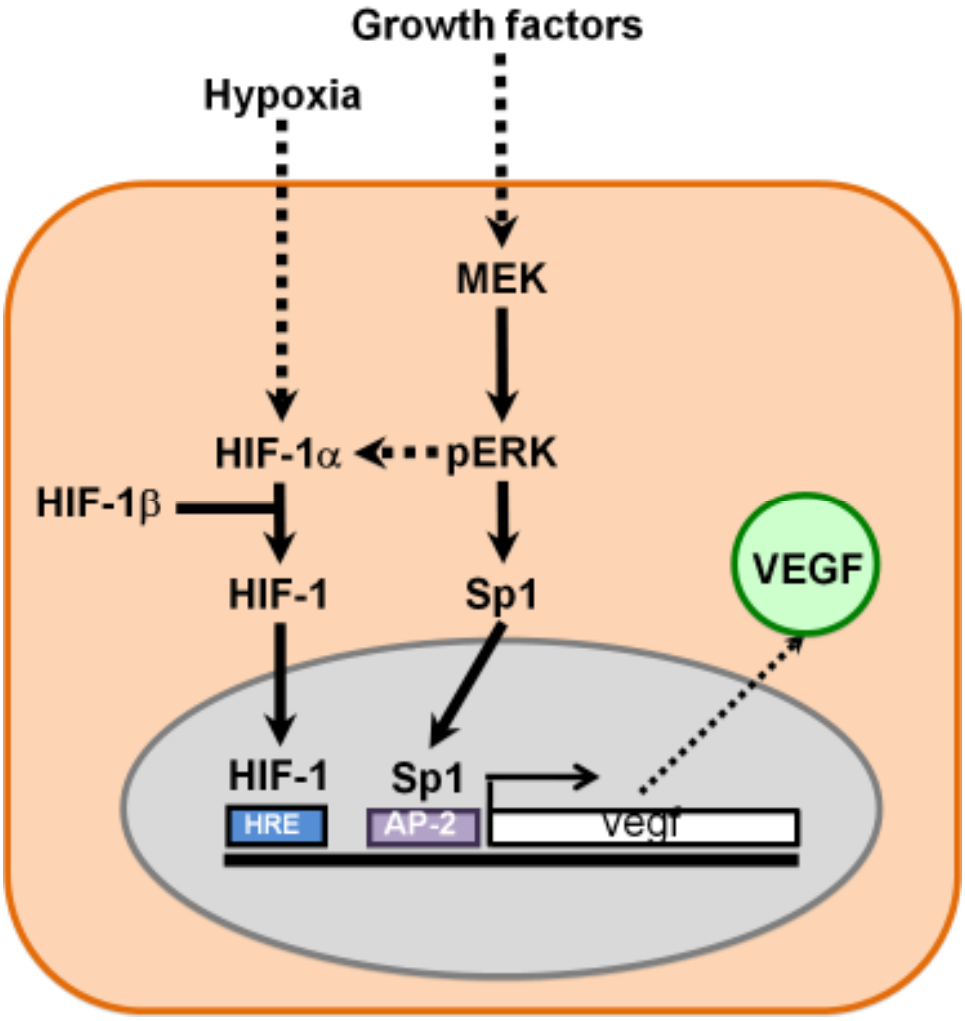

Figure 1.9. VEGF expression regulation. Schematic representing the influence of hypoxia and activation of the p42/p44 MAPK pathway by growth factors have on increasing VEGF expression. Stabilization of HIF-1α allows it to form a complex with HIF-1β where it binds to HRE located on the VEGF promoter and initiates transcription of VEGF.

Most research has focused on regulation of VEGF by its traditional receptors, VEGFR-1, VEGFR-2 and VEGFR-3, but little research has been conducted investigating nontraditional receptors such as integrins for VEGF regulation. As stated previously, under pathological conditions, the ECM is compromised and leads to proteolysis and the generation of ECM fragments. These ECM fragments are then able to influence the fate of neurovascular niche components by inducing the secretion of growth factors such as VEGF from cerebral endothelial cells. The complexity of interactions between integrins, VEGF and ECM has been investigated in retinal pigmented epithelial cells (Mousa et al., 1999). Mousa et al. demonstrated that blocking $\alpha_5\beta_1$ integrin leads to partial blockade of VEGF secretion induced by ECM fragments, suggesting VEGF secretion can be mediated by $\alpha_5\beta_1$ integrin and that ECM influences VEGF expression.

Research objectives

Objective 1

Angiogenesis along with neurogenesis is a key step in brain repair following ischemia. Studies following middle cerebral artery occlusion in this laboratory have revealed a significant increase in perlecan's DV fragment in brain lysate taken from the stroked hemisphere compared to brain lysate taken from contra-lateral hemisphere or sham surgery control brain tissue. This first confirmed that cleaved DV generation is increased following stroke and is potentially available to interact with the surrounding microvasculature. Because other previous results have demonstrated DV to be anti-angiogenic on human umbilical vein endothelial cells (Mongiat et al., 2003), the first

objective of this research was to investigate the in vitro angiogenic effects perlecan's DV fragment has on brain cerebral endothelial cells.

Objective 2

Previous reports investigating DV in vitro revealed DV interacts with the I-domain of the $\alpha_2\beta_1$ integrin (Bix et al., 2004). Furthermore, previous studies also demonstrate that the anti-angiogenic effects of DV in HUVEC do not occur in the absence of $\alpha_2\beta_1$ integrin (Woodall et al., 2008). In microvessel brain endothelial cells there is no staining of α_2 integrin (Rakic), (Wang and Milner, 2006b), (McGeer et al., 1990). Moreover, α_2 null mice have normal brain development and function (Chen et al., 2002). DV has also been shown to block endothelial cell adhesion to fibronectin (Mongiat et al., 2003) which suggests DV can bind to different receptors other than the previously reported $\alpha_2\beta_1$ integrin. The second objective of this research was to identify a new receptor present in microvessel brain endothelial cells required for DV's effect on in vitro angiogenesis.

Objective 3

Endothelial cells are known to modulate neurogenesis partly because of the secretion of soluble growth factors such as VEGF, NGF and BDNF. In the neurovascular niche, these diffusible growth factors help provide cross-talk between the closely associated brain endothelial cells and the neuronal precursor cells in a fashion that is consistent with the developmental association of neurogenesis and vasculogenesis (Wurmser et al., 2004). DV has previously been reported to activate tyrosine

phosphatase SHP-1 (Nystrom et al., 2009), which causes a widespread reduction of growth factor receptor phosphorylation and subsequent blockade of angiogenesis in vitro. The third objective of this research was to investigate the regulation of VEGF by DV in brain endothelial cells as a model for understanding the mechanism of DV mediated angiogenesis.

CHAPTER II

PERLECAN DV INDUCES A PRO-ANGIOGENIC EFFECT ON BRAIN MICROVASCULAR ENDOTHELIAL CELLS IN VITRO

Introduction

Among Americans, stroke occurs on average every 40 seconds and kills one person every four minutes. Currently, there is no therapeutic cure for stroke patients, but advances in the stroke therapy field have left researchers with a sense of optimism. Following ischemia, the affected brain area was once thought to be an irreversible site of injury, yet recent developments suggest this may not entirely be the case. Since Santiago Ramon's statement regarding adult neurogenesis, "everything may die, nothing may be regenerated," researchers have proven not only does neurogenesis take place following ischemia, but traumatic injuries such as ischemia can stimulate brain neurogenesis (Colucci-D' Amato, 2006). For complete and proper functional recovery following ischemia, neurogenesis must be coupled with angiogenesis. The induction of angiogenesis following ischemia has been proven beneficial, serving as a means of replenishing oxygen levels and nutrients to affected tissue but also to promote neurorepair processes such as neurogenesis and synaptogenesis.

Following development, angiogenesis no longer exists except during the female reproductive cycle. Under pathological conditions such as ischemia, angiogenesis commences in order to support processes such as wound healing. Given these two

separate scenarios, Hanahan and Folkman then postulated a “switch” governs the angiogenic process (Mahabeleshwar, 2008). When angiogenesis is required, pro-angiogenic proteins turn the switch on, and when angiogenesis is not required, anti-angiogenic proteins turn the switch off.

The extracellular matrix (ECM), which surrounds the neurovascular unit, contains multiple pro/anti-angiogenic proteins involved with angiogenic regulation. The ECM is critical for endothelial cell proliferation, migration, morphogenesis, survival and blood vessel stabilization (Davis and Senger, 2005). All of these stages are required for angiogenesis. Proteases that are upregulated following ischemia can initiate or “switch on” angiogenesis by degrading the ECM. Degradation of the ECM, once thought of as a negative process, can be beneficial under circumstances such as promoting angiogenesis. Breakdown of the ECM allows endothelial cells to migrate into the surrounding vascular space and proliferate into blood vessels. Breakdown of the ECM can cause the release of growth factors and bioactive fragments that were once sequestered in the ECM. These growth factors and bioactive fragments are now available to help promote endothelial cell migration, proliferation and stabilization during vascular remodeling.

ECM degradation is the primary step in initiating angiogenesis, but also causes the release of several proteolytic ECM fragments into the interstitial space surrounding the neurovascular unit. Research has lead to the discovery of ECM fragments such as endostatin, tumstatin and endorepellin. These fragments have been found to be

potentially beneficial for inhibiting angiogenesis during cancer (Bix and Iozzo, 2005). However, little research has been performed investigating these same ECM fragments and their role in regulating angiogenesis following ischemia in the brain. Accumulating research investigating the role of ECM components in the cerebral microvasculature following ischemia is setting the foundation for investigation of these ECM fragments following ischemia, (del Zoppo and Milner, 2006), (Takagi, 2002), (del Zoppo, 2008), (Milner et al., 2008a) opening up an entirely new and exciting field of research.

The heparan sulfate proteoglycan perlecan has been shown to be the most sensitive neurovascular ECM component following ischemia within the ischemic core. Studies have demonstrated a decrease in perlecan immunoreactivity as early as one hour and a continuous decrease up to two hours following MCAO (Fukuda et al., 2004). Perlecan has also been shown to harbor a C-terminal anti-angiogenic fragment, called endorepellin or DV. Previous research has investigated the regulatory mechanism by which DV inhibits angiogenesis in vitro and in vivo (Bix et al., 2004) (Bix et al., 2006). DV has been demonstrated to inhibit angiogenesis in vitro by blocking HUVEC migration, tube formation and proliferation and in vivo by blocking tumor angiogenesis and blood vessel formation in CAM and matrigel plug assays. DV's inhibitory effect on angiogenesis is due to interacting with the $\alpha_2\beta_1$ integrin (Woodall et al., 2008). Interestingly, results demonstrate that anti-angiogenic effects of DV in HUVEC do not occur in the absence of $\alpha_2\beta_1$ integrin (Woodall et al., 2008).

In microvessel brain endothelial cells there is no staining for the known DV receptor component α_2 integrin (Rakic), (Wang and Milner, 2006b), (McGeer et al., 1990). Moreover, α_2 null mice have no reported CNS abnormalities (Chen et al., 2002). Other data implies that there is an additional DV receptor, as DV can also block HUVEC adhesion to fibronectin (Mongiat et al., 2003). DV's role in post ischemic angiogenesis has currently not been elucidated, yet following ischemia there is an up-regulation of proteases such as cathepsin-L which causes proteolysis of perlecan and release of DV (Fukuda et al., 2004), (Cailhier et al., 2008). Until now, no research has been performed investigating what fragments of perlecan are upregulated following ischemia. As mentioned previously, research in our laboratory confirmed perlecan's DV fragment is significantly upregulated in the stroked hemisphere of mice and rats (**Figure1.7)**. These observations suggest a potentially new role and new receptor for DV following ischemia in the brain.

In this study, using three separate in vitro angiogenesis assays, I have identified DV as a pro-angiogenic stimulator in brain endothelial cells derived from mice. Our analysis demonstrates DV stimulates tube-like formation, proliferation and migration of brain endothelial cells suggesting DV can promote angiogenesis in the neurovascular environment following ischemia.

Materials and methods

Cloning and oligonucleotides

Genomic DNA was prepared by amplifying cDNA from HUVEC cDNA utilizing a GC-rich PCR system and dNTPack (Roche Applied Science, Indianapolis, IN). The human DV gene was amplified by PCR using forward and reverse oligonucleotides. Forward primers were designed with NHE1 restriction enzyme sites at the 5' end, and reverse primers were designed with XhoI restriction sites and His_6 tag at the 3' end. Following digestion, the gene was later ligated into pCepPu vector, kindly provided by Professor Maurizio Mongiat (Center for Cancer Research, Aviano Italy), between the Nhe1 and XhoI sites. DH5α cells were transformed with pCepPuDV, plated on LB+ amp plates and incubated at 37°C overnight. Colonies were selected and grown in 500mL of LB+ amp at 37°C overnight. pCepPuDV DNA was purified using Qiagen plasmid maxi prep kit and the sequence was confirmed (Gene Technologies Laboratory Texas A&M University, College Station). A cell line containing pCepPuDV was constructed by transfecting pCepPuDV plasmid into 293 EBNA cells, kindly provided by Maurizio Mongiat, using Lipofectamine™ 2000 as per the supplier's instructions (Invitrogen). Forward and reverse primers used are listed as follows:

NHEI DV Forward 5'-AGG CTA GCG ATC AAG ATC ACC TTC CGG C-3'

XHOI HIS DV REVERSE 5'-AGC TCG AGC ATG ATG ATG ATG ATG ATG CGA GG-3'

Domain V protein expression and purification

Transfected 293 EBNA cells containing pCepPuDV plasmid were transferred into a CelLine adhere 1000 bioreactor (Argos Technologies, Elgin, IL) and grown for seven days in complete media containing 10% FBS, 1x Antibiotic/Antimycotic, 1% G418 Sulfate, and 0.05ug Puromycin. After seven days the complete media was removed, the cells were washed five times with CD293 media containing 4mM L-glutamine, 1x Antibiotic/Antimycotic, 1% G418 Sulfate, and 0.05ug Puromycin to remove any serum, and then fresh CD293 media was added to the cells. After an additional seven days, the cells were harvested by centrifugation at 5000 X g for 10 minutes at 37C. The media was collected and filtered using a 0.45µM syringe filter. After filtration, protease inhibitors (Roche) were added to the media at one pill per 50 mL of media. 0.02% Triton X was added to the media and the pH was adjusted to 8.0. Two mL of Ni NTA Agarose Beads (Qiagen) were added to a Kontes FlexColumn (Fisher Scientific) and allowed to settle. Once the beads were settled, wash buffer A (125mM Na3PO4, 40mM NaCl, 20mM Imidazole pH 8.0) was added to the column in order to equilibrate the beads and provide proper packing of the beads. Once the column was ready, prepared media containing DV-His tagged protein was added to the column and allowed to flow through by gravity at one drop per 10 seconds. After the media had run through, the column was washed with 10mL of wash buffer A and collected. DV-His was eluted off the column by adding 10mL of elution buffer (125mM Na3PO4, 40mM NaCl, 350mM Imidazole, 10% glycerol pH 6.0) to the column. One mL fractions of eluted protein were

collected and stored at 4°C. Protein quantification was performed using a BCA kit (VWR) and fractions containing protein were pooled together and then dialyzed against 1X PBS. Following dialysis, if necessary, the DV-His protein was concentrated to the desired concentration by incubating DV-His in polyethylene glycol (VWR). The resulting protein preparation was methanol precipitated, run on a SDS gel and stained with Brilliant Blue G-colloidal stain (Calbiochem). Purified DV-His protein exhibited a single band at 85kDa. Purified protein was aliquoted and stored at -80C.

Cell culture

Human brain micro-vascular endothelial cells were purchased from Lonza (Basel, Switzerland) and Cell Systems (Kirkland, WA), and passaged as per the supplier's instructions. Mouse and rat brain micro vascular endothelial cells were kindly provided by Jane Welsh, Texas A&M University, and passaged as previously described (Sapatino et al.). Primary mouse dermal endothelial cells were purchased from Celprogen, Inc. (San Pedro, CA) and maintained initially as recommended by the manufacturer. After the second passage, cells were passaged to flasks pre-coated with 1mg/ml gelatin and fed with culture medium. Briefly, 500 ml M199 was supplemented with 15% FBS (Invitrogen), 200mg bovine hypothalamic extract, 50 mg heparin (Sigma-Aldrich), 5 ml antibiotics (Invitrogen) and 0.5ml gentamycin (Invitrogen). In all endothelial cells, the presence of endothelial cell markers von Willebrand Factor and VEGF receptor was confirmed via immunohistochemistry and western blot.

Proliferation assays

Mouse brain endothelial cells were seeded in 96-well plates at a concentration of 4 x 10^3 cells per well in IMDM media (Invitrogen) supplemented with 10% FBS (Invitrogen) 1x Antibiotic/Antimycotic and incubated overnight at 37C and 5% CO_2. Following overnight incubation, complete media was aspirated off and cells that underwent treatment were washed with plain IMDM media to remove residual all serum. Purified DV and 1% media was added back to each well to a final volume of 100µL and incubated for an additional 24 hours at 37C and 5% CO_2. After 24 hours, 20µL of MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), was added to each well and incubated for an additional two hours at 37C and 5% CO_2. Cell proliferation was measured following the two hour incubation by reading the 96-well plate at 490nm using plate reader. Proliferation was normalized to untreated 1% condition for each experiment.

$\alpha_2$integrin expression

Brain microvascular cells were transfected with a plasmid vector (pEGFP-N2, Clontech) containing a sequence encoding the α2-subunit integrin with a C-terminal RFP fusion protein (Texas A&M University Biomedical Engineering). Empty vector was used as a control. Cells were allowed to recover during 24hours in IMDM medium containing no antibiotics. Transfection efficiency was appreciated after 24hours using an inverted fluorescent microscope.

Migration assay

Cell migration was assessed with a modified Boyden Chamber (NeuroProbe, Gaithersburg, MD) following the instructions of the manufacturer. In the top chamber, brain endothelial cells were added to each well at a concentration of 50 x 10^3 cells per well. In the bottom chamber, VEGF (20ng/mL) or purified DV was added to each well. A polycarbonate membrane (PVD-free 8 micron pore) was coated with type I collagen and fitted in between the top and bottom chamber. After the chamber was set up, it was placed at 37C and 5% CO_2 and incubated for 6 to 8 hours. Following incubation, the polycarbonate membrane was scraped on the apex surface to remove any cells that did not actively migrate through the 8 micron pores in the membrane. Next the membrane was incubated in 0.1% crystal violet for one hour, rinsed 3 times with 1xPBS and cell number was counted using a microscope.

Capillary tube-like formation assays

Twenty four-well plates were incubated at -20^{o}C overnight along with 200µL pipette tips. Matrigel (VWR) was thawed on ice and approximately 50µL was aliquoted to each pre-chilled well in the 24-well plate. Even coating of the matrigel was accomplished using a pre-chilled pasture pipette. During cell collection, the matrigel coated plate was placed at 37C and 5% CO_2. Brain endothelial cells were seeded to each well at a concentration of approximately 50 x 10^3 cells per well in IMDM supplemented with 1% FBS to a final volume of 350µL. Purified DV was added to each well at a desired concentration. Experiments were run for 12 to 18 hours at 37C and 5%

Co_2. Following incubation, cells were fixed with 4% paraformaldehyde in 1x PBS. Tube formation was imaged and quantified as tube pixels/high power field, 10 areas per condition using Adobe Photoshop, CS.

Immunofluorescence of Actin Stress Fibers

Brain endothelial cells plated on type I collagen were treated with DV for 10 minutes followed by fixation with 4% paraformaldehyde (VWR). Immunocytochemistry was performed to stain for vinculin (Sigma-Aldrich) (a focal adhesion component and marker) and stained for F-actin with rhodamine conjugated phalloidin (VWR). Following staining, pictures were captured using a Zeiss spinning disk confocal microscope and Retiga EXi Fast 1394 CCD Camera.

Results and discussion

Domain V increases angiogenesis in vitro

The induction of angiogenesis following cerebral ischemia has been proven to be beneficial by replenishing oxygen levels and nutrients to affected tissue and also in promoting neurorepair processes such as neurogenesis and synaptogenesis. Endothelial cells migrate, proliferate and form new capillaries during angiogenesis in order to restore blood supply and nutrients and to help promote neurogenesis in the ischemic environment following stroke. Within the ischemic environment, the angiogenic process is greatly influenced by bioactive fragments and growth factors that are released from the ECM following proteolysis. C-terminal bioactive fragments derived from the ECM are currently being exploited as anti-angiogenic therapies for

diseases such as cancer (Rodrigues, 2010) (Bix et al., 2006) (Colorado et al., 2000). These studies are currently proving ECM fragments can be used as a means for regulating angiogenesis in diseased states. However, investigations of these fragments promoting angiogenesis in cases such as stroke are sparse, leaving an entirely new field of research open for investigation. Therefore, I sought to investigate one proven anti-angiogenic bioactive fragment, Perlecan's DV fragment, and monitor its effect on brain micro vascular endothelial cells migration, proliferation and tube morphogenesis.

Because DV's previously reported receptor for its anti-angiogenic effect is not present in brain micro-vascular endothelial cells, I expected to observe a different effect induced by DV on in vitro angiogenesis assays. In order to investigate DV's effect on brain micro vascular angiogenesis in vitro, I used the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay, modified Boyden Chamber and matrigel assays to monitor proliferation, migration and capillary tube-like morphogenesis, respectively.

Proliferation is one of the first steps endothelial cells undergo once active angiogenesis is initiated. Studies evaluating cell cycle proliferation have incorporated several techniques such as BrdU or ^{3}H-Thymidine incorporation or non radioactive assays such as MTS. MTS is reduced to formazan by dehydrogenase enzymes produced by metabolically active cells (**Figure 2.1**). An increase in the number of cells correlates to an overall increase in activity of mitochondrial dehydrogenases in the sample.

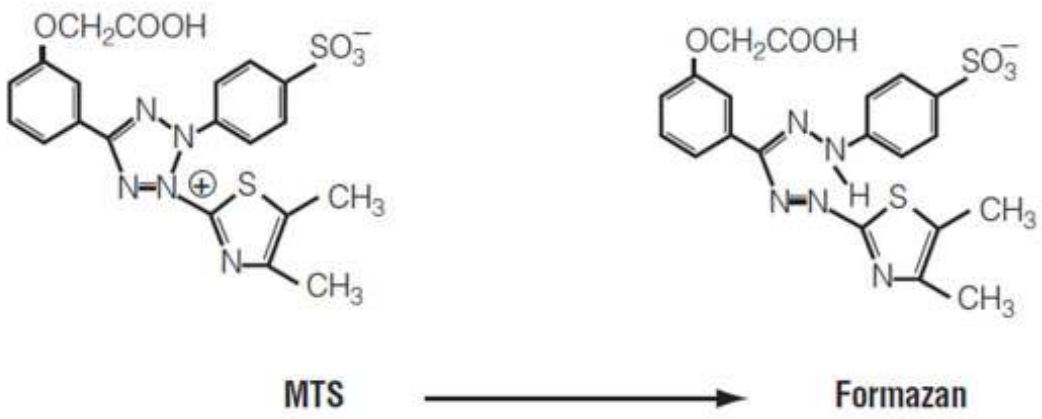

Figure 2.1. Structure of MTS and its soluble product formazan. Dehydrogenase enzymes that are present in metabolically active cells convert MTS to formazan. The amount of Formazan is directly proportional to the amount of proliferating cells and can be measured by reading its absorbance at 490nm. Modified from Promega, 2010.

The amount of soluble formazan detected at 490nm is directly proportional to the number of living cells in culture. Brain endothelial cells grown in IMDM supplemented with 1% FBS media were used as the baseline control population. To control for DV's previously reported anti-angiogenic effects, mouse dermal endothelial cells were also treated under the same conditions. Dermal endothelial cells showed a significant inhibition of proliferation because of the presence of soluble DV. DV was able to inhibit dermal endothelial cell proliferation by 40% as compared to control cells grown in DMEM supplemented with 1% FBS (**Figure 2.2**). These results are consistent with the notion DV induces an anti-angiogenic response to dermal endothelial cells. Interestingly, quantification of brain endothelial cells treated with soluble DV had the opposite effect of dermal endothelial cells. DV significantly enhanced brain endothelial cell proliferation 40% when normalized to control brain endothelial cell proliferation (**Figure 2.2**). Because DV interacts with the α2 integrin to cause an anti-angiogenic effect on HUVEC and dermal endothelial cells (Bix et al., 2006) and brain endothelial cells do not express this integrin (Woodall et al., 2008), I next transfected brain endothelial cells with α2 integrin plasmid. Our hypothesis was if the α2 integrin is expressed in brain endothelial cells, an inhibitory effect induced by DV would be observed. Contrary to DV's pro-proliferative effect on WT brain endothelial cells, DV inhibited proliferation of brain endothelial cells expressing $\alpha_2\beta_1$ integrin. (**Figure 2.2**) This suggests that the absence of $\alpha_2\beta_1$ integrin from brain microvascular endothelial cells is essential to DV's pro-angiogenic effects in the brain.

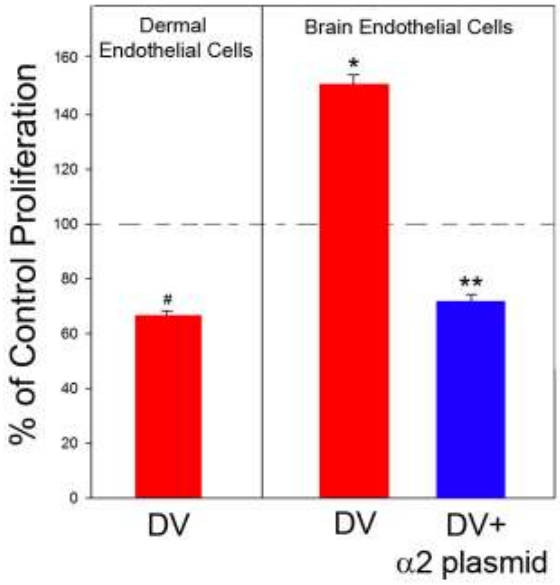

Figure 2.2. DV increases brain endothelial cell proliferation. Quantification of proliferation of dermal and brain endothelial cells ± the addition of the α_2 integrin plasmid following 48 hours ± DV in IMDM supplemented with 1% FBS media as measured via MTS assay. Values shown (n=15, mean ± standard deviation normalized to control proliferation arbitrarily set to 100%) demonstrate significant inhibition of dermal endothelial cell proliferation (* p=0.00005) and brain endothelial cells expressing α_2 integrin (**p=0.00009) and significant enhancement of brain endothelial cell proliferation (# p=0.002) after treatment with DV.

Endothelial cell proliferation and migration seem to occur simultaneously. Newly formed endothelial cells respond to stimuli such as VEGF and migrate towards these stimuli. These newly migrated endothelial cells will later form contacts with other endothelial cells and mature into newly formed capillaries. As stated previously, the ECM is constantly being degraded during angiogenesis in order to make way for newly migrating endothelial cells and their capillary networks. A byproduct of this process is the generation of ECM bioactive fragments that are now available to act upon local endothelial cells. DV has previously been shown to inhibit endothelial cell migration (Mongiat et al., 2003). I hypothesized DV would promote brain endothelial cell migration because of our finding DV promotes proliferation of brain endothelial cells rather than inhibit it, because of the absence of α_2 integrin. In order to asses this hypothesis, mouse brain and dermal endothelial cell migration was tested in the presence and absence of soluble DV using a modified Boyden chamber migration assay model. DV stimulated brain endothelial cell migration towards VEGF, with a 450% increase in migration compared to controls (**Figure 2.3**). Consistent with previous results (Mongiat et al., 2003), DV significantly inhibited dermal endothelial cell migration. These results suggest DV can act as a stimulus for migrating brain endothelial cells.

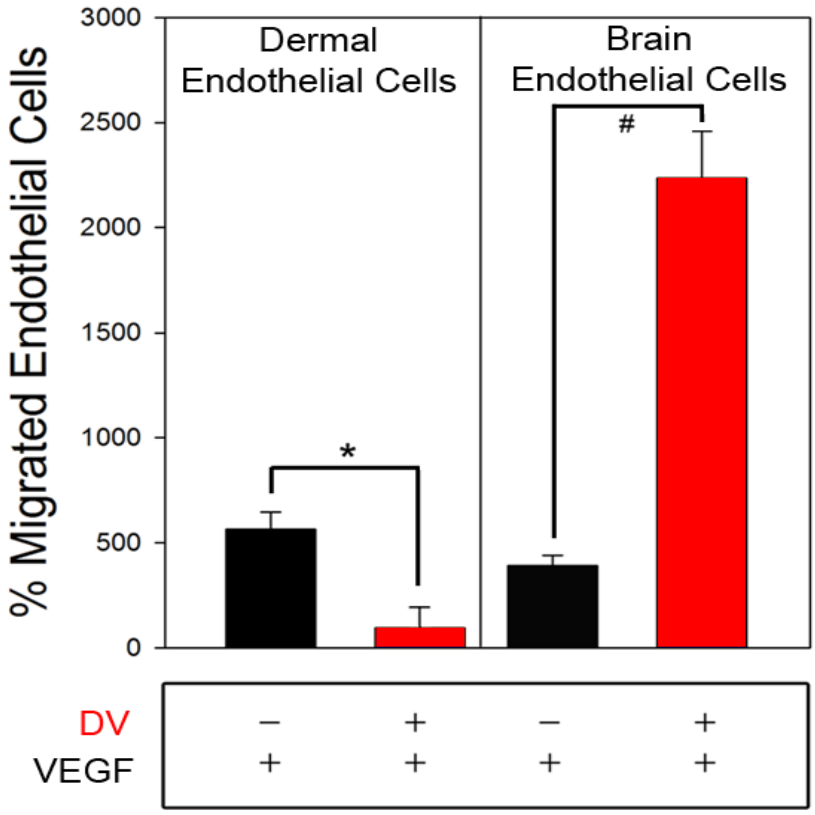

Figure 2.3. DV increases brain endothelial cell migration. Quantification of migration of dermal and brain endothelial cells towards VEGF (20 ng/ml) in a modified Boyden chamber migration assay ± direct exposure to DV (mean normalized values for n=15 ± standard deviation plotted), as normalized to random migration across the membrane in the absence of VEGF. DV significantly inhibited dermal endothelial cell migration (* p=0.0008 as compared to VEGF alone) but significantly enhanced brain endothelial cell migration (n=15, # indicates significance, p=0.000001 for DV).

To investigate DV's effect on brain endothelial cell tube-like morphogenesis, I stimulated brain endothelial cells with DV and plated them on matrigel. Matrigel is solubilized basement membrane isolated from Engelbreth-Holm-Swarm mouse sarcoma cells and composed of laminin, collagen type IV, heparan sulfate proteoglycans and nidogen. The composition of matrigel allows researchers to investigate tube-like formation in the presence or absence of angiogenic stimulators or inhibitors.

Previously, DV has been shown to inhibit HUVEC tube-like formation (Mongiat et al., 2003). Because of our results showing DV has opposite effects on brain endothelial cells than on HUEVECs, I hypothesized DV would increase tube-like formation of brain endothelial cells on matrigel. Micrographs (**Figure 2.4a)** show brain endothelial cells form tube-like structures in the presence of DV. I observed capillary tube-like formation within three hours of DV treatment where as control cells did not show visible capillary tube-like formation until six hours after being plated on matrigel. Dermal endothelial cells were also plated in the presence or absence of DV and showed an inhibition of capillary tube-like formation in the presence of DV consistent with that previously reported for HUEVECs (data not shown). These results suggest DV promotes tube like morphogenesis of brain endothelial cells.

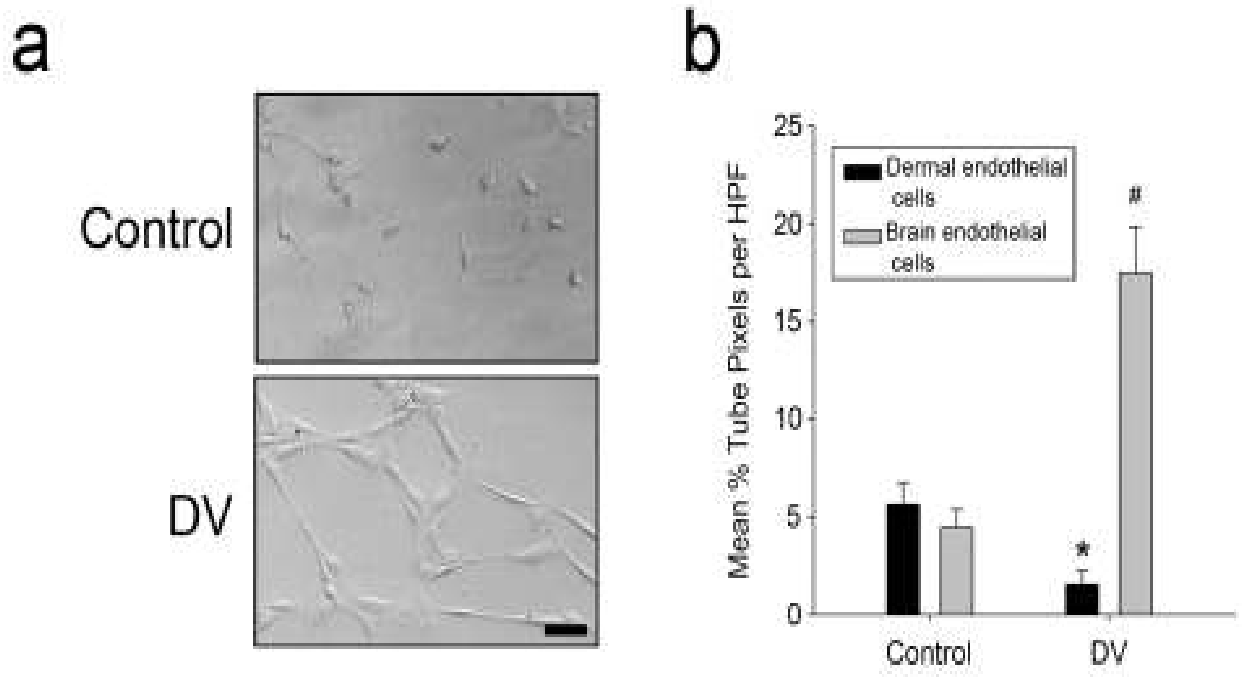

Figure 2.4. DV stimulates brain endothelial cell capillary tube-like formation. a) Micrographs of brain endothelial cell tube-like formation production for control cells or cells treated with DV. Bar in lower panel indicates 50 um. b) quantification of Matrigel capillary tube assays for mouse dermal and brain endothelial cells demonstrating significant enhancement of brain endothelial cell tube formation and inhibition of dermal endothelial cell tube formation (n=15, #p=0.0009, n=15, *p=0.001, as compared to corresponding control, HPF=high power field, error bars=standard deviation). Tube pixels per high power field for both in the presence or absence of DV were measured and quantified.

Finally, as DV has been shown to cause a collapse of the actin cytoskeleton as part of its anti-angiogenic mechanism (Bix et al., 2004); I next investigated if DV had any effect on the actin cytoskeleton of brain endothelial cells. brain endothelial cells seeded in eight well chamber slides were treated with DV for 10 minutes in IMDM media supplements with 1% FBS. Following fixation cells were stained in green for vinculin, a protein involved with linking integrins to the actin cytoskeleton and in red for filamentous actin (F-actin) the protein responsible for cell spreading and motility.

As shown in **Figure 2.5**, brain endothelial cells maintain prominent actin stress fibers when treated with DV. This result is consistent with brain endothelial cells that were not treated with DV. There is no collapse of the actin cytoskeleton which was previously reported in dermal endothelial cells (Bix et al., 2004). This result suggests that DV does not negatively affect the actin cytoskeleton of brain endothelial cells. Previous results (**Fig 2.3**) demonstrated DV has a positive effect on cell motility.

Because endothelial cell motility involves constant collapse and reformation and polarization of actin it could be suggested that the same phenomenon should be observed in the staining of brain endothelial cells. Yet, the cells observed in our migration model and the cells stained for f-actin are experiencing different conditions. The endothelial cells are being challenged to migrate where as in our f-actin staining experiments, the endothelial cells are in a subconfluent mono layer, and are not stimulated to migrate. Finally, the DV-induced collapse of the actin cytoskeleton previously observed in HUVEC (Bix et al., 2004) was also assayed in non-motile brain endothelial cells, further emphasizing the difference in DV function within and without the central nervous system. Collectively, these results demonstrate that DV surprisingly has pro-angiogenic effects on brain endothelial cells. These results were directly compared to those on mouse dermal endothelial cells where DV has been previously shown to inhibit proliferation, migration and tube formation (Bix et al., 2004).

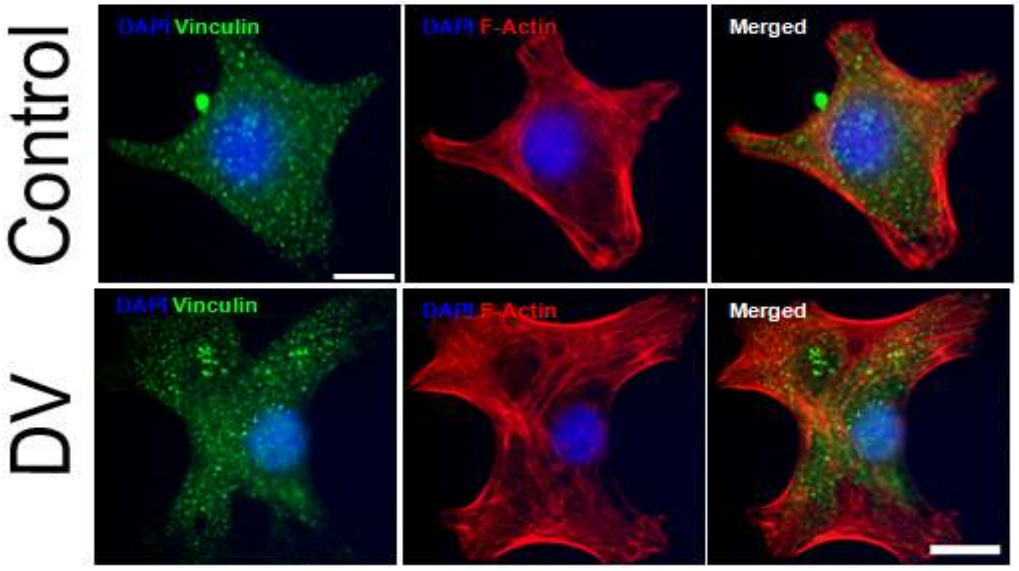

Figure 2.5. DV does not cause brain endothelial cell actin collapse. brain endothelial cells plated on type I collagen were treated with DV for 10 minutes followed by fixation with 4% paraformaldehyde. Immunocytochemistry was performed to stain for vinculin (a focal adhesion component and marker) and stained for F-actin with rhodamine conjugated phalloidin. Unlike in non-brain endothelial cells (Bix et al., 2004), prominent actin stress fibers were unchanged despite the presence of DV. Images are representative. Bar is 2 µm.

DV was capable of stimulating three separate assays involved with measuring angiogenesis in vitro: proliferation, migration and capillary tube-like morphogenesis. DV also had no effect on f-actin stabilization in non-migrating cells. These results also indicate that DV's effects on brain endothelial cells are distinct and different from those observed on non-brain endothelial cells. All three aspects of angiogenesis I investigated in vitro follow separate mechanisms involving the regulation of different signaling pathways. These data suggest DV could modulate the onset of angiogenesis by increasing the proliferation and migration of endothelial cells and also during the maturation of tube morphogenesis.

Figure 2.2 demonstrates DV is a positive regulator of cell cycle for brain endothelial cells by increasing their proliferation. The technique used to monitor their proliferation is the quantitative, well known and validated proliferative assay, MTS assay, which monitors proliferation by assessing the metabolic activity of the cells being treated. The amount of soluble formazan detected at 490nm is directly proportional to the number of living cells in culture. However, potential pitfalls to keep in mind while using this experiment are different conditions or chemical treatments can give different results that can increase or decrease the metabolic activity and the use of tetrazolium salts are generally cytotoxic. The variations arise under circumstances by which these chemicals or treatments change the activity of succinate dehydrogenase, the main enzyme involved with reducing MTS to formazan (Wang, 2010). My results indicate that brain endothelial cells treated with DV have significantly more soluble formazan than

brain endothelial cells not treated with DV. These results suggest DV increases the metabolic activity of brain endothelial cells and therefore implies DV increases brain endothelial cell proliferation. DV was still able to inhibit the proliferation of dermal endothelial cells suggesting a difference between the two cell lines used. As mentioned previously, brain endothelial cells do not have the α2 integrin, therefore when brain endothelial cells were made to express the α_2 integrin, proliferation was able to be blocked, indicating DV has a higher affinity for this integrin and its presence will dictate what kind of an angiogenic effect DV may have. Alternative assays to confirm DV is a pro-proliferative agent would be BrdU incorporation or ^{3}H-Thymidine incorporation.

My results also beg the question as to how DV is promoting an increase in proliferation of brain endothelial cells. One way to investigate how DV is affecting cell cycle is monitoring the activation of cyclin-dependent kinase enzymes (Cdk) by cyclins. When the concentration of cyclins increases, they form complexes with Cdk and in turn promote the phosphorylation of substrates responsible for cell cycle control. Currently four cyclins can be monitored to investigate cell cycle, Cyclin D for G1-phase, Cyclin E for S-phase, Cyclin A for G2-phase and Cyclin B for Mitoses. Therefore, in order to gain further insight into the mechanism DV stimulates cell division, experiments utilizing flow cytometry and staining for the different cyclins (D, E, A and B) involved with regulating cell cycle, could be conducted.

DV's ability to enhance brain endothelial cell migration towards VEGF in the modified Boyden chamber assay suggests DV is a chemotaxis agent. Consistent with previous results reported, DV inhibited dermal endothelial cell migration to VEGF. Other experiments performed using DV as the chemoattractant (data not shown) alone suggest DV stimulates migration by directly acting upon the cells. The stimulation would suggest two plausible mechanisms: DV is acting as a chemoattractant and directly promoting the endothelial cells to migrate, or VEGF is the chemoattractant but is potentiated by DV. In the latter case, DV could be up regulating VEGF receptors involved with migration and making the endothelial cells more sensitive and therefore more responsive to the stimulus VEGF provides.

Once endothelial cells have arrived at their final destination, the endothelial cells organize to form new capillary networks. This process requires the newly formed endothelial cells to align, connect to one another and morph into hollow lumens. The ECM is intimately involved during capillary morphogenesis by providing guidance cues or controlling intercellular signaling by interacting with cell surface receptors such as integrins (Davis, 2005). **Figure 2.4** demonstrates how DV induces rather than inhibits tube-like formation of brain endothelial cells. This observation was not only a surprise, but also interesting because of the increase in speed that the cells made tube-like structures compared to cells not treated with DV. Other matrix components such as collagen and fibronectin have been shown to promote endothelial cell formation of pre-capillary cords (Davis, 2005); antagonists to their receptors have blocked tumor

angiogenesis in vivo (Senger, 2002). It has already been demonstrated that DV could act as an antagonist to collagen's receptor inhibiting tube like formation in HUVECs (Bix et al., 2004). Our observation of an increase in tube formation rather than inhibition, suggests DV is activating an integrin responsible for tube morphogenesis, which would then potentially stimulate intercellular signaling cascades that are also involved with tube morphogenesis.

These results demonstrate DV has a completely opposite effect on HUVEC and brain endothelial cells. DV increased brain endothelial migration, tube formation and proliferation along with maintaining prominent actin stress fibers. These results may be because of differences in respective microenvironments, differences in expressed receptors, such as the presence or absence of $\alpha_2\beta_1$ integrin, or differences in signal transduction components. Indeed, angiogenic differences between brain and nonbrain endothelial cells have been reported. For example, Wnt/β-catenin signaling is required for CNS, but not non-CNS angiogenesis (Daneman et al., 2009). Another example is platelet-derived sphingosine-1-phosphate (S1P). Normally S1P is pro-angiogenic yet is anti-angiogenic in brain endothelial cells because of their lack of MT1-MMP expression (Pilorget et al., 2005). Additionally, the type XVIII collagen-derived inhibitor of angiogenesis, Endostatin, promotes angiogenesis in immature endothelial cells derived from differentiated embryonic stem cells (Schmidt et al., 2004).

CHAPTER III

THE $\alpha_5\beta_1$ INTEGRIN IS REQUIRED FOR PERLECAN DV'S PRO-ANGIOGENIC EFFECTS ON BRAIN MICROVASCULAR ENDOTHELIAL CELLS IN VITRO

Introduction

Cell surface receptors greatly influence cellular processes such as shape, mobility, and cycle. Mechanistically, a ligand attaches to the binding site of the receptor and causes an "outside-in" signaling event that induces a cellular response involved with shape, mobility, proliferation, etcetera. Integrins are heterodimeric transmembrane receptors involved with cell-to-cell and cell-to-ECM interactions. Integrins have the ability to control cell fate by numerous methods such as linking the matrix with the cell cytoskeleton, transducing extracellular stimuli to intracellular signaling (outside-in signaling), and increasing the receptor specificity by cellular activity (inside-out signaling) (del Zoppo and Milner, 2006).

Currently eight β and 14 α subunits of integrins have been identified (Laurens, 2009). Of those integrins, few are involved with developmental and pathological angiogenesis. Perlecan's DV fragment has previously been reported to negatively modulate angiogenesis by interacting with the $\alpha_2\beta_1$ on HUVECs, and this integrin is not present on brain microvascular endothelial cells. Therefore, I decided to investigate other potential integrins that are involved with inducing post stroke angiogenesis in order to understand DV's pro-angiogenic mechanism of action on brain endothelial

cells. During wound repair, there is an up-regulation of pro-angiogenic integrins responsible for regulating cell adhesive, migratory properties and cell cycle. There are three integrin receptors involved with angiogenesis in the brain that could be potential binding partners for perlecan's DV following MCAO: $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha V\beta_3$ (del Zoppo and Milner, 2006). Interestingly, change or loss in expression of these integrins also correlates to alterations in ECM ligands such as laminin-1, collagen type IV, fibronectin and perlecan (Hamann, 1995), (Fukuda et al., 2004) during cerebral ischemia in non-human primates. These observations further emphasize the direct relationship between ECM fragments and integrins and how this relationship dictates cell fate following ischemia.

Following ischemia, the $\alpha_V\beta_3$ and $\alpha_5\beta_1$ angiogenic integrins are re-expressed in order to promote angiogenesis and wound repair (Li, 2010), (del Zoppo and Milner, 2006). The exact roles $\alpha_V\beta_3$ and $\alpha_5\beta_1$ play in post ischemic angiogenesis are still being defined. Current research suggests that the $\alpha_V\beta_3$ plays a non-essential role in vasculogenesis and angiogenesis (Li et al., 2010), (Lee, 2009). Studies performed by Reynolds and co-workers have demonstrated mice lacking β_3 integrins have enhanced tumor growth and angiogenesis, (Reynolds, 2002). Bader and co-workers have demonstrated mice lacking α_V integrin maintain proper vasculogenesis and angiogenesis (Bader, 1998). More recently, the role of $\alpha_V\beta_3$ integrin in ischemic cerebral angiogenesis has been investigated in mice subjected to hypoxia (Li, 2010). In this work, Li and co-workers demonstrate that although the $\alpha V\beta 3$ integrin is strongly induced on

angiogenic brain endothelial cells in mice subject to hypoxia, knock out of β_3 integrin in mice show no obvious defects in cerebral angiogenesis following hypoxia. In fact, following hypoxia β_3 null mice have an up-regulation in α_5 integrin and an increase in proliferating cerebral endothelial cells. These observations further suggest the importance of $\alpha_5\beta_1$ integrin following ischemia and its role in the regulation of angiogenesis in the ischemic central nervous system.

During development, cerebral capillaries express high levels of $\alpha_5\beta_1$ integrin (Milner et al.). The importance of $\alpha_5\beta_1$ integrin during development has been demonstrated in α_5-null mice showing these mice have a phenotype of significant defects in blood vessel formation (Francis et al., 2002). Following maturation, there is a switch in expression from the $\alpha_5\beta_1$ integrin to the $\alpha_6\beta_1$ integrin, the receptor for laminin, in order to maintain a quiescent environment (Milner and Campbell, 2002). Directly following ischemic stroke in non-human primates, β_1 expression in endothelial cells and astrocytes in the ischemic core is lost (del Zoppo and Milner, 2006), (Milner et al., 2008b). Transcription of β_1 is increased in tissues surrounding the ischemic core, and, the ischemic penumbra (del Zoppo and Milner, 2006). Integrin expression following ischemia can be recapitulated in vitro using the oxygen-glucose deprivation (OGD) model. OGD limits the availability of oxygen and glucose needed for cell survival in vitro. Endothelial cells that have undergone OGD increase their expression of $\alpha_5\beta_1$ (Milner et al., 2008b), confirming this model is consistent with in vivo studies. Other studies in adult mice have also observed an increase in $\alpha_5\beta_1$ integrin expression using a

different hypoxic model, the hypobaric hypoxia model (Milner et al.). Taken together, these studies suggest that following ischemia there is an up-regulation in $\alpha_5\beta_1$ integrin expression that induces the pro-angiogenic environment required for tissue repair and angiogenesis.

Because I observed a pro-angiogenic effect induced by DV (Fig 2.2, 2.3 and 2.4), I reasoned that DV's pro-angiogenic effect on brain endothelial cells might be because of both the absence of $\alpha_2\beta_1$ and the presence of a distinct pro-angiogenic unidentified DV receptor. I decided to investigate whether DV's pro-angiogenic effect is due to interacting with the $\alpha_5\beta_1$ integrin. I chose this integrin because [1] perlecan, DV's parent molecule, greatly increases $\alpha_5\beta_1$ integrin expression in brain endothelial cells (Milner et al., 2008b), [2] perlecan supports β_1 integrin mediated cell adhesion via its DV region (Brown et al., 1997) and [3] DV can inhibit cell adhesion to fibronectin (without directly binding to the fibronectin) (Mongiat et al., 2003), a primary ligand for $\alpha_5\beta_1$.

In this study I have demonstrated DV interacts with the $\alpha_5\beta_1$ integrin and proven this integrin is required for DV's pro-angiogenic effect in vitro. Our results demonstrate that DV co-localizes with the $\alpha_5\beta_1$ integrin and binds to the $\alpha_5\beta_1$ integrin with a K_d of $1.6 \times 10^{-7} \pm 7.2 \times 10^{-8}$ M. DV also causes the up-regulation of $\alpha_5\beta1$ integrin mRNA expression. Lastly, DV is not capable of promoting angiogenesis in vitro when $\alpha_5\beta1$ integrin is negatively modulated. These results suggest proteolytic fragments of DV in the ischemic environment present in the cerebral fluid are able to interact with newly

expressed $\alpha_5\beta1$ integrin subunits. The interaction of DV with the $\alpha_5\beta1$ integrin induces a pro-angiogenic effect and subsequently promotes brain self-repair.

Materials and methods

DV and α_5 integrin binding assays

Binding assays were carried out using an optical biosensor (IAsys; Affinity Sensors, UK) as described (Brittingham et al., 2005). In brief, to covalently bind the $\alpha_5\beta_1$protein, designated as an acceptor, onto the surfaces of a sensor, carboxylate groups present on the surface were activated by injection of a 1:1 mixture of 0.1 M N-hydroxysuccinimide and 0.4 M N-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce). The acceptor dissolved in phosphate buffered saline (PBS) was then allowed to bind to the activated surface until a response plateau was reached. The residual active groups were blocked by an injection of 100 µL of 1 M Tris-HCl (pH 8.5).

A cuvette with immobilized α_5 was primed with the binding buffer (150 mM NaCl, 25 mM Tris-HCl, pH=7.4, and 1 mM $MnCl_2$) at 25°C for 10 min. A 100-µL sample containing free DV interactant dissolved in the binding buffer was added to the cuvette, and then the association phase was recorded. Subsequently, the sample was removed, analyte-free buffer was added to the cuvette, and the dissociation phase was recorded. After each assay, the surface of the cuvette was regenerated by a brief wash with 100 mM glycine, pH=4, and followed by equilibration with the binding buffer. During regeneration cycles attention was paid to complete removal of the surface-bound

analyte, and the washing continued until a response equal to a baseline value was reached.

For binding assays, free DV was added at concentrations ranging from 8.0×10^{-8} M to 4.0×10^{-7} M. Data from the biosensor were analyzed by the global fitting method described by Myszka and Morton (Myszka and Morton, 1998). For each assay, the association rate constants (k_{on}) and the dissociation rate constants (k_{off}) were obtained, and the equilibrium dissociation constants (K_d) values were calculated from a ratio of k_{off}/k_{on}. In addition, control binding of bovine serum albumin (BSA) at the molar concentration of 8.0×10^{-7} (double of the highest concentration for DV) was also performed.

Immunocytochemistry

Brain endothelial cells plated on type I collagen were treated with DV for 10 minutes followed by fixation with 4% paraformaldehyde (VWR). $\alpha_5\beta_1$ co-localization with administered DV was examined with antibodies directed to $\alpha_5\beta_1$ (Millipore) and the 6X HIS tag on the human recombinant DV (Calbiochem). Appropriate secondary antibodies were used to stain for DV (Alexa fluor 488) and $\alpha_5\beta_1$ (Alexa fluor 594) purchased from Invitrogen.

$\alpha_5\beta_1$ *integrin knockdown*

Brain microvascular endothelial cells at 50% confluency were transferred from normal growth media to Optimem (Gibco) for 20 minutes. Lipofectamine 2000 (Invitrogen) and siRNA oligos targeted against the human α_5 integrin (Mission siRNA;

Sigma) were diluted in Optimem media individually for 5 minutes. Tubes were combined and incubated at room temperature for 20 minutes. α_5 SiRNA-containing media was then added to the brain endothelial cells dropwise and incubated for 2 hours. After 2 hours, Optimem was replaced with antibiotic free growth media (M199, 10% FBS, 150 mg/ml bovine brain extract, 60 mg/ml heparin), and cells were allowed to recover overnight. After 24 hours, the media was changed to normal growth media containing antibiotics. α_5 integrin knockdown was confirmed by α_5 QT-PCR and $\alpha_5$western blot.

Proliferation assays

Mouse brain endothelial cells were seeded in 96-well plates at a concentration of 4 x 10^3 cells per well in IMDM media (Invitrogen) supplemented with 10% FBS (Invitrogen) 1x Antibiotic/Antimycotic and incubated overnight at 37C and 5% CO_2. Following overnight incubation, complete media was aspirated off, and cells that underwent treatment were washed with plain IMDM media to remove residual serum. Purified DV and 1% media was added back to each well to a final volume of 100µL and incubated for an additional 24 hours at 37C and 5% CO_2. After 24 hours, 20µL of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), was added to each well and incubated for an additional two hours at 37C and 5% CO_2. Cell proliferation was measured following the two hour incubation by reading the 96-well plate at 490nm using plate reader. Proliferation was normalized to untreated 1% condition for each experiment.

Migration assay

Cell migration was assessed with a modified Boyden Chamber (NeuroProbe, Gaithersburg, MD) following the instructions of the manufacturer. In the top chamber, brain endothelial cells were added to each well at a concentration of 50 x 10^3 cells per well. In the bottom chamber, VEGF (20ng/mL) or purified DV was added to each well. A polycarbonate membrane (PVD-free 8 micron pore) was coated with type I collagen and fitted in between the top and bottom chamber. After the chamber was set up, it was placed at 37C and 5% CO_2 and incubated for 6-8 hours. Following incubation, the polycarbonate membrane was scraped on the apex surface to remove any cells that did not actively migrate through the 8 micron pores in the membrane. Next the membrane was incubated in 0.1% crystal violet for one hour, rinsed 3x with 1xPBS and cell number was counted using a microscope.

Capillary tube-like formation assays

24-well plates were incubated at -20C overnight along with 200µL pipette tips. Matrigel (VWR) was thawed on ice and approximately 50µL was aliquoted to each pre-chilled well in the 24-well plate. Even coating of the matrigel was accomplished using a pre-chilled pasture pipette. During cell collection, the matrigel coated plate was placed at 37C and 5% CO_2. brain endothelial cells were seeded to each well at a concentration of approximately 50 x 10^3 cells per well in IMDM supplemented with 1% FBS to a final volume of 350µL. Purified DV was added to each well at a desired concentration. Experiments were run for 12-18 hrs at 37C and 5% Co_2. Following incubation, cells were

fixed with 4% paraformaldehyde in 1x PBS. Tube formation was imaged and quantified as tube pixels/high power field, 10 areas per condition using Adobe Photoshop, CS.

Results and discussion

Angiogenesis following ischemia involves the degradation of the ECM, and an integrin "switch" to go from a quiescent environment to a pro-angiogenic environment. DV has been reported to regulate angiogenesis by interacting with the $\alpha_2\beta_1$ integrin which is not present on microvascular brain endothelial cells. Data reported in the previous chapter suggest DV is inducing a pro-angiogenic effect on brain endothelial cells. Therefore, I asked if DV could interact with a new integrin that is essential in promoting angiogenesis and re-expressed following ischemia. The $\alpha_5\beta_1$ integrin is critical for vascular development (Francis et al., 2002) and promotes post-stroke brain angiogenesis (Milner et al.), but is otherwise down-regulated in the mature brain until re-expressed in brain endothelial cells after hypoxia (Milner et al.), (Milner et al., 2008b). Given my previous results and these observations, I reasoned that DV's

pro-angiogenic effect could be because of interaction with the $\alpha_5\beta_1$ integrin.

DV co-localizes with and binds to the $\alpha_5\beta_1$ integrin

In order to investigate whether DV's pro-angiogenic mechanism of action requires the $\alpha_5\beta_1$ integrin I first set out to investigate whether DV co-localizes with the $\alpha_5\beta_1$ integrin. Immunocytochemistry was incorporated to investigate the cell surface relationship between $\alpha_5\beta_1$ and DV (**Figure 3.1**). brain endothelial cells were treated in the presence or absence of DV (300nm) for 30 minutes and stained for $\alpha_5\beta_1$ (green) and anti his-DV (red). In the control panel, cells not treated with DV show only surface staining of $\alpha_5\beta_1$ and no staining of his-DV. $\alpha_5\beta_1$ surface localization is spread out and uniform. In contrast to those results, cells treated with DV show staining of both $\alpha_5\beta_1$ integrin and DV, indicating the presence of DV. Cells treated with DV demonstrate clustering of $\alpha_5\beta_1$ integrin, a sign of integrin activation (Berrier, 2007) and co-localization of DV and $\alpha_5\beta_1$. To further demonstrate that DV's interaction with brain endothelial cells is mediated by the presence of the $\alpha_5\beta_1$ integrin, brain endothelial cells were subjected to $\alpha_5\beta_1$ shRNA and assayed for staining of DV. As expected, there

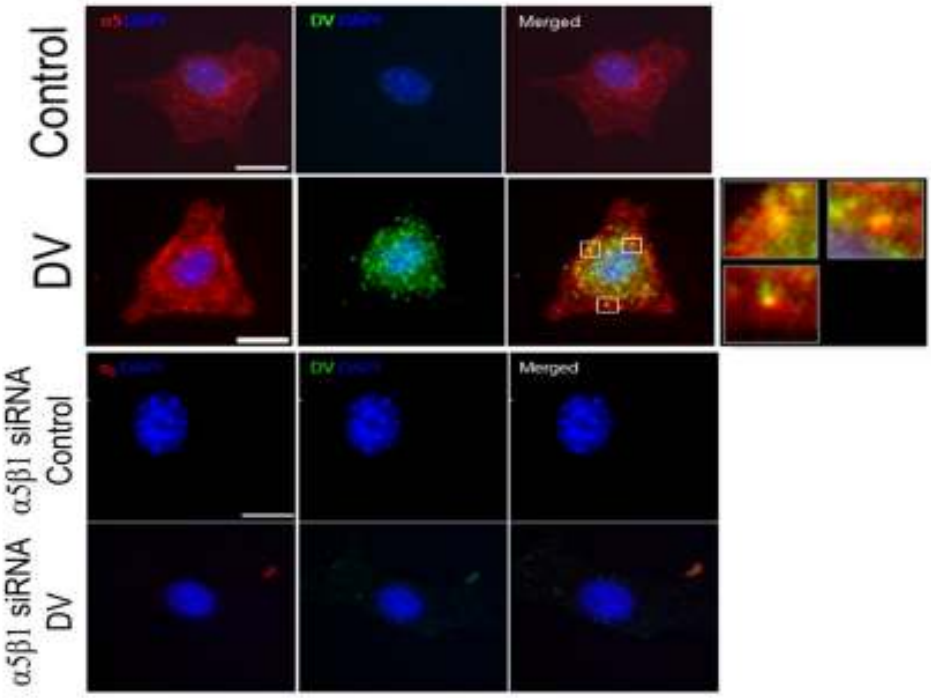

Figure 3.1. DV co-localizes with $\alpha_5\beta_1$ integrin. brain endothelial cells were stained for $\alpha_5\beta_1$ (red) and DV (anti-HIS, green) in order to investigate whether DV and $\alpha_5\beta_1$ co-localize. Top panel, Cells treated with DV (± DV treatment for 30 minutes) demonstrate DV and $\alpha_5\beta_1$ integrin colocalization (yellow/orange color in merged field and as magnified in three boxes) and $\alpha_5\beta_1$ clustering. Bottom panel, brain endothelial cells with shRNA mediated knockdown of the $\alpha_5\beta_1$ integrin show no detectable staining of DV which suggests the $\alpha_5\beta_1$ integrin is required for DV's interaction with brain endothelial cells.

was no staining of $\alpha_5\beta_1$ integrin in the cells treated with $\alpha_5\beta_1$ shRNA (**Figure3.1**). shRNA cells treated with DV also showed no staining for DV, indicating the $\alpha_5\beta_1$ is required for DV's interaction with these brain endothelial cells. Collectively, these data suggest DV interacts with brain endothelial cells by interacting with the $\alpha_5\beta_1$ integrin.

To further investigate the molecular interaction between $\alpha_5\beta_1$ and DV, I next incorporated optical biosensor analysis. The method is used to quantify the molecular interaction between two species by monitoring a change in mass without needing to label or modify the two molecular species being tested. The angle of extinction of light, reflected after polarized light impinges upon the film, is altered and monitored as a change in detector position for the dip in reflected intensity (Drescher, 2009). In my experimental model, soluble $\alpha_5\beta_1$ integrin was immobilized to the cuvette and free DV was added at various concentrations (**Figure 3.2**). Under these conditions, DV was determined to bind the $\alpha_5\beta_1$ integrin with a k_{on}, k_{off} and K_d of $3.8 \times 10^6 \pm 2.7 \times 10^5$/M-s, $7.2 \times 10^{-1} \pm 1.1 \times 10^{-1}$/s and $1.6 \times 10^{-7} \pm 7.2 \times 10^{-8}$ M, respectively. BSA was run as a negative control and showed no binding to the $\alpha_5\beta_1$ integrin, indicating DV binding to $\alpha_5\beta_1$ integrin was specific.

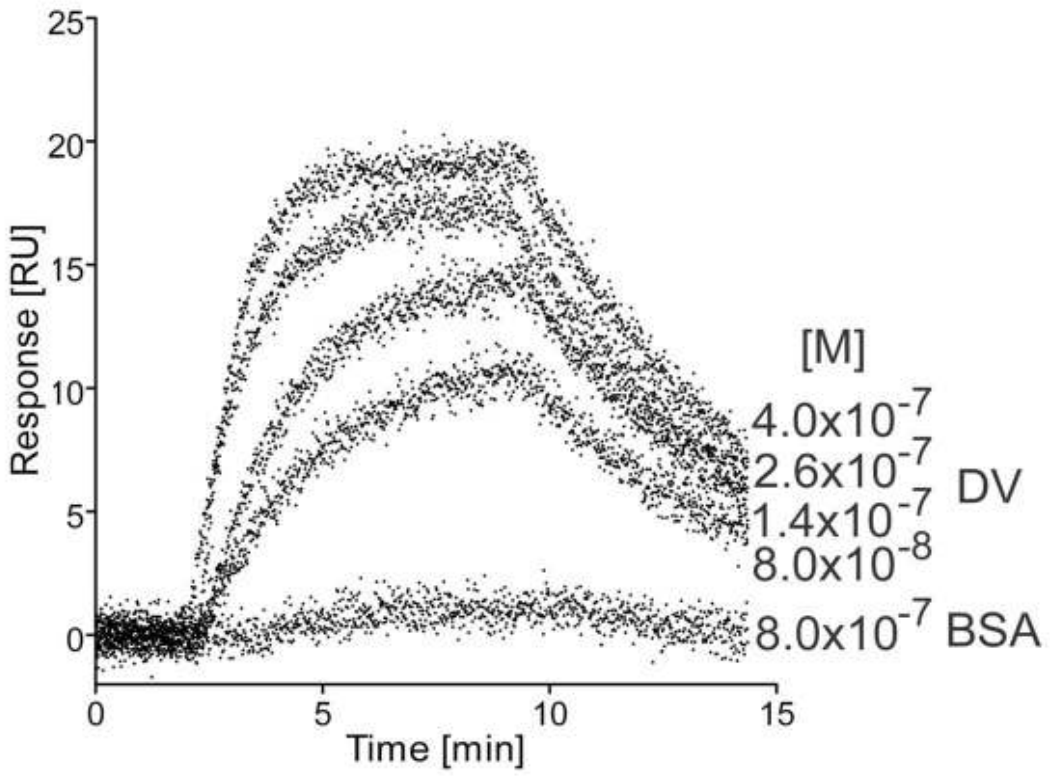

Figure 3.2. DV binds $\alpha_5\beta_1$ integrin. Optical biosensor traces showing the association and dissociation of DV and BSA (control) with immobilized α_5 integrin. Concentrations of DV (determined by Bradford assay) and BSA used are listed in molarity on the right hand side. DV was determined to bind the $\alpha_5\beta_1$ integrin with a k_{on}, k_{off} and K_d of 3.8 x 10^6 ± 2.7 x 10^5/M-s, 7.2 x 10^{-1} ± 1.1 x 10^{-1}/s and 1.6 x 10^{-7} ± 7.2 x 10^{-8} M, respectively.

As the presence of integrin ligand is known to increase that integrin's intercellular expression (Milner et al., 2008b), and our data suggests DV is a ligand of α_5, I next asked whether DV might also increase α_5 integrin mRNA expression in brain endothelial cells in vitro. **Figure 3.3** demonstrates that by qPCR, after 1.5h or 3h of DV exposure, α_5 mRNA expression increases by approximately 2 fold and 1.8 fold, *p=0.0001, and **p=0.006 respectively, as compared to cells not treated with DV. These results suggest DV increases mRNA expression of the α_5 in brain endothelial cells. Collectively, these experiments demonstrate DV binds to, co-localizes with, and increases the mRNA expression of the $\alpha_5\beta_1$ integrin.

Negative modulation of $\alpha_5\beta_1$ integrin abolishes DV's pro-angiogenic effect

With the knowledge that DV co-localizes with and could directly bind to the $\alpha_5\beta_1$ integrin, I next set out to determine whether negatively modulating the $\alpha_5\beta_1$ integrin would abolish DV's pro-angiogenic effect on brain endothelial cells. In order to negatively modulate the interaction of DV and $\alpha_5\beta1$ integrin, I incorporated two

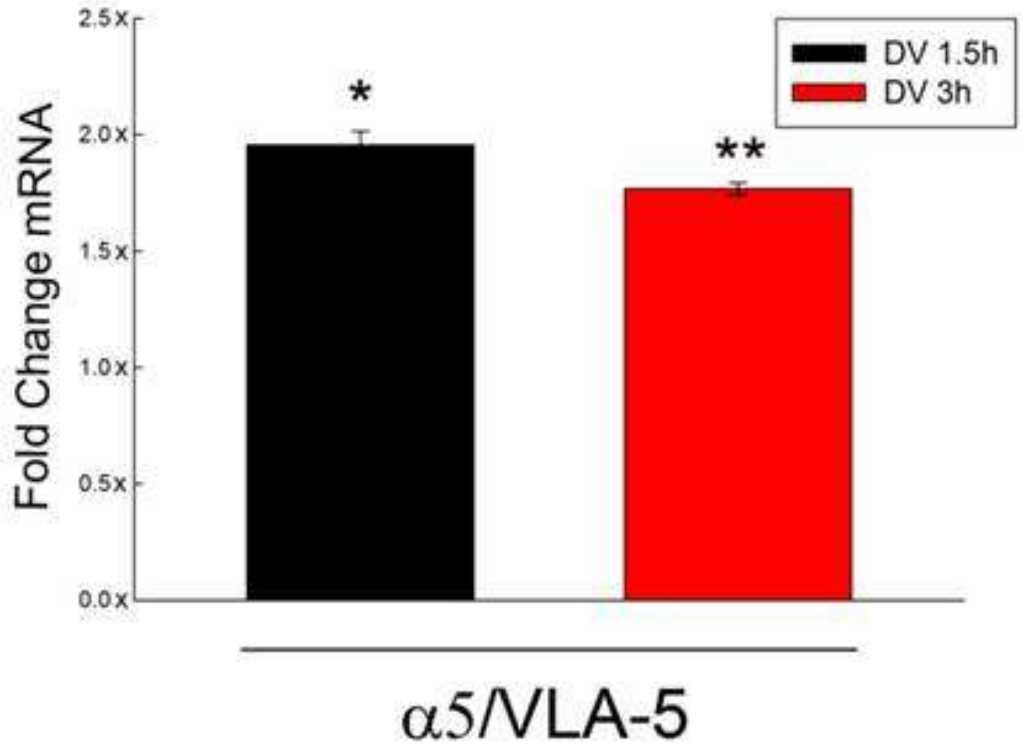

Figure 3.3. DV increases α_5 integrin mRNA expression in brain endothelial cells. Fold change in mRNA of α_5 integrin isolated from cells treated ± DV for 1.5 hours and 3 hours. Control mRNA levels were normalized to one and the natural log was then reported of this data. After 1.5 hours or 3h of DV exposure, α_5 mRNA expression increases by approximately 2 fold and 1.8 fold, *p=0.0001, and **p=0.006 respectively, as compared to cells not treated with DV. (Error bars indicate standard error)

methods. The first method used short hairpin RNA constructs (shRNA) to knockdown α_5 integrin, the second method was using a soluble $\alpha_5\beta_1$ integrin-GST and third method used synthetic peptides designed specifically for the binding pocket of $\alpha_5\beta_1$. By inhibiting the $\alpha_5\beta_1$ using these techniques, I was able to investigate the role $\alpha_5\beta_1$ has in DV's pro-angiogenic mechanism on brain endothelial cells in vitro.

Gene silencing of α_5 integrin was accomplished using shRNA. To confirm α5 integrin was successfully knocked down, qPCR was performed to demonstrate a reduction in α_5 integrin mRNA and western blot analysis was performed to confirm a reduction in protein level (**Figure 3.4**). Brain endothelial cells treated with lentiviral shRNA targeted towards α5 integrin showed a 75% reduction in both α_5 integrin mRNA and α_5 integrin protein level as compared to control. Once α_5 integrin was successfully knocked down in brain endothelial cells, I then incorporated these cells in the previously used in vitro angiogenic assays: MTS proliferation assay and modified Boyden Chamber migration assay in order to investigate whether this integrin plays a role in DV's pro-angiogenic effect in vitro.

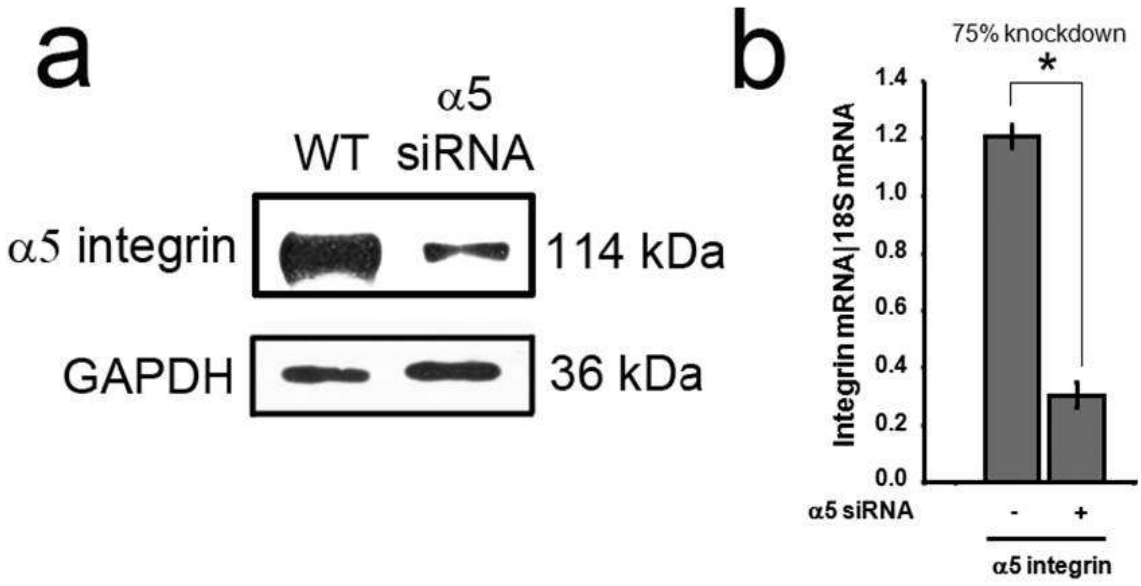

Figure 3.4. Confirmation of α_5 knockdown. Representative western blot and qPCR analysis of α_5 integrin knockdown. Western blot (a) and (b) qPCR of wild type and α_5 shRNA treated brain endothelial cells demonstrating a 75% knockdown of α_5 protein, and mRNA, respectively (GAPDH loading control also shown). *p=0.0061.

Wild type brain endothelial cells grown in IMDM supplemented with 1% FBS media were used as the baseline control population and their proliferation was arbitrarily set to 100%. α_5 knockdown brain endothelial cells grown in IMDM media supplemented with 1% FBS were treated incubated with or without DV [20 ug/mL] (**Figure 3.5**). α_5 mediated knocked down brain endothelial cells do not show significant inhibition of proliferation as compared to wild type brain endothelial cells grown in 1% FBS supplemented media. This result suggests that, in reduced serum conditions, knockdown of $\alpha_5\beta_1$ integrin has no effect on brain endothelial cell proliferation. This result also implies shRNA mediated knockdown of $\alpha_5\beta1$ integrin was not toxic to brain endothelial cells because the MTS assay monitors both cell proliferation and cell death by monitoring cell metabolic activity. These results demonstrate DV significantly enhanced proliferation of wild type brain endothelial cells. Brain endothelial cells with α_5 knocked down were no longer responsive to DV pro-proliferative effect. These results suggest DV's pro-proliferative effects are because of the presence of the $\alpha_5\beta1$ integrin on brain endothelial cells, suggesting DV induces its pro-proliferative signal through the $\alpha_5\beta1$ integrin.

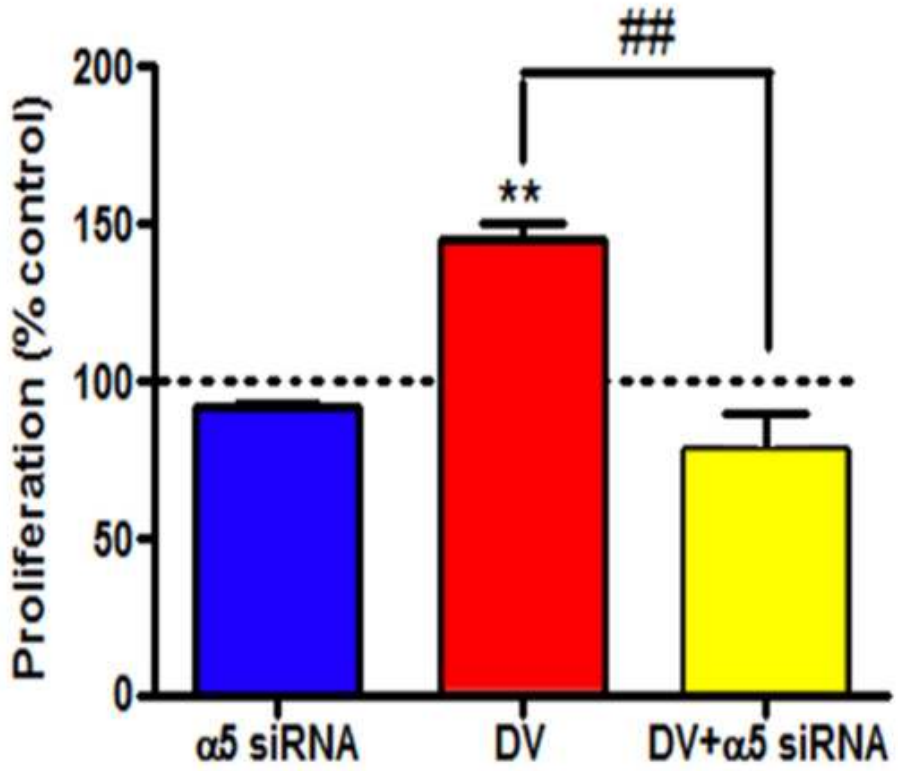

Figure 3.5. Blocking $\alpha_5\beta_1$ inhibits DV's effect on brain endothelial cell proliferation. Quantification of proliferation of brain endothelial cells ± addition of the α_5 integrin plasmid after 48 hours ± DV in serum free media as measured via MTS assay. Values shown (mean ± standard deviation normalized to control proliferation arbitrarily set to 100%) demonstrate that α_5 siRNA did not significantly inhibit brain endothelial cell proliferation (p=0.6), but did inhibit the positive (**p=0.002) proliferative effect of DV (##p=0.0001).

Next, I asked if negative modulation of $\alpha_5\beta_1$ integrin would inhibit DV's pro-migratory effect on brain endothelial cells (**Figure 3.6**). In order to investigate this hypothesis, two separate experimental models were used. First, soluble α5β1-GST integrin was co-incubated with DV in the bottom wells of the modified Boyden chamber. This model was used to test the hypothesis that soluble DV co-incubated with soluble α5β1-GST integrin will block DV's pro-migratory effect because of competition for binding of DV between soluble α5β1-GST and cell surface $\alpha_5\beta_1$ integrin. As shown in **Figure 3.6,** endothelial cells migrating towards VEGF were used as a positive control. Interestingly, endothelial cells migrated towards DV suggesting DV is just as much of a chemoattractant as VEGF. Incubation of DV with soluble $\alpha_5\beta_1$-GST blocked DV's pro-migratory response of brain endothelial cells. To further demonstrate this integrin is required for DV's effect on brain endothelial cell migration, brain endothelial cells with $\alpha_5\beta_1$ knocked down were also treated with DV. Under this condition, DV was no longer able to promote migration of brain endothelial cells. Collectively, these data indicate the α5β1 integrin is required for DV's pro-migratory effect on brain endothelial cells.

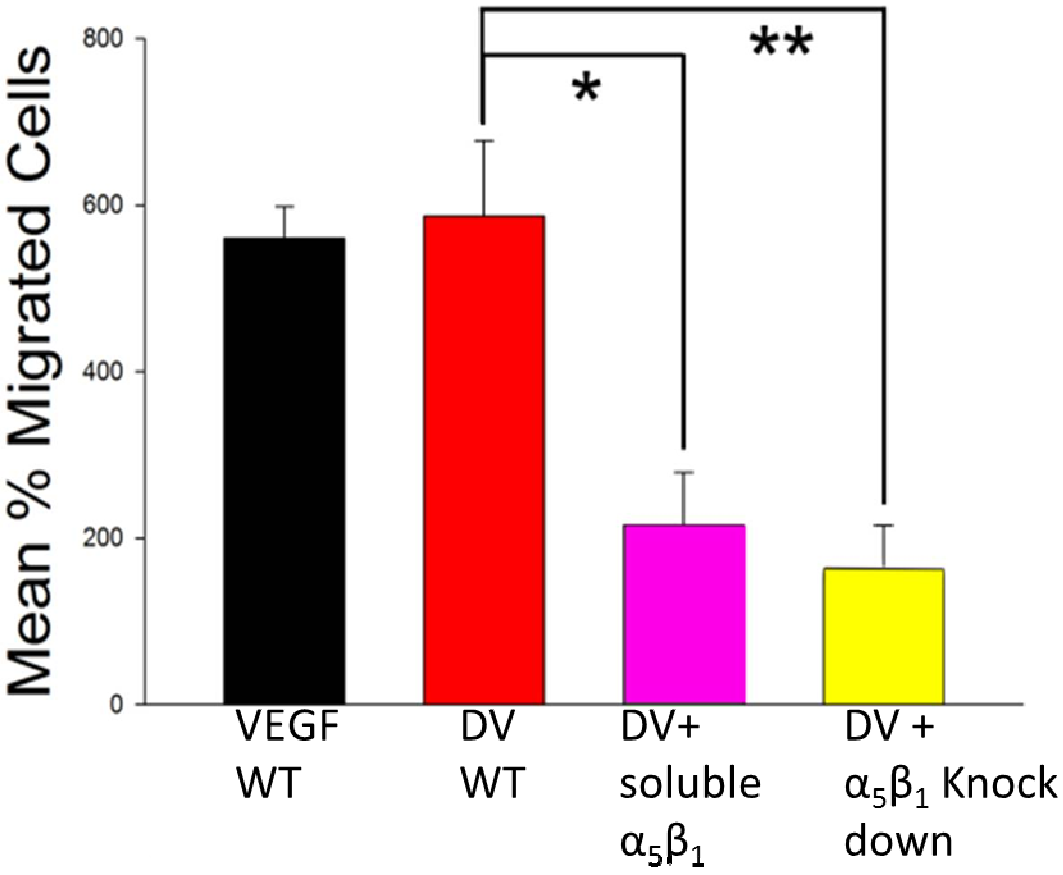

Figure 3.6. Blocking $\alpha_5\beta_1$ inhibits DV's effect on brain EC migration. Quantification of mean number cells (± standard deviation) migrating towards 3% fetal bovine serum (control) or DV ± $\alpha_5\beta_1$--GST or α_5 knockdown with α_5 siRNA (as normalized to negative control (no chemotractant) demonstrating that DV was as powerful a chemotractant as 3% serum which could be significantly inhibited by $\alpha_5\beta_1$-GST (*p=0.02) or α_5 knockdown (**p=0.009)

Lastly, I determined if blocking the $\alpha_5\beta_1$ integrin with the CRRETAWAC ligand, a ligand specific for a binding pocket in $\alpha_5\beta_1$ integrin, would block DV's effect on inducing tube-like structures and morphogenesis of human D3 cells. The CRRETAWAC ligand shares the same or overlapping binding site in $\alpha_5\beta_1$ as RGD-containing peptides (Koivunen et al., 1994). Because the CRRETAWAC ligand is specific for the human $\alpha_5\beta_1$ integrin, I utilized the human D3 brain endothelial cell line to investigate DV's effect on tube morphogenesis (**Figure 3.7**). Control D3 brain endothelial cells did not form tubes in the absence of DV. As expected, DV induced D3 brain endothelial cells to form tube like structures. This suggests DV's effect is not species specific, as it has now been observed to have pro-angiogenic effects in murine and human brain endothelial cells. When D3 brain endothelial cells were pre-incubated with the CRRETAWAC ligand, DV's effect on promoting tube like structures was greatly inhibited. However, I did not observe complete inhibition of DV induced tube morphogenesis when co-incubated with the CRRETAWAC peptide. These data suggest DV may also share the same or overlapping binding site the CREETAWAC peptide has in $\alpha_5\beta_1$, yet also suggests DV binds to other parts of the integrin that is substantial enough to promote some tube formation. Another explanation may be because of the fact that only one concentration for the peptide was used for this experiment. Future experiments would investigate if higher concentrations of the CREETAWAC peptide could completely block DV's tube like formation.

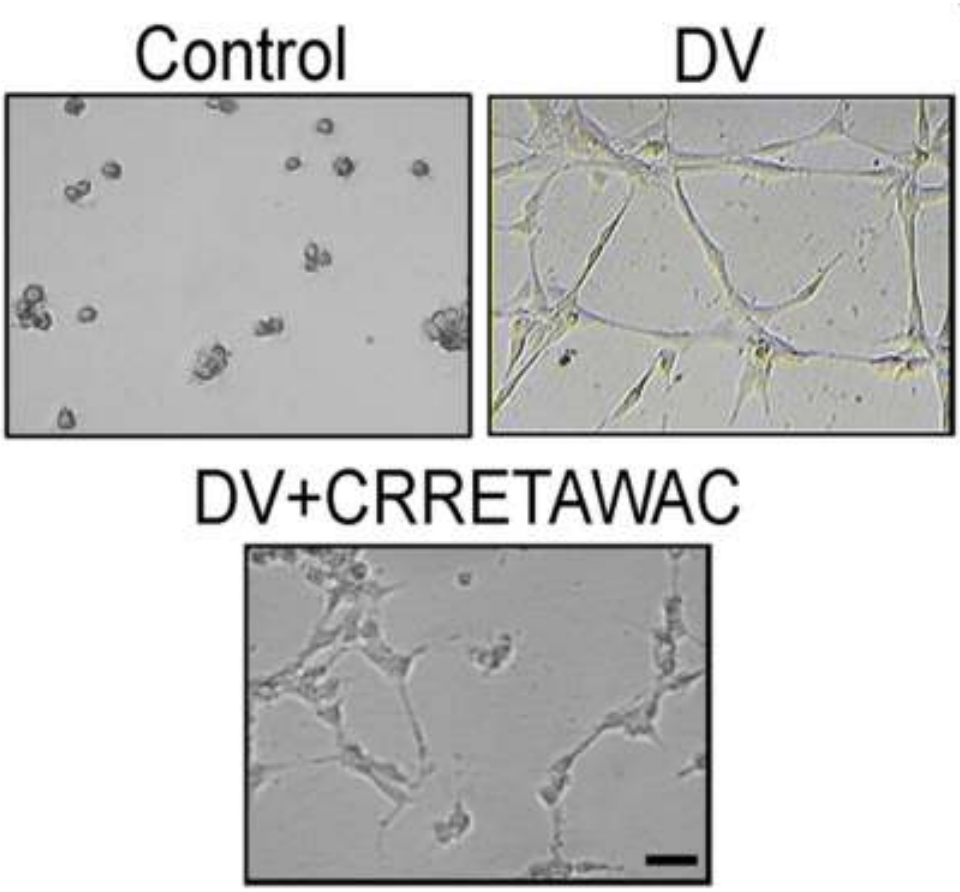

Figure 3.7. Blocking $\alpha_5\beta_1$ blocks DV's pro-angiogenic effect on human D3 brain endothelial cell tube formation. Human D3 brain endothelial cells were treated ±DV 300nm and ±DV CRRETAWAC peptide. DV induced D3 brain endothelial cells to make tube like structures and promote morphogenesis. In the presence of the $\alpha_5\beta_1$ human specific blocking peptide, CRRETAWAC, DV's capability to promote tube like structures and morphogenesis was inhibited.

In order to dissect the mechanism by which DV is promoting angiogenesis on brain endothelial cells in vitro, I first set out to identify a new receptor that is essential in promoting angiogenesis following ischemia in the brain. To date, little research has been performed linking integrins with angiogenesis in the post ischemic brain. Luckily, I did have some evidence to go on that led me to identify a new receptor responsible for DV's pro-angiogenic effect on brain endothelial cells. First, in murine, rat and non-human primates, angiogenic brain endothelia experience an integrin receptor switch after brain hypoxia from mature integrin receptors such as the $\alpha_6\beta_1$, back to developmental integrin expression, $\alpha_5\beta_1$, $\alpha_V\beta_3$, and β_1, in order to promote angiogenesis (Lathia et al., 2009), (Milner and Campbell, 2002), (Milner et al., 2008a), (del Zoppo and Milner, 2006). Second, new evidence suggests that the $\alpha_V\beta_3$ integrin is non essential for post stroke angiogenesis, while the $\alpha_5\beta_1$ integrin promotes brain endothelial cell proliferation following ischemia in a mouse model (Li, 2010). Third, perlecan, DV's parent molecule, greatly increases $\alpha_5\beta_1$ integrin expression in brain endothelial cells (Milner et al., 2008b), perlecan supports β_1 integrin mediated cell adhesion via its DV region, (Brown et al., 1997) and DV can inhibit cell adhesion to fibronectin (without directly binding to the fibronectin) (Mongiat et al., 2003), a primary ligand for $\alpha_5\beta_1$.

Immunocytochemistry and Surface Plasmon resonance experiments suggest DV co-localizes with and binds to the $\alpha_5\beta_1$ integrin with a k_{on}, k_{off} and K_d of $3.8 \times 10^6 \pm 2.7 \times 10^5$/M-s, $7.2 \times 10^{-1} \pm 1.1 \times 10^{-1}$/s and $1.6 \times 10^{-7} \pm 7.2 \times 10^{-8}$ M, respectively. These data

would suggest that, following post stroke proteolysis of perlecan, DV is released into the vascular cerebral space. Following DV release, my data support the hypothesis that free DV is able to bind to the $\alpha_5\beta_1$ integrin expressed on brain endothelial cells and promote brain angiogenesis. Moreover, my results also imply that DV could play a role in inducing the integrin receptor "switch" (Milner and Campbell, 2002) from a quiescent environment to a pro-angiogenic environment because DV increases mRNA expression of $\alpha_5\beta_1$ integrin in brain endothelial cells. Other work performed in our laboratory supports the hypothesis that DV could play a role in inducing the integrin receptor "switch" because animals treated with DV following middle cerebral artery occlusion and resultant cerebral ischemia express significantly higher integrin levels of $\alpha_5\beta_1$ in post-stroke brain tissue compared to contralateral tissue, (data not shown). Indeed, DV binds to $\alpha_5\beta_1$ with a K_d of 160 nM which is weaker than its interaction with its previously reported receptor, $\alpha_2\beta_1$ (K_d 80 nM) (Bix et al., 2004), yet these results could explain how DV induces its anti-angiogenic effect on brain endothelial cells transfected with $\alpha_2\beta_1$ plasmid. As DV's affinity for each receptor is different, DV has a binding preference for $\alpha_2\beta_1$. Therefore, if $\alpha_2\beta_1$ integrin is expressed, the anti-angiogenic response will dominate.

Once I had identified $\alpha_5\beta_1$ as a plausible receptor for DV on brain endothelial cells, I investigated whether this interaction was essential for modulating DV's pro-angiogenic effect. Therefore, DV's pro-proliferative, migratory and tube like formation effect was blocked by negatively modulating the $\alpha_5\beta_1$ integrin through shRNA mediated

knockdown, soluble $\alpha_5\beta_1$-GST and CRRETAWAC peptide. From these experiments, I am able to conclude that DV requires the $\alpha_5\beta_1$ integrin in order to induce its pro-angiogenic response on brain endothelial cells.

Future experiments will investigate how DV interacts with the $\alpha_5\beta_1$ integrin. Previous reports indicate DV interacts with α2β1 integrin via its I-domain (Bix et al., 2004) yet the $\alpha_5\beta_1$ integrin does not contain such domain (Diamond, 1994). My data with the CREETAWAC peptide may offer some clues as to where DV is binding to the $\alpha_5\beta_1$ integrin since this peptide was able to inhibit DV's effect on tube like formation and morphogenesis by brain endothelial cells. CRRETAWAC has been demonstrated to directly compete with Arg-Gly-Asp (RGD) containing peptides and mAb 16, which do not share the same binding sites (Mould et al., 1998). As DV does not contain an RGD binding motif and the CREETAWAC peptide blocks DV's enhancement of tube formation one could suggest DV may share the same binding pocket as the CREETAWAC peptide on the $\alpha_5\beta_1$ integrin. Interestingly, opposite of what has been reported (Woodall et al., 2008), my surface Plasmon resonance binding experiments had to be performed in the presence of Mn^{2+}. These data suggest DV binding to the $\alpha_5\beta_1$ integrin is cation-dependent. As bivalent cations have been shown to induce conformational relaxation that leads to exposure of ligand-binding sites in the $\alpha_5\beta_1$ integrin (Mould, 1998), it is plausible that $\alpha_5\beta_1$ must be "primed" in order for DV to bind to it.

CHAPTER IV

PERLECAN DV INDUCES THE RELEASE OF VEGF FROM BRAIN ENDOTHELIAL CELLS VIA A MAP-KINASE DEPENDENT SIGNALING PATHWAY INVOLVING THE $\alpha_5\beta_1$ INTEGRIN

Introduction

Regulatory mechanisms involved in angiogenesis are essential for restoration of tissues in and out of the CNS. A better understanding of the mechanisms that inhibit angiogenesis would be beneficial for diseases such as cancer, whereas a better understanding of the mechanisms for promoting angiogenesis would be beneficial in cases such as limb ischemia or myocardial infarction (Navaratna, 2009). Studies performed by Krupkinski suggest that active angiogenesis following stroke is beneficial for the ischemic brain (Krupinski et al., 1993). Induction of angiogenesis in the CNS following ischemia would increase blood flow to affected tissues, reduce infarct size, promote neurogenesis and provide support for new neuronal networks (Navaratna, 2009). Current evidence suggests active angiogenesis following ischemia occurs as soon as 12 to 24 hours in mice models and three to four days in humans (Navaratna, 2009). The discrepancy in time may have to do with when the samples are available to analyze in human models. However, how angiogenesis is induced following ischemia in the CNS remains to be answered.

Angiogenesis and neuroprotection following stroke involves the release and binding of growth factors such as epidermal growth factor, basic fibroblast growth factor, transforming growth factor beta and VEGF (Slevin, 2000) (Krupinski et al.,

1994a). VEGF is a homodimeric protein that plays a critical role in angiogenesis during development, wound healing, cancer, cardiovascular diseases and ischemia (Zachary, 2001). Five iso-forms of VEGF-A exist, differing in amino acids and affinity to their receptors. The most abundant iso-form found in the brain, $VEGF_{165}$, has been shown to regulate proliferation, migration and tube formation in endothelial cells (Kaji et al., 2006), (Katsumata, 2010). VEGF expression is regulated at the transcriptional, translational and post transcriptional level. Secretion of VEGF following ischemia is related to activation of hypoxia-inducible factor pathways and intercellular signaling pathways. The majority, if not all VEGF, signaling is mediated by or involves VEGFR2 (Zachary, 2001). Perlecan's DV has previously been demonstrated to inhibit angiogenesis in HUVECs by negatively modulating VEGFR2, implicating a role for perlecan's DV in growth factor downstream signaling (Nystrom et al., 2009).

VEGF is regulated at the transcriptional level by activating PI-3-K/AKT and MAPK signaling pathways (Hermann and Zechariah, 2009) and HIF-1α under normoxic and hypoxic conditions (Berra et al., 2000), (Levy, 1996) (Karni, 2002). The MAP kinase cascade is triggered by multiple extracellular molecules, is conserved from yeast to man and is involved in cell proliferation, cell differentiation and cell behavior (Pouyssegur, 2002). Under normoxic conditions, mRNA levels of VEGF are rapidly induced by the p44/p42 MAP kinase cascade (Mazure, 2003). Under normoxic conditions, ERK has been demonstrated to directly phosphorylate HIF-1α. This leads to increased levels of

the HIF-1α, and therefore HIF-1β and HIF-1α heterodimer complex, and activation of VEGF expression (Berra et al., 2000).

It was not until recently, that investigators began to link ECM ligands with integrin binding for modulation of angiogenic growth factors. This becomes an interesting area of research for stroke because degradation of the ECM following ischemia produces bioactive fragments that are available to interact with cell surface receptors and subsequently regulate cell fate. ECM-specific ligands binding to the $\alpha_5\beta_1$ integrin have been linked to activating the ERK and PI 3-K cascades and promote retinal endothelial proliferation (Wilson, 2003). Research performed by Mousa et al. demonstrates that ECM fragments induce VEGF secretion by retinal pigmented epithelial cells, linking ECM degradation and promotion of angiogenesis.

Perlecan's C-most terminal fragment, LG3, is linked to activation of the ERK pathway (Soulez, 2010). Therefore, it would be logical to think perlecan's DV could induce $VEGF_{165}$ secretion by activating the ERK pathway. Yet, no work has been performed investigating this hypothesis. $VEGF_{165}$ has also been linked to the induction of perlecan synthesis in brain endothelial cells (Kaji et al., 2006). Collectively, these data suggest a positive feedback loop where DV causes VEGF secretion and subsequent perlecan synthesis by activating the ERK signaling pathway. Therefore, because DV was promoting a pro-angiogenic effect on brain endothelial cells, and because VEGF is up-regulated following ischemia, I investigated whether DV's pro-angiogenic effect

involved VEGF up-regulation in brain endothelial cells. I later investigated if this effect involved the $\alpha_5\beta_1$ integrin and activation of ERK and HIF-1α.

I was first able to demonstrate that DV induces VEGF secretion by brain endothelial cells in a dose dependent manner due to DV up-regulation of VEGF mRNA levels. I later investigated the intercellular signaling cascades involved in DV up-regulation of VEGF and demonstrated that DV activates AKT and ERK signaling cascades that lead to an increase in HIF-1α stabilization, thus increasing transcription of VEGF message. To investigate the significance of $\alpha_5\beta_1$ integrin in DV's pro-angiogenic effect, I utilized brain endothelial cells that were knocked down for the $\alpha_5\beta_1$ integrin via shRNA and demonstrate these cells were no longer responsive to DV's effect on VEGF's mRNA levels or activation of AKT, ERK and HIF-1α. My results demonstrate that DV is pro-angiogenic, and this effect involves DV signaling through the $\alpha_5\beta_1$ integrin in order to activate AKT, and ERK signaling cascades that are responsible for increasing VEGF expression and secretion by stabilizing HIF-1α. $VEGF_{165}$ induces perlecan synthesis in human brain endothelial cells and perlecan is the most sensitive ECM component following ischemia. Therefore my data suggests a positive feedback loop model by which fragments of perlecan, particularly DV, following proteolysis can replenish levels of perlecan inadvertently by causing the release of VEGF.

Materials and methods

Construction of stable cells with $\alpha_5\beta_1$ integrin knockdown

Bacterial glycerol stocks with shRNA mediated clones to α_5 integrin constructed within the lentivirus plasmid vector pLKO (Sigma) were streaked out onto agarose plates containing Ampicillin and grown overnight. Single colonies were picked and grown in 50mL LB-Amp media and grown overnight. Plasmid DNA was purified using Qiagen maxi prep kit. Recombinant lentiviral particle were made following purification of plasmid DNA. Plasmids were transfected into 293 EBNA cells using Lipofectamine™ 2000 (Invitrogen) and Mission® Lentiviral Packaging Mix (Sigma) as per the supplier's instructions. Once lentiviral particles were made, they were collected and lentiviral transduction was performed following lentiviral transduction protocol developed by Sigma Mission® RNAi team. brain microvascular endothelial cells were grown to 70% confluency. Media was changed and fresh media with 20µL of Lentiviral particles were added to the appropriate wells. Plate was gently swirled and incubated an additional 18 hours at 37C and 5% CO_2. Following this incubation, media was removed and fresh media was added back to each well and incubated another 18 hours at 37C and 5% CO_2. Next, the media was replaced with fresh puromycin (2.5µg/mL) containing media and incubated another 18 hours at 37C and 5% CO_2. After this, media was replaced with fresh puromycin containing media every 3-4 days until resistant colonies were identified (five days). In order to confirm appropriate knockdown of α_5 integrin knockdown in these cell lines, qPCR and western blot analysis was performed.

VEGF secretion ELISA analysis

Confluent microvascular brain endothelial cells, and D3, were serum-starved for 12 hours prior to experiments. Cells were washed with PBS, then fresh serum free media was added back to each well with desired concentrations of DV and incubated at 37C and 5% CO_2 for various time points up to nine hours. Following incubation, conditioned medium was collected and spun down at 10,000 X g for five minutes. After centrifugation, VEGF in the conditioned medium was detected following manufacturer's instructions (RayBio® Mouse/Human VEGF-A ELISA Kit for serum, plasma and cell culture supernatant). A standard curve was constructed and VEGF concentrations were plotted using SigmaPlot10.0. In some conditions, cells were pretreated the $\alpha_5\beta_1$ integrin activating antibody SNAKA51 for 30 minutes prior to DV incubation (kindly provided by Martin Humphries) (Clark et al., 2005).

Gene expression analysis using qPCR

A 12 well plate of cells was grown to confluency. Prior to the experiment, cells were serum starved overnight in 1% FBS IMDM media. The cells were then washed with PBS and fresh 1%IMDM +/-DV (20µg/mL) was added back to the wells and incubated at 37C and 5% CO_2 for various time points. At the end of the experiment, the media was aspirated, cells were rinsed with PBS, and the protocol from RNEASY MiniKit was followed. (Cat. #74104). Samples were quantified using a spectrophotometer and qualitative analysis was performed by running samples on a 1% agarose gel. First-strand

cDNA synthesis used cloned AMV RT for RT-PCR. cDNA from each sample was prepared following the Invitrogen cloned AMV Reverse Transcriptase protocol (Cat. No.12328-019). Briefly, the following components were mixed in a RNASE-Free eppendorf tube: 1uL oligo(dT)20 (500ug/mL), 1ug total RNA, 2uL 10mM dNTP Mix. Samples were incubated at 65C for 5 min collected by centrifugation and the following components were added: 4uL 5x cDNA Synthesis buffer, 1uL 0.1M DTT, 1uL Cloned AMV RT (15units/uL). The final volume was adjusted to 20uL and the samples were mixed and incubated at room temp for 10 min. The samples were then incubated at 45C for 1hr. The reaction was terminated by heating the samples at 85C for 5min. The samples were then adjusted to a final volume 200uL with RNA-FREE H_2O. qPCR was performed using the following products: TaqMan® Fast Universal PCR Master Mix (2X), No AmpErase® UNG Cat. No. 4352042, MicroAmP® Fast 96-Well Reaction Plate, 0.1mL Cat. No. 4346907 (AppliedBiosystems). Primer and probe sets designed for Vascular Endothelial Growth factor A. Assay ID Mm00437304_m1, Glyceraldehyde-3-phosphate dehydrogenase Assay ID Mm99999915_g1 and Integrin alpha-5. Assay ID Mm00439797_m1 were used. The following were mixed to a final volume of 25uL and added to TaqMan® Fast 96-well Reaction Plate: Fast Universal PCR Master Mix, gene expression assay mix, cDNA and H20. The amount of cDNA to add for each gene expression was optimized so that the dCT was around 18cycles. Once the reaction plate was complete, ΔCT and ΔΔCT values were calculated using Applied Biosystems 7500 Fast Reverse transcriptase PCR software.

Cell signaling western immunoblot

Confluent mouse brain microvascular endothelial cells or the same cells with shRNA mediated knockdown of $\alpha_5\beta_1$ integrin were serum-starved for 24h prior their exposure to DV (20µg/mL). In experiments involving inhibitors, drugs were diluted in DMSO and incubated for 1hour at 37C and 5% CO_2 prior DV addition. In all cases, the DMSO maximum concentration reached was not over 0.1%. PI3Kinase, MEK1 and ERK inhibition experiments were performed by incubating cells in the presence of 10µM LY294002, 10µM U0126 or 10µM PD98059 (Cell Signaling Technology), respectively 1h prior to DV exposure. Cells were exposed to DV for 0, 5, 15 and 30 minutes. Following DV exposure, cells were washed with ice-cold PBS and homogenized in cell lysis buffer (Cell Signaling Technology) complemented with protease inhibitor cocktail (Calbiochem, EMD Chemicals, San Diego, CA). Accurate protein concentration was determined using BCA protein assay reagent (Thermo Scientific). 20 µg/lane of total protein from each sample were loaded in a 10% SDS-PAGE gel. Following electrophoresis (170 volts for 1 hour) samples were transferred on PVDF membranes. Membranes were incubated in blocking buffer (5% BSA/ TBS) for 1h at room temperature followed by an overnight incubation at 4ºC in the presence of antibodies directed against phospho-Akt (1:1000, Cell Signaling), pan-Akt (1:1000, Cell Signaling), phospho-ERK1/2 (1:1000, rabbit, R&D Systems), pan-ERK1/2 (1:1000, R&D Systems), phospho-eIF4E (Cell Signaling), eIF4E (Cell Signaling), HIF-1α (1:500, Novus Biologicals) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000, Sigma-Aldrich). Membranes were washed with TBS-

0.1% Tween 20 and incubated in presence of horseradish peroxidase (HRP) conjugated secondary antibody (GeneTex, Irvine, CA). Band detection was performed by enhanced chemiluminescent substrate (Picowest Signal, Thermo-Fisher Scientific) and captured by X-ray films. Blot quantification was performed using ImageJ software (ImageJ, NIH, Bethesda, MD).

Statistical analysis

Data are presented as Mean +/- standard deviation (unless otherwise stated). Statistical significance ($p<0.05$) was determined for all experiments by *Student's* unpaired t-test with the Sigmastat software package.

Results and discussion

DV induces VEGF secretion in brain endothelial cells by increasing VEGF mRNA expression

In order to investigate how DV is promoting angiogenesis on brain endothelial cells, I investigated the possibility that DV regulates growth factors that are involved with angiogenesis following ischemia. I decided to determine if DV's pro-angiogenic effect is due to the positive regulation of VEGF because [1] Hypoxia leads to a transient increase in expression of VEGF on several cell types, including endothelial cells, in the brains of both rats and mice (Ogunshola et al., 2000). [2] $VEGF_{165}$ has been linked to induction of perlecan synthesis in human brain

microvascular endothelial cells (Kaji et al., 2006), suggesting a possible mechanism for how the brain replenishes the ECM following ischemia. [3] Interestingly, DV's anti-angiogenic effect is linked to the suppression of VEGF signaling in HUVEC due to the dephosphorylation of VEGFR2 (Nystrom et al., 2009). Therefore, I hypothesized that DV's pro-angiogenic effect on brain endothelial cells involves $VEGF_{165}$ (hereafter referred to as VEGF) stimulation. To the best of my knowledge, VEGF synthesis and release have not been previously linked to the activation of the $\alpha_5\beta_1$ integrin.

First I asked whether DV could increase levels of mRNA for VEGF and its receptor (VEGFR2) in brain endothelial cells in vitro using qPCR. Brain endothelial cells were treated with DV for 1.5 and 3 hours. VEGF mRNA levels were significantly increased at these time points, (8.2 fold and 2 fold, p=0.001 and p=0.003, respectively) and VEGFR2 mRNA levels (1.5 and 1.8 fold, p=0.002 and p=0.007, respectively) **(Figure 4.1).** Earlier time points show no significant increase with DV treatment yet, later time points demonstrated that these levels go back to normal (data not shown). These results indicate that DV positively regulates both VEGF and VEGFR2 mRNA levels.

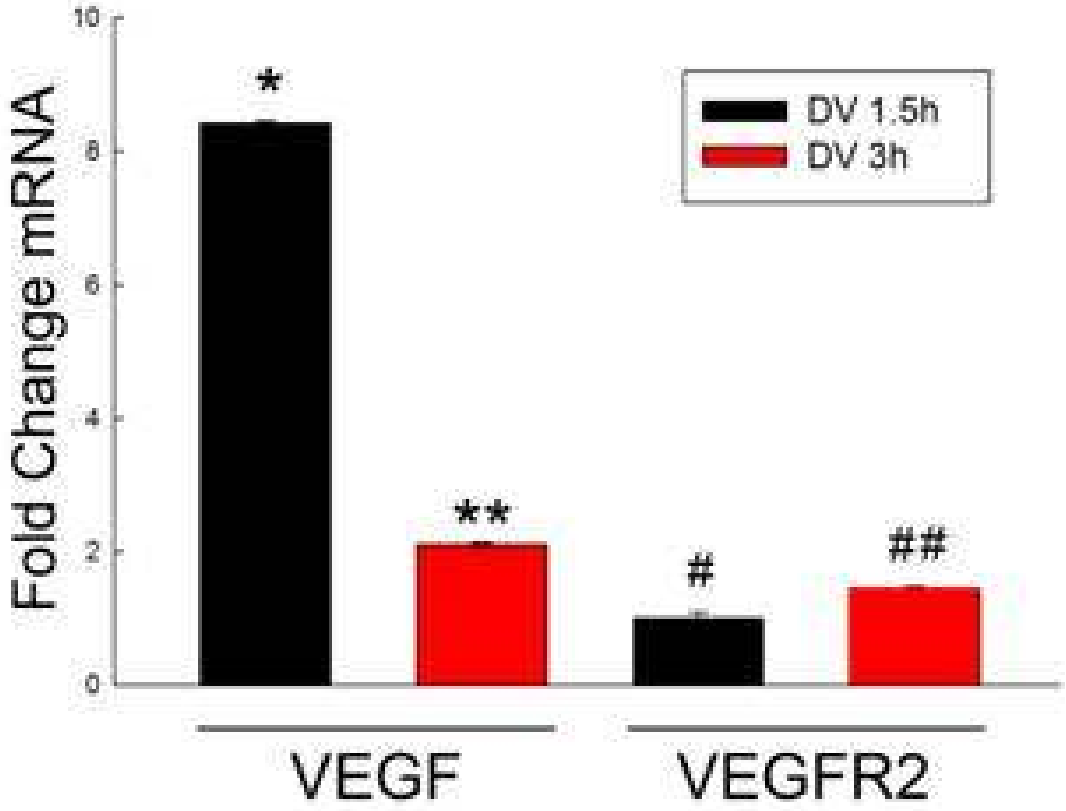

Figure 4.1. DV increases VEGF and VEGFR2 mRNA expression. qPCR from mouse brain endothelial cells ±DV treatment for 1.5 or 3 hours demonstrates a significant increase in VEGF mRNA (*p=0.001, **p=0.003) and VEGFR2 mRNA (#p=0.02, ##p=0.007) as compared to control (normalized to one and then shown in natural log scale).

To determine whether the increase in VEGF expression results in an increase in VEGF secretion, brain endothelial cells were treated with DV [20ug/mL] for 1.5 to 24 hours, and the cultured media was analyzed for VEGF concentration using a VEGF ELISA kit. DV significantly increased VEGF secretion at all time points, and its effect peaked by nine hours, after which VEGF levels started to decline (**Figure 4.2**). My results also demonstrate that DV significantly increases VEGF secretion in a dose dependent fashion (**Figure 4.3**) over the course of nine hours. Collectively, these results demonstrate DV significantly increases VEGF protein secretion by brain endothelial cells. I next examined whether the VEGF secreted in the media by DV plays a role in DV's in vitro pro-angiogenic effect. In order to investigate this hypothesis, I performed proliferation and matrigel tube-like formation experiments (previously described) using a VEGF neutralizing antibody. C75BL6 brain endothelial cells treated with the VEGF neutralizing function blocking antibody alone had no significant effects on proliferation or tube like formation. I was able to significantly block DV's pro-proliferative effect using the VEGF neutralizing antibody (**Figure 4.4**).

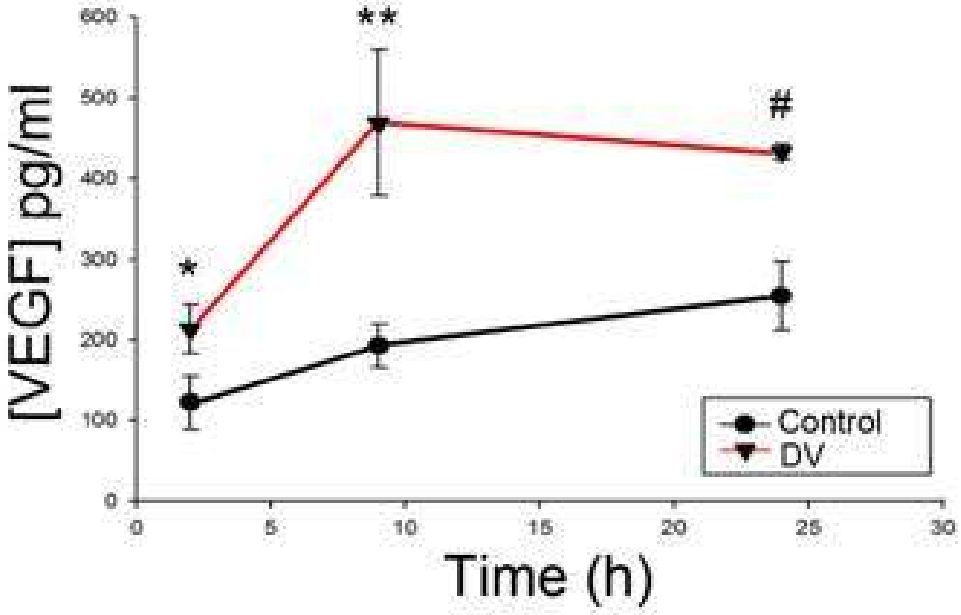

Figure 4.2. DV causes a significant release of VEGF from brain endothelial cells. VEGF ELISA of conditioned media from brain endothelial cells treated with DV [20ug/mL] at times indicated. N=3 for each time point measured. Values are mean ± standard deviation. DV caused a significant increase in measured VEGF levels at all time points measured (*p=0.01, **p=0.002, #p=0.006).

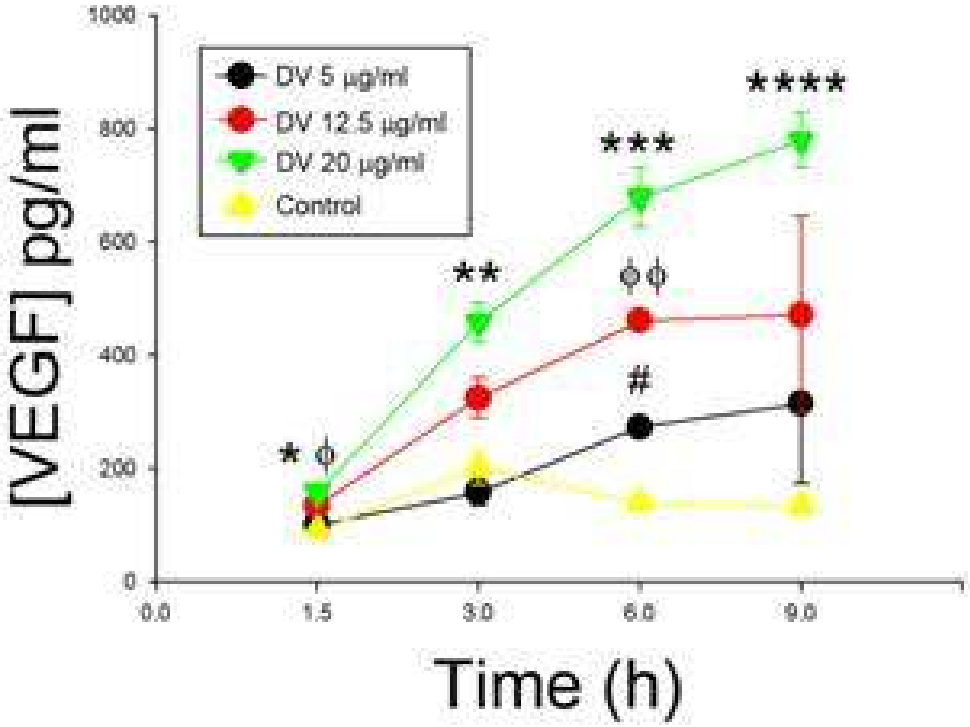

Figure 4.3. DV causes brain endothelial cells to secrete VEGF in a dose dependent manner. VEGF ELISA of conditioned media from cells treated with DV at the times indicated. N=3 for each point. Values are mean ± standard deviation. DV causes a significant increase in measured VEGF levels at all time points measured indicating DV's response is dose dependent (*p=0.04, **p=0.001, ***p=0.005, ****p=0.00004, ϕp=0.03, ϕϕp=0.002, #p=0.003) Values are mean ± standard deviation.

However, brain endothelial cells treated with VEGF function blocker and DV had minimal effects on tube like formation compared to cell treated with DV alone (**Figure 4.5**). These results suggest DV's pro-angiogenic effect in vitro is only partly due to VEGF secretion induced by DV. Other possibilities that could be involved with DV's pro-angiogenic effect include DV's ability to secrete other growth factors that are involved with regulating angiogenesis such as BDNF, FGF and EGF.

Collectively, these results suggest DV can induce the secretion of VEGF by brain endothelial cells by increasing VEGF mRNA. These results also imply that DV increases VEGFR2 protein levels because DV causes an increase in mRNA levels of VEGFR2. The ability of the VEGF neutralizing antibody to block DV's pro-proliferative activity as well as mitigate its pro-tube-like formation effect, implies that the pro-angiogenic effect of DV is a two step mechanism. DV induces VEGF secretion, and newly secreted VEGF causes the pro-angiogenic response effect in vitro. Taken together, these results imply DV can promote a pro-angiogenic environment by increasing soluble growth factors and their receptors. My results also suggest a positive feedback loop mechanism by which the ECM is restored following injury. Fragments of perlecan are released and cause the secretion of VEGF. Newly secreted VEGF is able to bind to VEGFR2 and cause the subsequent synthesis of perlecan, where it can now be secreted into the ECM for restoration and remolding.

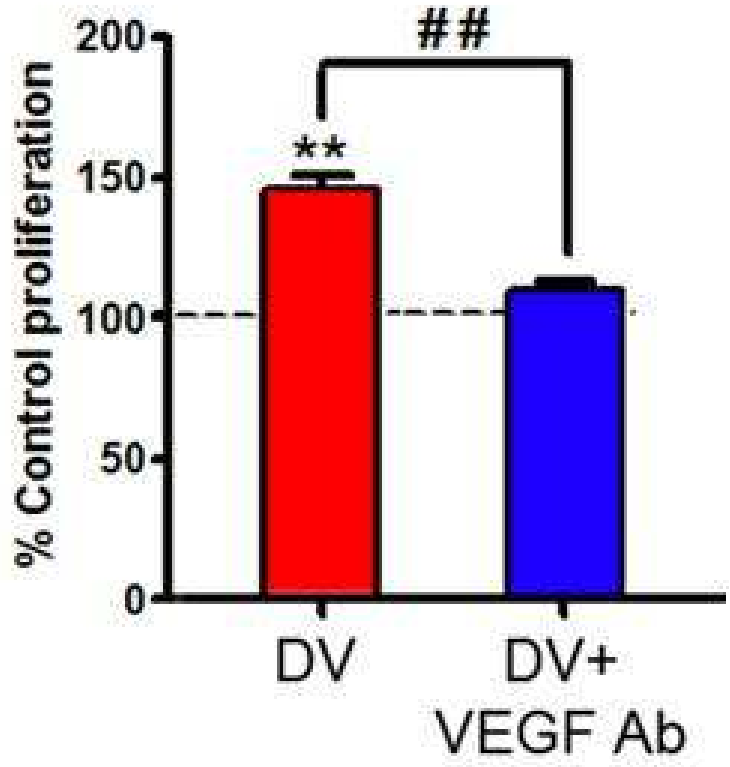

Figure 4.4. VEGF function blocking antibody inhibits DV's pro-proliferative effect. brain endothelial cells were treated in the presence or absence of DV [20 ug/mL] ±VEGF [20 ug/mL] neutralizing antibody. These results indicate that DV's pro-proliferative effect is due to DV's induction of VEGF secretion.

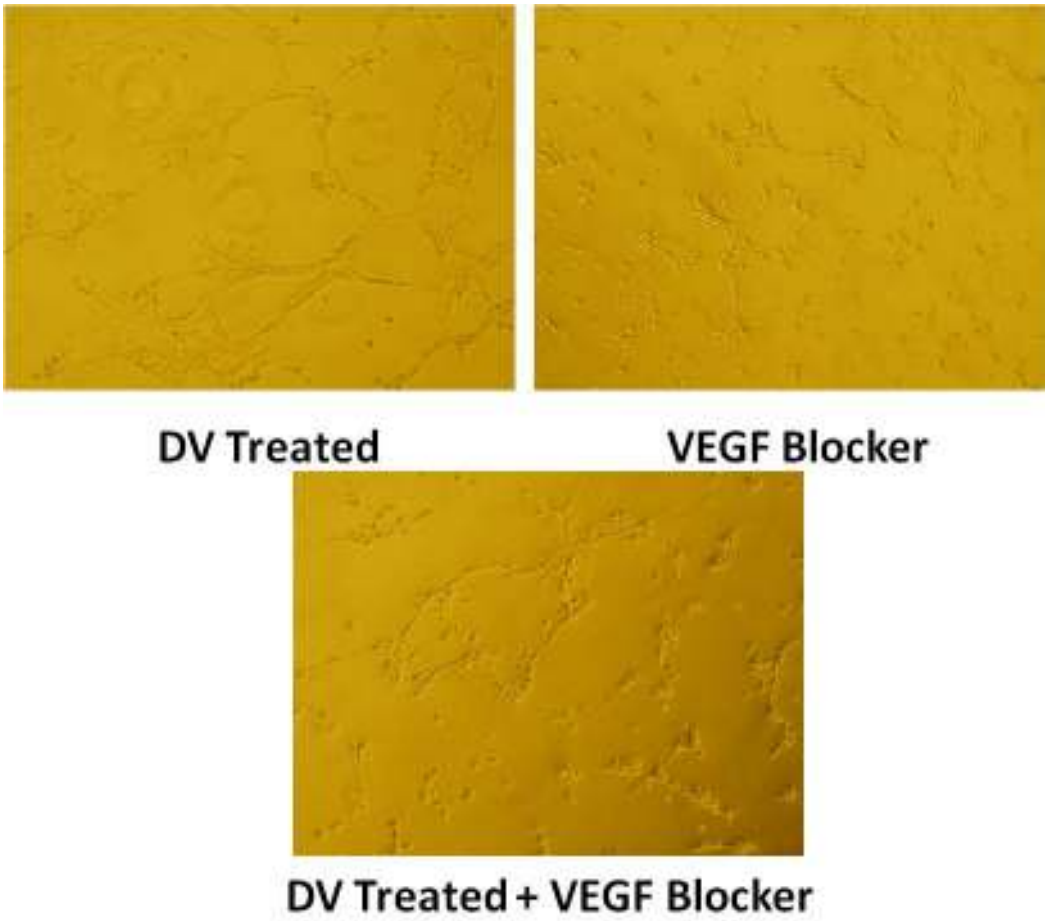

Figure 4.5. VEGF function blocker retards DV's effect on tube-like morphogenesis. brain endothelial cells were treated in the presence and absence of DV [20 ug/mL] ± VEGF [20 ug/mL] neutralizing antibody. DV promoted tube-like morphogenesis while VEGF neutralizing function blocking antibody retarded the DV effect.

The α5β1 integrin is involved with DV's effect on VEGF expression and secretion

Having demonstrated that DV's pro-angiogenic effects are $\alpha_5\beta_1$ integrin and VEGF dependent, I next hypothesized DV's effect on VEGF expression and secretion could be due to DV's interaction with the $\alpha_5\beta_1$ integrin. In order to test this hypothesis, I utilized the brain endothelial cell $\alpha_5\beta_1$ integrin knockdown cell line and the SNAKA-51 antibody. The SNAKA-51 antibody was used to convert the $\alpha_5\beta_1$ integrin into a ligand-competent form. Because the maximal effect of VEGF expression was seen at three hours and DV's most potent concentration observed thus far was 20 μg/mL, brain endothelial deficient in $\alpha_5\beta_1$ integrin were treated ± DV (20 μg/mL) for three hours, and VEGF expression was quantified. VEGF expression was not induced by DV in brain endothelial cells with $\alpha_5\beta_1$ integrin knockdown (**Figure 4.6**). This suggests that the interaction of DV and $\alpha_5\beta_1$ integrin is necessary for DV's induction of VEGF expression. In an experiment complementary to $\alpha_5\beta_1$ integrin knockdown, I activated the $\alpha_5\beta_1$ integrin with the SNAKA-51 antibody, which activates $\alpha_5\beta_1$ integrin by binding outside the ligand binding domain (Clark et al., 2005), and assayed VEGF secretion (**Figure 4.7**). Interestingly, activating the $\alpha_5\beta_1$ integrin with the SNAKA-51 antibody significantly induced VEGF secretion by itself suggesting that activation of $\alpha_5\beta_1$ integrin can cause VEGF expression or some contamination in the media is causing VEGF secretion that is not significant when the integrin is not activated. Importantly, when SNAKA-51 antibody was added in combination with DV, even more VEGF was secreted by brain endothelial cells than by either addition alone, suggesting that activation of $\alpha_5\beta_1$

integrin with SNAKA-51 enhances DV-induced VEGF secretion. These results also suggest most cells have the inactive form of this integrin and therefore, DV is slightly able to induce VEGF secretion because some active forms of this integrin exist due to cations present in the media. Yet when there is an abundance of activated integrin, DV is able to induce more VEGF secretion. DV could also be acting as an activator of the $\alpha_5\beta_1$ integrin yet in this case I would expect to observed SNAKA-51 and DV to have similar effects on VEGF secretion. Suggesting DV probably activates this integrin and causes downstream signaling that is sufficient for VEGF secretion. Because VEGF release has also been tied to the $\alpha_V\beta_3$ integrin (Mousa et al., 1999), I investigated if this integrin played a role in DV's effect on VEGF secretion. Human D3 brain endothelial cells were treated with DV ± $\alpha_V\beta_3$ integrin neutralizing antibody (1 μg/mL). This antibody was unable to inhibit DV-induced VEGF secretion suggesting that DV-induced VEGF release was unlikely the result from DV interacting with $\alpha_V\beta_3$ integrin (**Figure 4.8**).

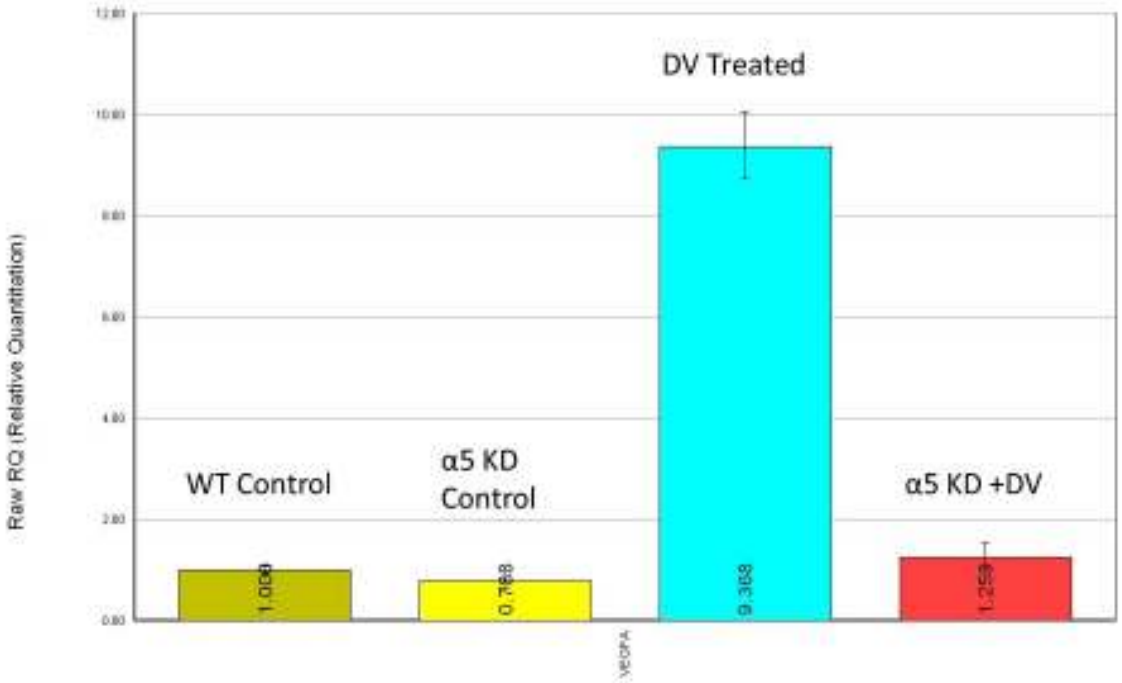

Figure 4.6. DV interaction with the $\alpha_5\beta_1$ integrin is crucial for DV's effect on VEGF expression. WT brain endothelial and $\alpha_5\beta_1$ integrin null brain endothelial cells were treated with DV (20ug/mL) for three hours. Knockdown of $\alpha_5\beta_1$ integrin significantly blocks DV's induction of VEGF mRNA expression as compared to WT brain endothelial cells treated with DV. Untreated WT brain endothelial cells were normalized to zero.

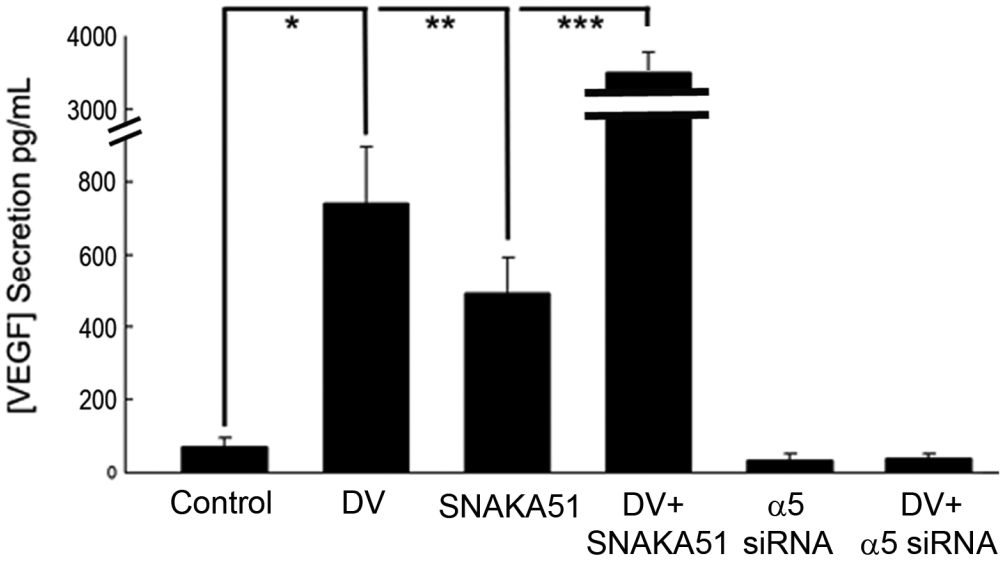

Figure 4.7. DV induced VEGF secretion is $\alpha_5\beta_1$ integrin dependent. Brain endothelial cells treated with SNAKA-51 activating $\alpha_5\beta_1$ integrin antibody [20 μg/mL] significantly increases VEGF secretion. Combination of SNAKA-51 activating $\alpha_5\beta_1$ integrin antibody and DV [20 μg/mL] significantly increases DV's induction of VEGF secretion as compared to DV alone. Brain endothelial cells with knockdown of $\alpha_5\beta_1$ integrin are nonresponsive to DV treatment.

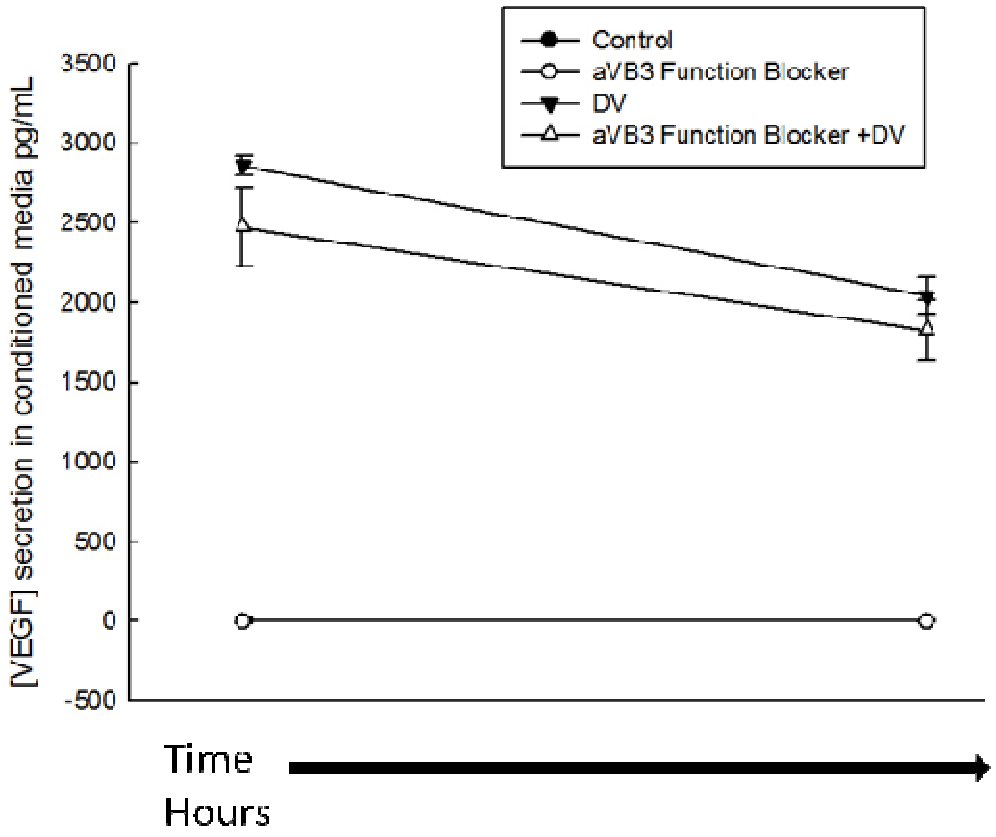

Figure 4.8. $\alpha_V\beta_3$ integrin does not play a role in DV induced VEGF secretion. Human D3 brain endothelial cells were treated with DV (20 µg/mL) for three and nine hours. DV was able to significantly induce VEGF secretion from human D3 brain endothelial cells. Co-incubating $\alpha_V\beta_3$ function blocking antibody (1 µg/mL) with DV had no significant effect on DV's induction of VEGF secretion. Control and $\alpha_V\beta_3$ function blocker did not have detectable amounts of VEGF in the media.

DV activates a novel signaling pathway to regulate VEGF expression

I next investigated whether several cell signal transduction components known to be involved in VEGF release might be activated by DV exposure and whether inhibition of these factors could inhibit DV-induced VEGF release. As Akt and ERK activation have been implicated in VEGF production and release (Berra et al., 2000), I determined whether DV could activate Akt and ERK in brain endothelial cells. **Figure 4.9** demonstrates that, as assayed by western blot, DV addition to brain microvascular endothelial cells results in AKT phosphorylation/activation. AKT phosphorylation remained elevated for at least 30 minutes (**p=0.02) as normalized to total Akt compared to controls. Furthermore, this DV-induced Akt activation could be completely inhibited by the addition of the PI3K inhibitor LY-294002. Multiple concentrations of total protein lysate were run to demonstrate a significant difference between untreated and treated cells. Next, I showed that DV phosphorylates/activates ERK in brain microvascular endothelial cells after 5 minutes (*p=0.001). ERK phosphorylation also remained elevated for at least 30 minutes (**p=0.02) as normalized to total ERK signal (**Figure 4.10**). This phosphorylation could be inhibited with the PI3K inhibitor LY-294002 (#p=0.003, ##p=0.009 as compared to DV changes). These data suggest DV activates AKT which then leads to the activation of ERK.

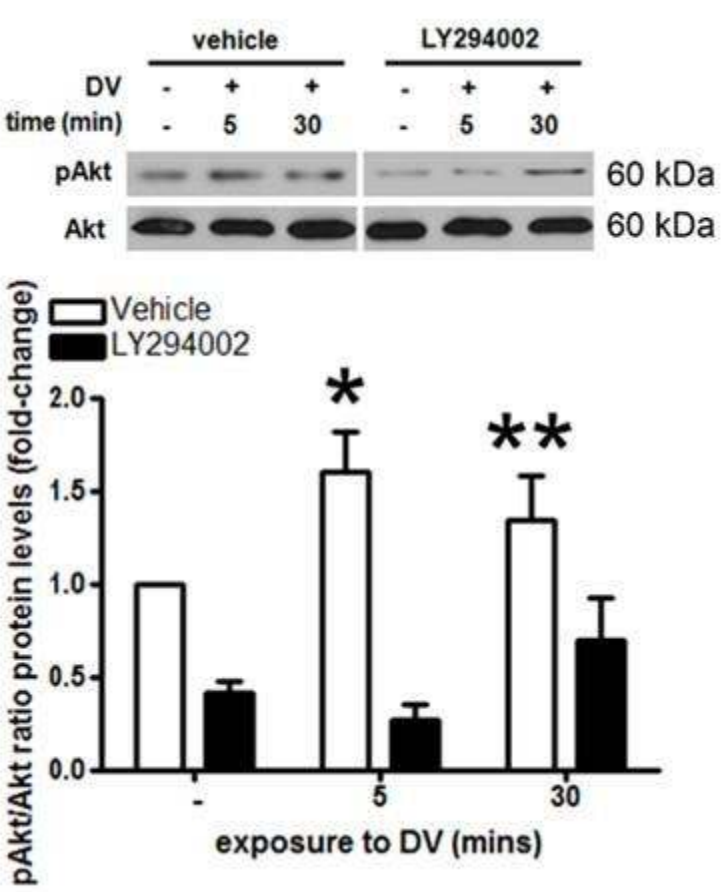

Figure 4.9. DV activates AKT by phosphorylation. DV phosphorylates/activates brain endothelial cell Akt after 5 minutes (*p=0.005 versus control) and 30 minutes (**p=0.02 versus control) as demonstrated with representative western blot and plot (mean fold-change +/- standard deviation plotted) as normalized to total Akt signal. This activation is inhibited with LY294002.

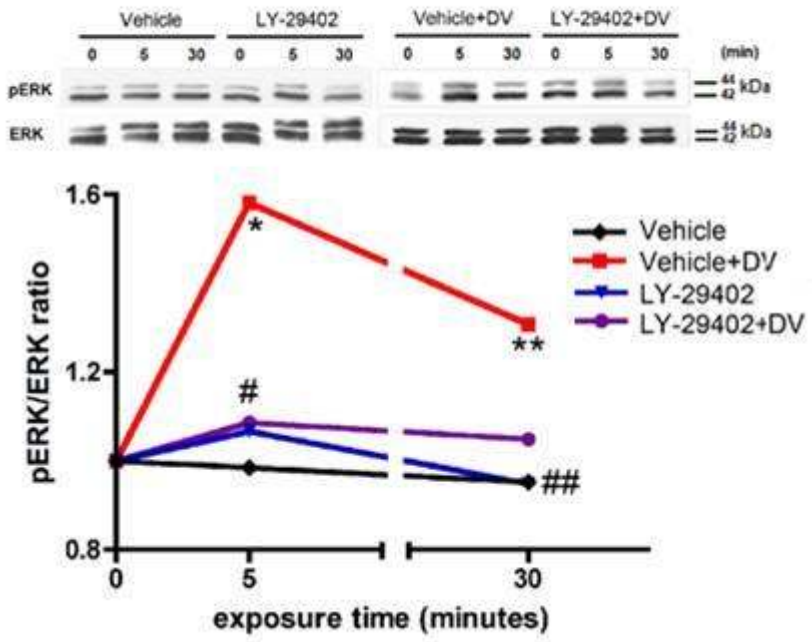

Figure 4.10. DV causes phosphorylation of ERK. DV phosphorylates/activates ERK in brain microvascular endothelial cells after 5 minutes (*p=0.001, which persists to at least 30 minutes, **p=0.02) as demonstrated with representative western blot and plot as normalized to total ERK signal. This transient phosphorylation could be inhibited with the PI3K inhibitor LY-294002 (#p=0.003, ##p=0.009 as compared to DV changes). The inhibitor by itself had no significant effect on ERK phosphorylation.

Under both normoxic and hypoxic conditions (Karni, 2002), VEGF regulation has been linked to hypoxia-induced factor-1α (HIF-1α) activation. Under normoxic conditions, HIF-1α is constantly degraded and almost nonexistent, (Kaur, 2005) yet studies performed by Karni et al. demonstrate HIF-1α expression can also exist under normoxic conditions (Karni, 2002). Therefore, I investigated whether DV treatment could causes changes in HIF-1α levels in brain endothelial cells (**Figure 4.11**). Application of 0.1% DMSO (vehicle) transiently stabilized HIF-1α which then rapidly became undetectable. Interestingly, DV induced rapid stabilization of HIF-1α which remained stable for up to 15 minutes, followed by a rapid decrease to barely detectable levels after 30 minutes. Because eukaryotic translation initiation factor 4E (eIF4e) activation is linked to MAP kinase and PI-3 kinase signaling and activation of HIF-1α (Fukuda, 2002), the next logical factor to look at that is involved with HIF-1α expression was eIF4e activation (**Figure 4.12**). HIF-1α stabilization correlated with increased phosphorylation of eIF4E induced by DV. In addition, usage of U0126, a potent MEK inhibitor, reduced phosphorylation of eIF4e, but did not have an effect on DV's stabilization of HIF-1α.

Because DV mediates VEGF expression through the $\alpha_5\beta_1$ integrin, I next investigated the activation of the same signaling molecules in my $\alpha_5\beta_1$ integrin knockdown brain endothelial cells. Surprisingly, in $\alpha_5\beta_1$ integrin knockdown brain endothelial cells (**Figure 4.12**) I observed constitutively HIF-1α stabilization. However, $\alpha_5\beta_1$ integrin null brain endothelial cells in the presence of DV did not further significantly enhance HIF-1α levels suggesting that HIF-1α stabilization by DV is strictly regulated by $\alpha_5\beta_1$ upstream signaling. Along the same lines as these results, $\alpha_5\beta_1$ integrin null brain endothelial cells had constitutively activated eIF4E (Figure 4.12). DV had no significant effect on phosphorylation of eIF4E in $\alpha_5\beta_1$ integrin null brain endothelial cells. Phosphorylation of eIF4E in $\alpha_5\beta_1$ integrin null brain endothelial cells was not blocked by U0126 inhibitor. This suggests a separate pathway besides MAP kinase involved with the phosphorylation of eIF4E. Yet, because I did not see any significant changes in phosphorylation of eIF4E by DV in $\alpha_5\beta_1$ integrin null brain endothelial cells plus U0126 inhibitor, my data suggests that $\alpha_5\beta_1$ integrin is still required for DV's phosphorylation of eIF4E and stabilization of HIF-1α.

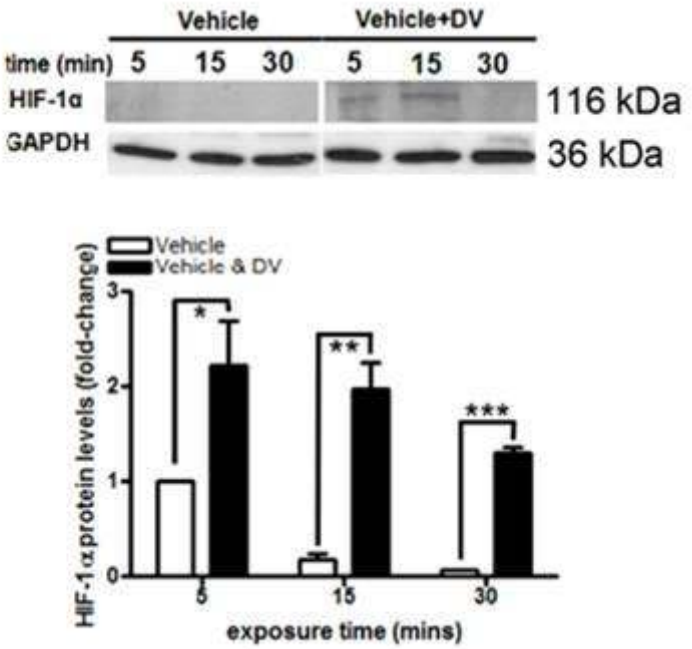

Figure 4.11 DV increases accumulation of HIF-1α. DV treatment results in a sustained (from 5 to 30 min) increase of HIF-1α levels in brain endothelial cells as demonstrated with representative western blot and plot (mean fold change as normalized to GAPDH loading control +/- standard deviation, *p=0.04, **p=0.01, ***p=0.001).

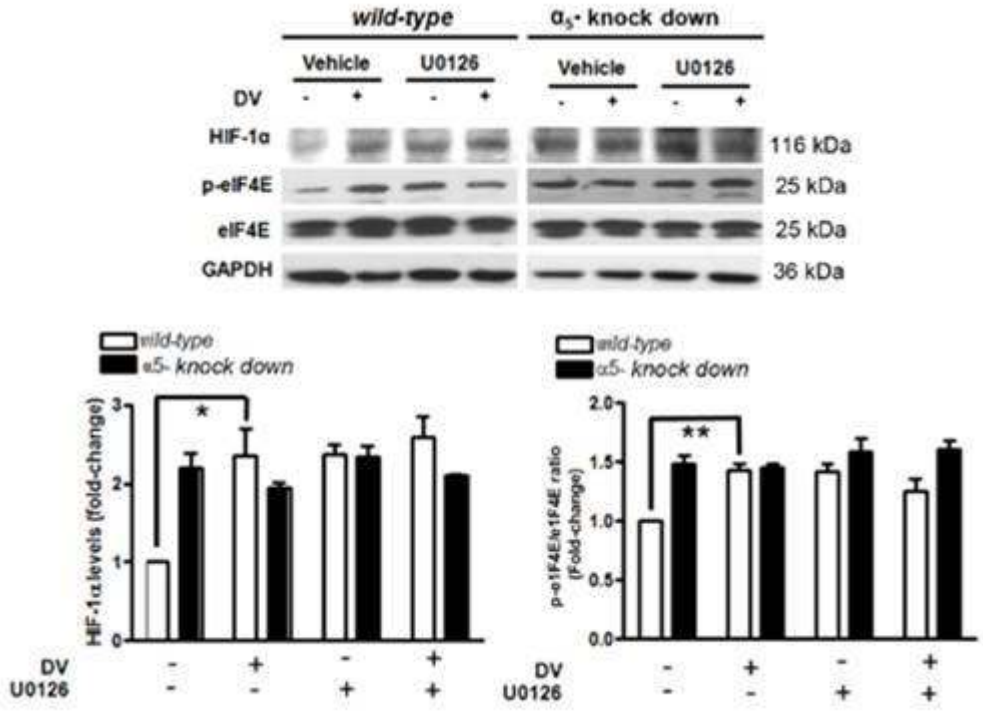

Figure 4.12 $\alpha_5\beta_1$ integrin knockdown mediates DV activation of HIF-1α and eIF4E. DV increases HIF-1α levels and phosphorylation/activation of e1F4E in an $\alpha_5\beta_1$ integrin dependent fashion as demonstrated by representative western blots and respective plots (for HIF-1a, *p= 0.02, plotted as mean fold change +/- standard deviation) for e1F4E, **p=0.004, plotted a fold change in the ratio of phosphorylated e1F4E to total e1F4E. In both cases, DV-induced changes were absent in α5β1 knocked down cells and were blocked in wild type cells by the MEK inhibitor U0126.

I next investigated changes in phosphorylated Akt and ERK in $\alpha_5\beta_1$ integrin null brain endothelial cells following exposure to DV and/or to U0126 (**Figure 4.13**) to investigate if DV activation of these molecules was dependent upon the presence of this integrin. As previously observed, DV treatment significantly increased both phospho-Akt (**Figure 4.13**) and phospho-ERK (**Figure 4.13**) in wild-type cells. Whereas U0126 treatment significantly affected DV-induced ERK phosphorylation, I observed a slight decrease in phospho-Akt levels. Interestingly, $\alpha_5\beta_1$ integrin null brain endothelial cells, displayed significantly high levels of phospho-Akt whereas phospho-ERK levels were significantly low compared to wild-type in control conditions. In contrast to wild-type cells, DV did not significantly increase phosphorylation of AKT or ERK in $\alpha_5\beta_1$ integrin null brain endothelial cells. U0126 significantly blocked ERK phosphorylation in $\alpha_5\beta_1$ integrin null brain endothelial cells without affecting phospho-AKT levels suggesting that HIF-1α stabilization in knockdown cells may be controlled by Akt, rather than ERK.

Finally, ERK signaling pathway inhibition by U0126 (**Figure 4.14**) significantly blocked DV-induced VEGF mRNA levels as well as VEGF secretion (**Figure 4.15**). Collectively, these studies demonstrated the ability of DV to independently activate Akt and ERK in mouse brain endothelial cells. This results in HIF-1α stabilization through a eIF4E dependent mechanism and ultimately leads to the secretion of VEGF.

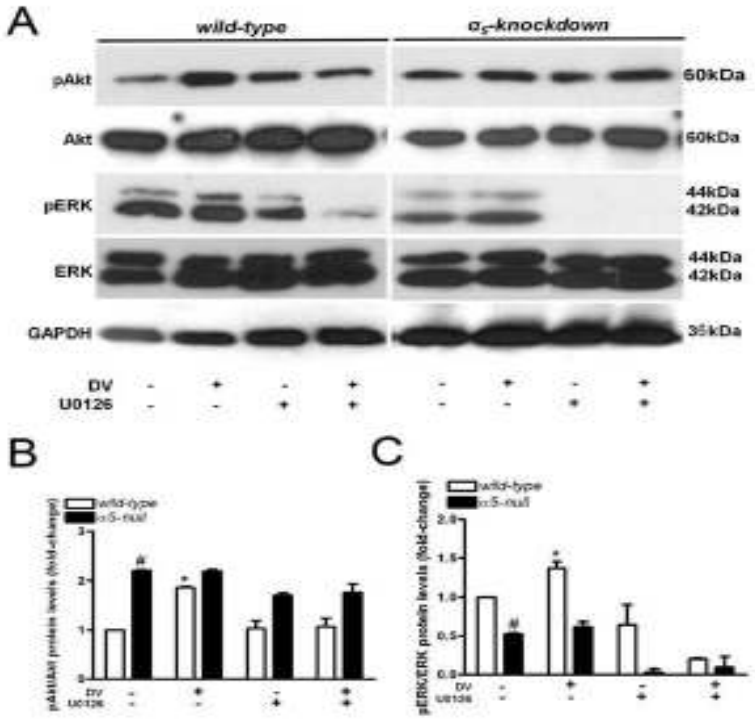

Figure 4.13. $\alpha_5\beta_1$ integrin suppression decreases phospho-ERK levels and increases phospho-Akt levels. (a) Representative Western-blot of phospho-Akt and phospho-ERK levels in wild-type and α_5-knockdown cells. Cells were treated without or with 20µg/mL of DV and/or 10µM U0126 (MEK inhibitor). Pan-Akt and ERK were used to quantify changes in phosphorylation levels, GAPDH was used as an internal loading control. (b) Akt phosphorylation (pAkt) is induced by DV in wild-type cells, not in knockdown cells. Note the significant increase in pAkt levels compared to baseline (*p=0.078) in wild-type, whereas α_5-knockdown showed constitutive high pAkt levels under basal conditions ($^{\#}$p=0.0344). (c) ERK phosphorylation is up-regulated by DV and down-regulated in α_5-knockdown cells. Note the significant increase (*p=0.0336) following DV treatment. Treatment with U0126 abolishes DV-induced ERK phosphorylation (pERK). Surprisingly, α_5-knockdown cells showed hypophosphorylated pERK levels ($^{\#}$p=0.0002). DV was not able to increase pERK levels in knockdown cells.

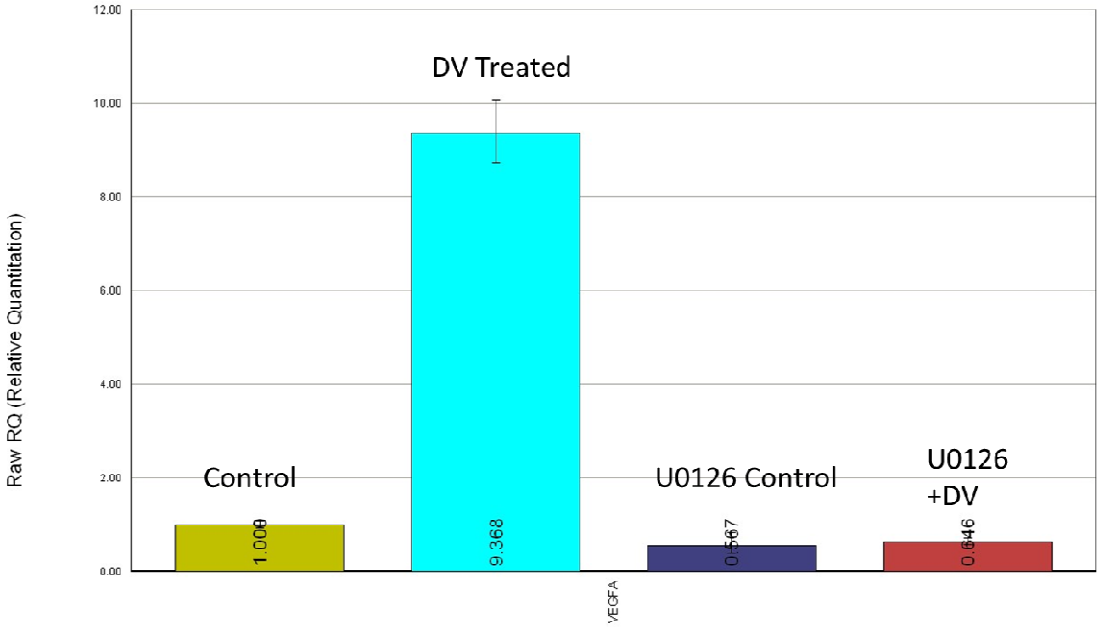

Figure 4.14.Blockade of ERK by inhibition blocks DV's positive effect on VEGF mRNA expression. Inhibition of ERK with U0126 inhibits VEGF mRNA production in brain microvascular endothelial cells (*p=0.02) which could not be prevented with the addition of DV (**p=0.05). Mean values +/- standard deviation shown.

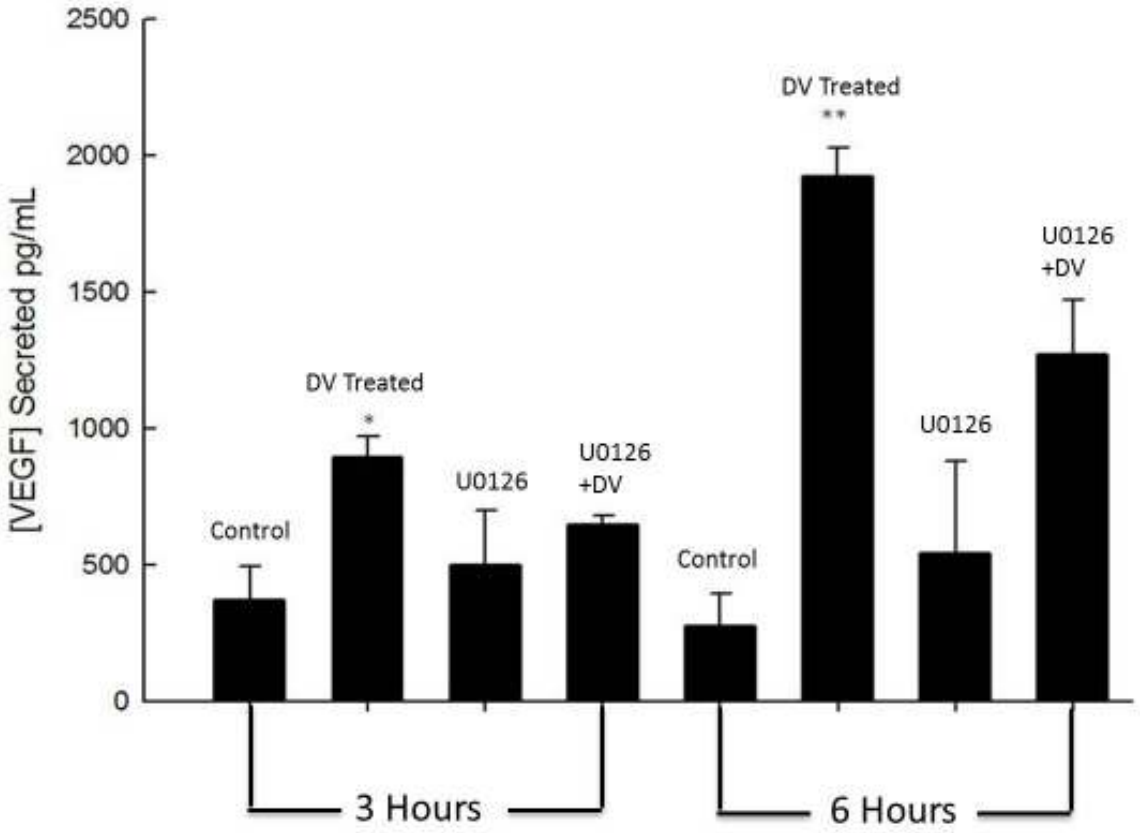

Figure 4.15. ERK inhibitor U0126 significantly blocks DV's effect on VEGF secretion. Inhibition of ERK with U0126 inhibitor significantly blocks DV's effect on VEGF secretion at three (*p=0.01) and six hour incubation (**p=0.04). Mean values +/- standard deviation shown.

VEGF has been implicated in regulating vascular remodeling in post-ischemic neurovascular coupling (Hermann and Zechariah, 2009). Five separate iso-forms of VEGF-A exist, differing in the number of amino acids and affinity to their receptors. The most abundant iso-form found in the brain, $VEGF_{165}$ (investigated in this dissertation), has been shown to regulate proliferation, migration and tube formation in endothelial cells (Kaji et al., 2006), (Katsumata, 2010). This iso-form, along with $VEGF_{121}$, are more diffusible variants allowing these two VEGF forms to act over long distances compared to $VEGF_{189}$ and $VEGF_{206}$, which are matrix bound and can only provide short distance guidance clues (Kaji et al., 2006). Current evidence suggests extracellular matrix proteolysis, i.e. the generation of matrix fragments such as DV, induces VEGF following ischemia (Hermann and Zechariah, 2009). Interestingly, VEGF has emerged as a "double-edged sword" in stroke research. If given too soon after the onset of stroke, it promotes a leaky blood brain barrier, edema, hemorrhagic transformation and can ultimately worsened brain injury (Zhang et al., 2000). However, when administered later and more chronically, such as 24 hours post-stroke, VEGF it is pro-angiogenic, neuroprotective, and enhances neurogenesis (Sun et al., 2010) (Ferrara, 2003). Therefore the timing of when VEGF is administered or released is critical in the overall outcome of brain repair.

Current evidence linking VEGF secretion to ECM components is limited, especially in brain endothelial cells following ischemia. The work performed by Zong-Mei et al and Mousa et al does provide some evidence that ECM components can

induce the secretion of VEGF in retinal pigmented endothelial cells. Zong-Mei et al have discovered that thrombin is able to induce VEGF secretion in retinal epithelial cells (Zong-Mei, 2007). Research performed by Mousa et al demonstrate VEGF induced secretion, using retinal pigmented endothelial cells, is differentially affected by normoxia, hypoxia, ECM components and integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ (Mousa et al., 1999) . Indeed, as mentioned earlier, there is matrix proteolysis and up-regulation of VEGF following ischemia that happens simultaneously, yet no research has been performed to determine what role matrix fragments, such as DV, play in the brain following ischemia.

My results indicate that DV can significantly and dose-dependently increase the secretion of VEGF by brain microvascular endothelial cells in vitro. This up-regulation of VEGF secretion induced by DV is correlated to an increase in mRNA expression of the VEGF gene. DV significantly increases VEGF mRNA as early as 1.5 hours and tapers off after six hours with DV treatment. It was not until VEGF was significantly upregulated at the mRNA level, 1.5 hours, until I began to observe a significant increase in VEGF that was secreted into the media. Suggesting DV is not effecting protein stability within the cell yet instead affecting protein production. Soluble VEGF secreted into the media accumulated for nine hours before degradation of VEGF was detected at 24hrs. An explanation for this observation is the biological half-life of VEGF. The biological half-life of “free” $VEGF_{165}$ is 90 minutes, and VEGF release from collagen plateaus after 12 hours and no longer detectable following 24 hours (Kleinheinz, 2010). As my mRNA data are

only “freeze frames” in time, it would be interesting to see how long VEGF is actually expressed in cells treated with DV and if there is a correlation of VEGF expression and DV presence. In order to do such an experiment, one could incorporate luciferase-based assays to monitor VEGF expression in real time and soluble DV by means of western blot.

Due to my observations that DV increases VEGF secretion into the media and DV promotes endothelial cell proliferation and tube like morphogenesis, I next investigated if DV’s pro-angiogenic effect was due to the subsequent secretion of VEGF. In order to investigate the correlation of these phenomena, proliferation and tube like morphogenesis were assayed with DV in the presence of VEGF neutralizing antibody. These experiments suggest when VEGF is neutralized, DV’s effect on promoting brain endothelial cell proliferation is significantly reduced and brain endothelial cell tube like morphogenesis is inhibited as well. Collectively, these data suggest DV’s pro-angiogenic effect is due to the secretion of VEGF.

To the best of my knowledge, my discovery of a direct association between $\alpha_5\beta_1$ ligand binding and VEGF release in brain endothelial cells has not previously been shown. Specifically, I have demonstrated that $\alpha_5\beta_1$ integrin is essential for DV-induced VEGF mRNA expression via shRNA knockdown of $\alpha_5\beta_1$ integrin. In the absence of the $\alpha_5\beta_1$ integrin, DV no longer induces a significant increase in VEGF mRNA. Additionally, the $\alpha_5\beta_1$specific activating antibody SNAKA-51 results in increased VEGF release and profoundly augmented DV-induced VEGF release (Clark et al., 2005). These results

suggest that activation of $\alpha_5\beta_1$ integrin leads to a more pronounced effect of DV and possible synergism between activating $\alpha_5\beta_1$ integrin and DV for VEGF release. Collectively with shRNA and activation of $\alpha_5\beta_1$ integrin, I have demonstrated this integrin is important in DV's effect on VEGF expression and secretion. Mousa et al have previously demonstrated that the addition of the $\alpha_5\beta_1$ integrin ligand fibronectin to retinal pigmented epithelial cells could increase their secretion of VEGF, but this response was only partially inhibited with function blocking $\alpha_5\beta_1$ antibody suggesting that other, non- $\alpha_5\beta_1$ dependent mechanisms were involved (Mousa et al., 1999). Whether this link between $\alpha_5\beta_1$ integrin and VEGF release is an additional distinguishing characteristic of brain endothelial cells versus other endothelial cells remains to be proven. To investigate if other integrins such as the $\alpha_V\beta_3$ integrin could be related to VEGF modulation by DV, blockade of $\alpha_V\beta_3$ integrin was performed. $\alpha_V\beta_3$ integrin blocking experiments, with function blocking antibody and soluble $\alpha_V\beta_3$ integrin demonstrated that this integrin is not significantly involved with DV's induction of VEGF. This further underscores that DV's effect on VEGF induction is $\alpha_5\beta_1$ mediated.

Subsequent to ischemia a "pro-angiogenic environment" is reported partly due to the re-expression of developmental receptors (Milner et al., 2008a), (Milner and Campbell, 2002), (Milner et al., 2008b). As breakdown of the ECM is a vital step in promoting angiogenesis following ischemia in the brain, I next investigated if treatment of brain endothelial cells with soluble DV could increase pro-angiogenic receptors such as the $\alpha_5\beta_1$ integrin and VEGFR2. This data would give insight as to whether DV plays a

role in promoting the pro-angiogenic switch following ischemia. Interestingly, DV up-regulated $\alpha_5\beta_1$ integrin and VEGFR2 mRNA expression in brain endothelial cells. Other experiments in our laboratory also confirmed that DV increases $\alpha_5\beta_1$ integrin expression in vivo in mice and rats subject to hypoxia (data not shown). The actual mechanisms by which DV increases $\alpha_5\beta_1$ integrin and VEGFR2 mRNA expression remains to be proven, yet these preliminary experiments suggest DV plays a significant role in not only promoting angiogenesis by increasing migration, proliferation and tube formation of brain endothelial cells, but also by increasing pro-angiogenic receptors on brain endothelial cells allowing other pro-angiogenic factors to bind and promote angiogenesis.

In this study, I demonstrated that DV activated both ERK and Akt signaling pathways in brain endothelial cells, in agreement with the literature (Alghisi and Ruegg, 2006). Erythropoietin, a growth factor that stimulates blood vessel formation, has also been suggested to increase secretion of VEGF by activating PI3K/AKT and ERK pathways (Hermann and Zechariah, 2009). DV has been previously shown to activate FAK, p38MAPK and HSP27 but not ERK in HUVECs (Bix et al., 2004). The activation of those signaling molecules via binding of DV to □□□ integrin led to an anti-angiogenic effect and actin disassembly. Because I observed the opposite effect, i.e. a pro-angiogenic effect subsequent to AKT and ERK activation in brain endothelial cells where □□□ integrin is absent, our results support the concept that DV anti-angiogenic effects do not occur in the absence of the $\alpha_2\beta_1$ integrin (Woodall et al., 2008).

I found that DV induced a rapid activation of both the ERK and Akt signaling pathways following a similar time course with a maximal phosphorylation at 5 minutes followed by a progressive decrease after 30 minutes. Such findings agree with a recent study by Chetty and colleagues (Chetty et al., 2009) who demonstrated that activation of $\alpha_v\beta_3$-induced VEGF secretion in lung carcinoma occurred through a PI3K/Akt dependent mechanism. However I observed that ERK signaling pathways dominated over the PI3K/Akt pathway to up-regulate VEGF expression as both PD98059 and U0126 (MEK inhibitors) could completely inhibit DV-induced VEGF secretion from brain endothelial cells.

Following these observations, I further investigated the possible molecular mechanisms by which DV-induced ERK activation could result in VEGF secretion. Hypoxia-induced factor-1α (HIF-1α) constitutes one of the major signaling pathways known to up-regulate VEGF (Forsythe et al., 1996). Although HIF-1α stabilization following hypoxic conditions (when O_2 availability is diminished) is well known and considered to be its main regulatory pathway, recent studies have suggested evidence that HIF-1 stabilization may also occur by certain oxygen-independent pathways such as the mammalian-target of rapamycin (mTOR) signaling pathway or downstream of PI3K/Akt signaling pathway (Hudson et al., 2002) (Zhong et al., 2000).

As PI3K inhibition was not sufficient to efficiently suppress DV-induced VEGF transcription and secretion (data not shown), I suggest that subsequent HIF-1α stabilization by activation of the PI3K/Akt/mTOR axis remains marginal compared to

the ERK signaling pathway. Interestingly eukaryotic initiation factor 4E (elF4E), a major protein involved in protein translation, constitutes a merging point for both ERK and Akt signaling pathways, as well as an upstream signal for oxygen-independent HIF-1α stabilization (Ye et al., 2010) (Berra, 2000) (Karni, 2002) (Jin, 2008). In my study, I demonstrated that DV significantly increased both ERK and eIF4E phosphorylation. Inhibition of DV-increased phosphorylation was only achieved by the presence of MEK inhibitors or by α_5-integrin silencing.

Although the mechanism by which α_5-integrin down-regulates HIF-1α under normoxic conditions remains unclear, a recent study from Ryu and colleagues (Ryu et al., 2010) demonstrated that HIF-1α induced both α_5-integrin and fibronectin expression in squamous cell carcinoma. Preliminary data obtained with my α_5-knockdown cells shows hyper-phosphorylated Akt levels under basal condition, whereas ERK appeared hypo-phosphorylated compared to wild-type. Such data raises the speculation that ERK and Akt may be differentially implicated in HIF-1α stabilization. Whereas ERK-induced HIF-1α stabilization in wild-type cells requires the presence of both DV and $\alpha_5\beta_1$ integrin receptor, HIF-1α stabilization in α_5 knockdown cells appears to be both ERK and DV-$\alpha_5\beta_1$ integrin dependent and suggests exclusive involvement of the PI3K/Akt signaling pathway via mTOR signaling. In addition, such results also suggest the presence of two different feedback loops by α_5-integrin towards Akt and ERK: a positive feedback loop towards the ERK pathway, further elicited by DV binding, and a negative feedback loop towards PI3K/Akt that can be

partially (DV-induced Akt phosphorylation) or completely waived (α5-knockdown). To the best of my knowledge, such a feedback loop has not previously been demonstrated. Therefore, further investigation is required to better discern whether HIF-1α inhibition by α_5-integrin relies on oxygen-dependent (prolyl-hydroxylases) or independent (e.g. mTOR, elF4E) pathways. Such studies will definitively shed new light on HIF-1α regulation by integrin signaling pathways.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

In my dissertation work, I set out to investigate the mechanistic role of perlecan's DV fragment in brain endothelial cells by 1) investigating the in vitro angiogenic effects of perlecan's DV fragment on brain cerebral endothelial cells 2) identifying whether the $\alpha_5\beta_1$ integrin, present in brain endothelial cells and critical for brain angiogenesis, could be a novel DV pro-angiogenic receptor and 3) determining the regulation of VEGF by DV in brain endothelial cells as a model for understanding the mechanism of DV mediated angiogenesis.

In Chapters I and II, I investigated the in vitro angiogenic effect DV had on brain endothelial cells in vitro and tied this effect to a new, unidentified receptor for DV, the $\alpha_5\beta_1$ integrin. A problem that occurs when interpreting in vitro angiogenic assay data is incorrectly classifying a molecule as anti/pro-angiogenic (Auerbach, 1991) (Auerbach et al., 2000) (Auerbach et al., 2003). This normally occurs when the molecule is tested in only one model for angiogenesis. As mentioned previously, angiogenesis occurs in several steps and therefore, the more models an angiogenic agent is tested in, the more convincing this agent is for regulating angiogenesis. In order to investigate in vitro the stages involved with angiogenesis, I incorporated three different in vitro angiogenic techniques: modified Boyden Chamber, matrigel and MTS assays to monitor brain endothelial migration, tube like morphogenesis and proliferation respectively. Problems that arise using in vitro techniques because of propagation in vitro include

differences in activated state, loss and gain of attributes found in vivo such as cell surface antigens, receptor activation and modification of karyotypes. To avoid these problems I ensured cells that were used still expressed cellular markers such as von willebrand factor, VE-Cadherin and CD31/PECAM by western blot and immunocytochemistry. I also incorporated multiple cell lines, ranging from human (Vu, 2009) to mouse (Sapatino et al., 1993), in order to confirm my results were not due to artifacts from a certain cell line. One of the key criteria for investigating an angiogenic response in vitro is confirming it with in vivo experiments (Jain, 1997). Our laboratory utilizes the mouse model because of its close genetic homology to humans and because mice are better tools for investigating physiological systems, such as the nervous, that mammals share. However, there are significant physiological, neuroanatomical and metabolic obstacles between humans and rodents we must overcome in order to translate bench to bedside results (Braeuninger, 2009).

In contrast to its anti-angiogenic effect on HUVECs (Bix et al., 2004), DV significantly enhanced migration, proliferation and tube like morphogenesis in brain endothelial cells in vitro, suggesting DV was interacting with a new, pro-angiogenic receptor. The $\alpha_5\beta_1$ integrin has been proven to be necessary for angiogenesis and up-regulated following ischemia (Milner et al., 2008a) (Li et al., 2010), and has been previously indicated to be influenced by perlecan, although the specific domain of perlecan involved with this process remains unknown (Mongiat et al., 2003) (Milner et al., 2008b). Therefore, to maintain consistence with in vivo and in vitro observations, I

narrowed my search down to the $\alpha_5\beta_1$ pro-angiogenic receptor and confirmed this receptor was responsible for DV's pro-angiogenic effects. In order to investigate whether DV's pro-angiogenic mechanism of action required the $\alpha_5\beta_1$ integrin, two methodologies were incorporated, immunocytochemistry and optical biosensor analysis. Using these two separate methodologies, I was able to demonstrate 1) the $\alpha_5\beta_1$ integrin is required for DV's interaction with brain endothelial cells and 2) DV binds to the $\alpha_5\beta_1$ integrin with a K_d of 160 nM. Indeed, most other endothelial cells express both the $\alpha_2\beta_1$ and $\alpha_5\beta_1$ integrin, begging the question of why DV is anti-angiogenic in these co-expressing cells. The answer most likely lies in DV's varying affinity for each receptor. As stated above, Bix et al (Bix et al., 2004), using the same techniques as I did in this dissertation, have previously demonstrated that DV binds to $\alpha_2\beta_1$ with a K_d of 80 nM, exactly twice as strong of an interaction as that between $\alpha_5\beta_1$ and DV. Therefore, in the presence of both receptors, DV will have a binding preference for the $\alpha_2\beta_1$ integrin and because of this preference the anti-angiogenic response will predominate. I was able to support this hypothesis by making brain endothelial cells express the $\alpha_2\beta_1$ integrin, (Figure 2.2) which resulted in DV inhibiting rather than enhancing their proliferation.

The presence of integrin ligand is also known to increase that integrin's intracellular expression (Milner et al., 2008b). Because my data suggested DV was a ligand for $\alpha_5\beta_1$ integrin, I next investigated whether DV might also increase α_5 integrin mRNA expression in mouse brain endothelial cells in vitro. DV significantly increased

mRNA expression of α_5 in mouse brain endothelial cells which further suggested DV is a plausible ligand for the $\alpha_5\beta_1$ integrin.

After identifying $\alpha_5\beta_1$ integrin as a new receptor for DV, I next investigated if the interaction between $\alpha_5\beta_1$-DV was responsible for DV's pro-angiogenic effect. In order to answer this question, I revisited the previous in vitro angiogenic assays performed in Chapter I and negatively modulated the $\alpha_5\beta_1$ integrin by either shRNA mediated knockdown, soluble $\alpha_5\beta_1$-GST or a CREETAWAC peptide specific for the binding pocket in the $\alpha_5\beta_1$ integrin. My results demonstrated that brain endothelial cells subjected to $\alpha_5\beta_1$ integrin shRNA mediated knockdown no longer showed an increase in proliferation when treated with DV. To ensure these results were not because of the effects of shRNA transduction and RNAi activation, a non-targeting control was shown to have no effect. Indeed, the $\alpha_5\beta_1$ integrin can cause proliferation in endothelial cells. Therefore, the absence of this integrin may negatively influence endothelial cell proliferation. But cells with $\alpha_5\beta_1$ knocked down showed no significant difference in proliferation compared to wild type brain endothelial cells. Therefore, I was able to conclude that the induction of DV's pro proliferative effect is due to the presence of the $\alpha_5\beta_1$ integrin.

In order to link this receptor to DV's pro-migratory effect, I conducted competition studies with the $\alpha_5\beta_1$ integrin. DV incubated with soluble $\alpha_5\beta_1$ integrin no longer promoted endothelial cell migration. This suggests that when co-incubated, DV and soluble $\alpha_5\beta_1$ integrin interact with one another thereby preventing soluble DV from binding to $\alpha_5\beta_1$ on the cell surface and inducing its pro-migratory effect on brain

endothelial cells. One could argue that in this case, there is no more DV available to induce a pro-migratory effect with other plausible receptors. Yet, brain endothelial cells with $\alpha_5\beta_1$ integrin knocked down were unresponsive to DV as a migratory stimulus. This result further supports the hypothesis that the $\alpha_5\beta_1$ integrin is involved with DV's pro-migratory effect. Lastly, brain endothelial cells pre-treated with the CRRETAWAC peptide, a peptide specific for the binding pocket of $\alpha_5\beta_1$ integrin (Mould et al., 1998), demonstrated an inhibitory effect on DV's pro-tube-like morphogenesis.

These results also provide insight into how ECM proteolysis following ischemia is beneficial for brain repair by providing bioactive ligands responsible for inducing angiogenesis. As mentioned previously, fibronectin is up regulated following ischemia and interacts with the $\alpha_5\beta_1$ integrin (Milner et al., 2008a). Fibronectin also has been demonstrated to promote brain endothelial cell survival and proliferation through the $\alpha_5\beta_1$ integrin by activating the MAP kinase signaling pathway (Wang and Milner, 2006a). These results suggest DV and fibronectin share similar characteristics when promoting angiogenesis in brain endothelial cells. Yet, there still exist differences between DV and fibronectin in regards to binding to the $\alpha_5\beta_1$ integrin. DV and fibronectin have different affinities for the $\alpha_5\beta_1$ integrin, K_d of 160nM and K_d of 1.5nM, respectively. Fibronectin also utilizes the RGD binding domain in the $\alpha_5\beta_1$ integrin binding pocket. My results utilizing the CREETAWAC peptide suggests the $\alpha_5\beta_1$ integrin has multiple binding sites for DV. The exact interaction as to how DV binds to the $\alpha_5\beta_1$ integrin is left to be determined.

Collectively, these results demonstrated DV has an unexpected, opposite role on brain endothelial cells involving a newly identified receptor for DV. These data suggest following ischemia, DV is released into the cerebral vasculature and promotes angiogenesis by interacting specifically with newly expressed $\alpha_5\beta_1$ integrin on brain endothelial cells. Indeed, other cells within the neurovascular unit, for example neurons and astrocytes, express DV's previously reported receptor, the $\alpha_2\beta_1$ integrin. Yet preliminary studies in our laboratory suggest DV has no negative effects on these cell types, suggesting DV has multiple roles in brain repair following ischemia.

Based upon my observations that DV and fibronectin share the same receptor, I used fibronectin as a model to gain insight in to how DV could induce a pro-angiogenic response. Fibronectin promotes proliferation of brain endothelial cells by interacting with the $\alpha_5\beta_1$ integrin and activating the MAP kinase signaling cascade (Wang and Milner, 2006a). Therefore, I investigated if the MAP kinase and upstream activator AKT were activated by DV. DV addition to brain microvascular endothelial cells resulted in AKT phosphorylation/activation which could be completely inhibited by the addition of the PI3K inhibitor LY-294002, demonstrating DV is activating P I3 kinase in order to activate AKT. My results also demonstrated DV phosphorylates/activates ERK in brain endothelial cells. This was AKT dependent, because the PI3K inhibitor LY-294002 blocked the phosphorylation of ERK indicating DV signals through AKT in order to lead to the activation of ERK.

Under both normoxic and hypoxic conditions (Karni, 2002), VEGF regulation has been linked to hypoxia-induced factor-1α (HIF-1α) activation, therefore, I next set out to determine whether DV treatment could cause changes in HIF-1α levels in brain endothelial cells. Typically under normoxic conditions, HIF-1α is constantly degraded and present at very low concentrations (Kaur, 2005). Yet, studies performed by Karni et al. demonstrate that HIF-1α expression can also exist under normoxic conditions when HIF-1α synthesis is faster than its degradation (Karni, 2002). My results demonstrated DV induced rapid stabilization of HIF-1α which was linked to phosphorylation of eukaryotic translation initiation factor 4E (eIF4e), a downstream activator of MAP kinase and PI-3 kinase signaling (Fukuda, 2002). In addition, usage of U0126, a potent MEK inhibitor, reduced phosphorylation of eIF4e, but did not have an effect on DV's stabilization of HIF-1α suggesting that although DV causes an increase in ERK phosphorylation and eIF4E, other signaling cascades must be involved with DV's stabilization of HIF-1α which are yet to be determined.

As Akt and ERK activation have been implicated in VEGF production and release, (Berra et al., 2000) and VEGF causes the synthesis of perlecan in brain endothelial cells, I next set out to investigate whether DV regulated VEGF expression and secretion. From my results, I was able to conclude DV increased VEGF mRNA levels and increased VEGF secretion in a dose-dependent and time-dependent fashion in vitro. After observing that DV induced an up-regulation in transcription and subsequent release of VEGF into the media, I set out to determine if this effect was modulated by the $\alpha_5\beta_1$ integrin. To

the best of my knowledge, the only integrins expressed following ischemia that have been linked to VEGF secretion are the $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins (Choi, 2009) (Mousa et al., 1999). Utilizing the $\alpha_5\beta_1$ integrin knockdown cells that I constructed, I determined that DV no longer had an effect on increasing VEGF expression and secretion, demonstrating this integrin is required for DV-mediated regulation of VEGF.

I followed this experiment up by asking if activating the $\alpha_5\beta_1$ integrin could increase DV's effect on VEGF secretion by utilizing the SNAKA-51 antibody (Clark et al., 2005). The proposed mechanism of action induced by the SNAKA-51 antibody is that it causes the legs of the integrin to change conformation and thereby primes the integrin to bind to the ligand. Interestingly, cells treated with SNAKA-51 antibody alone significantly induced the secretion of VEGF into cultured media compared to non-treated cells. This observation suggests a ligand present in the media that would typically not induce VEGF secretion is able to do so once the $\alpha_5\beta_1$integrin has been primed for binding. Alternatively, this implies that activation alone of the $\alpha_5\beta_1$integrin can cause VEGF secretion, an observation that has not been published as of yet. If this were the case, then changes in intercellular signaling molecules would provide insight to support or deny this hypothesis. This data coincides with DV's previous effect on platelet activation where differences in integrin activation/ligand affinity state mitigated DV's effect [Bix 2007]. These data also suggest the majority of this integrin on the cell surface is primarily in a bent conformation state. Suggesting that activating the $\alpha_5\beta_1$ integrin provides a synergistic effect with DV treatment by putting the integrin in a

ligand competent form. Future studies would investigate intercellular signaling cascades in the presence or absence of SNAKA-51 antibody and also investigate if SNAKA-51 co-incubated with DV would make DV's effect more prominent. When cells were treated with DV and SNAKA-51, there was a significant increase in VEGF secretion, more than DV treatments and with SNAKA-51 alone.

As mentioned previously, the $\alpha_V\beta_3$ integrin has also been linked to VEGF secretion. Therefore, I next set out to rule out that this integrin played a role in DV's effect on VEGF secretion. In order to do this, I incorporated two separate scenarios. The first scenario was blocking the $\alpha_V\beta_3$ integrin with a function blocking antibody and incubating brain endothelial cells with DV. Under this scenario, DV still significantly induced VEGF secretion in the presence of $\alpha_V\beta_3$ function blocking antibody and this effect was not significantly different from DV treated alone. To further rule out that the $\alpha_V\beta_3$ integrin is not involved with DV's induction of VEGF secretion, DV was co-incubated with soluble $\alpha_V\beta_3$. Under this scenario, I hypothesized that if DV bound to soluble $\alpha_V\beta_3$, DV would no longer be able to bind to $\alpha_v\beta_3$ on the cell surface and induce VEGF secretion. My results indicated that DV was still able to induce VEGF secretion when co-incubated with soluble $\alpha_V\beta_3$, suggesting DV does not bind to this integrin. In order to support the hypothesis that DV does not bind to the $\alpha_V\beta_3$ integrin, I performed solid phase ELISA binding experiments and demonstrated DV did not bind to $\alpha_V\beta_3$ integrin.

Collectively, these experiments confirm DV's effect on VEGF expression and secretion involves the $\alpha_5\beta_1$ integrin and this effect does not involve the $\alpha_V\beta_3$ integrin. It is important to note that Mousa et al also demonstrated that the addition of fibronectin, the main ligand for the $\alpha_5\beta_1$ integrin, to retinal pigmented epithelial cells could increase their secretion of VEGF. However, this response could not be inhibited with function blocking $\alpha_5\beta_1$ antibody, suggesting that other, non $\alpha_5\beta_1$ dependent mechanisms were involved with VEGF secretion (Mousa et al., 1999).

Because my results suggested that the DV mediated increase in VEGF expression was $\alpha_5\beta_1$ dependent, I next investigated the activation of the same signaling molecules in $\alpha_5\beta_1$ integrin knocked down brain endothelial cells. Interestingly, $\alpha_5\beta_1$ integrin knocked down mouse brain endothelial cells displayed significantly high levels of phospho-Akt, whereas phospho-ERK levels were significantly low compared to wild-type in control conditions. In contrast to wild-type cells, DV did not significantly increase phosphorylation of AKT or ERK in $\alpha_5\beta_1$ integrin knocked down mouse brain endothelial cells, showing DV requires this integrin cause phosphorylation of these molecules. I also observed a constitutive stabilization of HIF-1α and eIF4E. However, DV did not further significantly increase HIF-1α levels or phosphorylation of eIF4E. Although I observed constitutive activation of Hif1α and eIF4E, VEGF expression and secretion were not significantly increased in $\alpha_5\beta_1$ integrin knockdown brain endothelial cells. These data suggest the downstream signaling cascade responsible for VEGF secretion via the $\alpha_5\beta_1$ integrin does not entirely consist of HIF-1α stabilization and

phosphorylation of eIF4E. Because I observed a decrease in phosphorylation of ERK in these knocked down cells and because the U0126 ERK inhibitor blocked DV's effect on VEGF expression and secretion, I was able to conclude DV primarily signals through the MAPK signaling pathway to induce VEGF production. My data also suggests that when the MAPK signaling pathway is blocked, other signaling molecules are activated in order to compensate and still allow DV to induce some of its pro-angiogenic effect.

Whether this link between $\alpha_5\beta_1$ integrin and VEGF release is an additional distinguishing characteristic of brain endothelial cells versus other endothelial cells remains to be proven. Given these results, as VEGF regulates perlecan synthesis in brain endothelial cells (Kaji et al., 2006), it is tempting to speculate that DV induced VEGF release from brain endothelial cells could result in a positive feedback loop that results in increased perlecan synthesis, which in turn restores the levels of perlecan deposits within the ECM following ischemia.

Relative importance and future implications

Understanding the mechanisms involved with regulating angiogenesis can be beneficial for clinical applications in numerous fields such as ischemia, coronary vascular disease, wound healing and tumorigenesis. As ischemic stroke is the leading cause of long term disability and the third leading cause of death in the United States, a better understanding of brain repair following ischemic stroke can significantly increase the recovery of patients who have suffered from ischemic stroke. The data presented in my dissertation provides yet another avenue of therapy for ischemia with the novelty

of using bioactive fragments that are native to the brain following a post-ischemic response. The use of matrix fragments has already been incorporated in studies for inhibiting angiogenesis in cases such as cancer, macular degeneration and diabetic retinopathy (Hamano and Kalluri, 2005; O'Reilly et al., 1997) (Bix et al., 2006). The benefits to using angiogenesis inhibitors derived from circulation and/or the ECM include decreased toxicity effects and a lower risk for drug resistance, making these inhibitors great candidates for therapeutic approaches (Folkman, 2004). Another benefit to incorporating bioactive fragments derived from the ECM involves the ability to modify these proteins in order to increase their half-life or stability thereby decreasing dose requirements (Meng-jie, 2009). The notion of using native fragments produced by the brain following ischemia instead of drugs may also seem to be beneficial as they may not experience much resistance crossing the blood brain barrier as most pharmacological studies still struggle with overcoming. Also, drugs are artificial substances to the body and therefore subject to all of the problems associated with such foreign substances including side effects, tolerability, toxicities, etc.

Little research has been spent investigating whether matrix fragments can promote angiogenesis for cases such as brain ischemia. It is plausible that the reason for this is an assumption that anti-angiogenic molecules will be anti-angiogenic in the brain. In addition to my results, there are several circumstances in which this is not the case. For example, endostatin promotes angiogenesis in immature endothelial cells derived from differentiated embryonic stem cells (Schmidt et al., 2004) and platelet-

derived pro-angiogenic sphingosine-1-phosphate (S1P) is anti-angiogenic in brain endothelial cells due to their lack of MT1-MMP expression (Pilorget et al., 2005). An explanation for these different responses may be due to endothelial heterogeneity, whereby endothelial cells in different vascular beds, in this case brain versus non-brain, respond differently to angiogenic factors. Differential responses may also be due to differences in respective microenvironments, differences in expressed receptors, such as the presence or absence of $\alpha_2\beta_1$ integrin, or differences in signal transduction components.

A number of animal studies within the past few years have demonstrated that improving neuroprotection or post-stroke angiogenesis/neurogenesis can improve stroke outcome. Current stroke therapies generally fit into three different categories; pharmacological agents, growth factors and stem cell therapies. In particular, therapeutics that induce angiogenesis following ischemia have proven to be a promising area of research for future clinical stroke therapy as it promotes both blood flow and nutrients back to dying tissue along with promoting neurogenesis (Fan, 2007).

Currently, there are only four molecules, VEGF, basic fibroblast growth factor (bFGF), granulocyte-colony stimulating factor (G-CSF) and Epo which have made it to pre-clinical trial for functional recovery in humans (Matthias, 2009). Pro-angiogenic therapy for stroke using VEGF has emerged as a “double-edged sword” in stroke research. Deng et al. have shown that 24 hours post-rat MCA stroke administration of bone marrow-derived mesenchymal stem cells resulted in improved neurological

function, reduced neuronal apoptosis and the promotion of neuronal proliferation via the release of VEGF (Deng et al., 2010). These results suggest VEGF is also neuroprotective. Yet, if VEGF is given too soon after the onset of stroke, it promotes a leaky blood brain barrier, edema, hemorrhagic transformation and an ultimately worsened brain injury (Zhang et al., 2000). However, when administered later and more chronically, such as 24 hours post-stroke, VEGF is pro-angiogenic, neuroprotective, and enhances neurogenesis (Sun et al., 2010) (Ferrara, 2003). Therefore the timing in which VEGF is administered is detrimental for obtaining positive results.

For the first time ever, my results link VEGF expression and secretion from brain endothelial cells to an ECM fragment. My results demonstrated DV causes an increase in VEGF secretion in brain endothelial cells, suggesting besides being pro-angiogenic, DV can also potentially be neuroprotective by causing the secretion of VEGF. Current experiments in our laboratory performed by Dr. Lee have investigated the administered effects of DV in vivo. After one day post stroke, DV administration did not cause an increase in permeability, suggesting DV at this time point does not induce negative permeability effects caused by VEGF. Yet, future studies should investigate the exact timing when DV administration causes VEGF production in vivo. Future experiments should also investigate the differences between VEGF expression and secretion in perlecan-hypomorph animals and wild type animals. Because perlecan null animals lead to embryonic lethality, preliminary studies utilizing perlecan-hypomorph animals would

allow us to investigate whether the presence of perlecan affects VEGF expression following ischemia.

The mechanism by which VEGF is neuroprotective requires the VEGFR2 (Matthias, 2009). Preliminary data using a VEGF neutralizing antibody suggest DV directly causes the increase in expression of VEGFR2. It is interesting to note that in HUVECs, DV causes a de-phosphorylation in VEGFR2 by activation of SHP-1 (Nystrom et al., 2009). Therefore investigating the mechanism by which DV causes an increase in VEGFR2 expression may also prove to be beneficial in understanding the complexity of DV's direct and indirect pro-angiogenic effect. Nonetheless, my data demonstrates that DV increases VEGFR2 expression in vitro, indicating DV could potentially promote neuroprotection two-fold by inducing the secretion of VEGF and by increasing the expression VEGFR2. The latter scenario would be beneficial for VEGF that is already present in the environment.

DV increased VEGF secretion by activating the $\alpha_5\beta_1$ integrin. Linking VEGF secretion with $\alpha_5\beta_1$ integrin activation has never been shown thus far and therefore provides yet another pathway for the brain to promote angiogenesis following ischemia. Nine integrins have been reported to be expressed on endothelial cells (Laurens, 2009). Of those nine, there are three integrin receptors involved with angiogenesis in the brain that could be potential targets for promoting angiogenesis following ischemic stroke: $\alpha_5\beta_1$, $\alpha_v\beta_5$ and $\alpha_v\beta_3$ (del Zoppo and Milner, 2006). To the best of my knowledge, only the $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins have been linked to VEGF secretion

(Choi, 2009) (Mousa et al., 1999). Yet the role of $\alpha_v\beta_3$ integrin in ischemic cerebral angiogenesis appears to be complex. Li and co-workers recently demonstrated that although the $\alpha_v\beta_3$ integrin is strongly induced on angiogenic brain endothelial cells in mice subject to hypoxia, β_3 integrin null mice demonstrate no obvious defects in cerebral angiogenesis following hypoxia. Furthermore, β_3 null mice following hypoxia exhibited an up-regulation in α_5 integrin and an increase in proliferating cerebral endothelial cells (Li, 2010), suggesting the $\alpha_v\beta_3$ integrin is not detrimental for promoting angiogenesis following ischemia. My results also support the hypothesis that the $\alpha_v\beta_3$ integrin is not involved with the up-regulation of VEGF. Collectively, our data further demonstrates that the $\alpha_v\beta_3$ integrin plays a minimal role in supporting angiogenesis and neuroprotection following ischemia by having no effect on growth VEGF secretion.

In vivo experiments performed by Dr. Lee in our laboratory confirm administration of DV enhances both neuroprotection and angiogenesis in rodents following ischemia resulting in normalization of stroke affected motor function. Dr. Lee's experiments also supported my findings in vivo by demonstrating DV increases post stroke $\alpha_5\beta_1$ integrin levels and correlated this finding with increased neuroprotection and angiogenesis in the brain. Therefore, developing therapies that can promote angiogenesis by signaling through this integrin and/or induce the expression of this integrin would hypothetically induce a pro-angiogenic environment and promote brain repair. Anti-angiogenic agents targeting integrins have been

exploited for therapeutic application in gliomas, melanoma, and prostate cancer (Idbaih, 2008) (Silva, 2008). However, few investigations have been performed to address targeting integrins for promoting angiogenesis in cases such as brain ischemia. As mentioned previously, there is an angiogenic "switch" following hypoxia that involves the up-regulation of $\alpha_5\beta_1$ integrin. My results with the SNAKA-51 antibody, demonstrate activating the $\alpha_5\beta_1$ integrin promotes VEGF secretion. Furthermore, my results also demonstrate DV promotes angiogenesis by signaling through the $\alpha_5\beta_1$ and DV up-regulates the expression of this integrin. Collectively, my results demonstrate targeting the $\alpha_5\beta_1$ integrin following ischemia could be exploited for therapeutic application to help promote angiogenesis and neurogenesis through its involvement with inducing VEGF production.

Collectively my results, along with Dr. Lee's results, demonstrate extracellular matrix components generated following ischemia can lead to potential neuroprotective and angiogenic therapeutic agents for patients who suffer from ischemia. Yet our results are only the beginning in describing DV's role in brain angiogenesis and neuroprotection following ischemia. For starters, DV contains two Leu-Arg-Glu (LRE) tripeptides within its sequence (Murdoch et al., 1992). LRE tripeptides are binding ligands for neurons and control neurite outgrowth and migration (Hunter et al., 1991). Therefore, in the case of nerve injury such as ischemia or spinal cord injury, it would be interesting to investigate DV's role in regulating neurite outgrowth and if these tripeptide sequences within DV serve as a recognition signal for neuronal migration.

The data presented in this dissertation suggests DV has a positive effect on endothelial migration. An explanation for this effect could be because of DV binding to α-dystroglycan (Whitelock, 2008) a receptor responsible for linking the ECM to intracellular actin. The association between perlecan and dystroglycan has been investigated and provides yet another role for perlecan in controlling cell polarity (Lindner, 2007) (Mirouse, 2009). Yet, in order to investigate the above explanations for DV's effect on neuronal and endothelial migration, site directed mutagenesis targeting the LRE tripeptides and knockdown of dystroglycan would be the first steps I would perform to investigate their role in DV's effect on cell migration.

As mentioned previously, perlecan is the most sensitive vascular matrix component following ischemia (del Zoppo et al., 2007). In my dissertation I only discussed proteases that could potentially cleave DV from full length perlecan. Other proteases exist such as thrombin, plasmin, collagenase, and stromelysin, which can release other fragments from perlecan besides DV. Thrombin activity is elevated following MCAO (Hua, 2003). Perlecan is sensitive to proteolysis by thrombin at four separate sites along its sequence. Proteolysis of perlecan could be beneficial as perlecan's domain I contains multiple glycosaminoglycan (GAG) attachment sites, responsible for sequestering growth factors, and thrombin can cleave domain I from perlecan. Therefore, when thrombin levels are elevated growth factors, such as VEGF 189, once anchored to the vascular basement membrane by perlecan's GAG chains are now released and able to interact with the surrounding vascular environment.

In summary, I have successfully identified a new role for perlecan's DV fragment following brain ischemia. My research has opened up a new field in brain recovery following stroke by linking ECM degradation and the promotion of angiogenesis. These results open up new avenues for understanding the mechanisms involved with brain self-repair following ischemia. One could hypothesize the following working model illustrated in **Figure 5.1** that occurs following ischemia: DV is released from perlecan due to up-regulation of proteases after ischemia and this free DV interacts with different cell surface receptors such as the $\alpha_5\beta_1$ integrin. Interaction with the $\alpha_5\beta_1$ integrin leads to phosphorylation of MAPK and HIF-1 stabilization and the subsequent induction of VEGF expression and secretion. Newly secreted VEGF is now capable of affecting the surrounding neurovascular unit where it can modulate angiogenesis during brain repair and promote synthesis of perlecan.

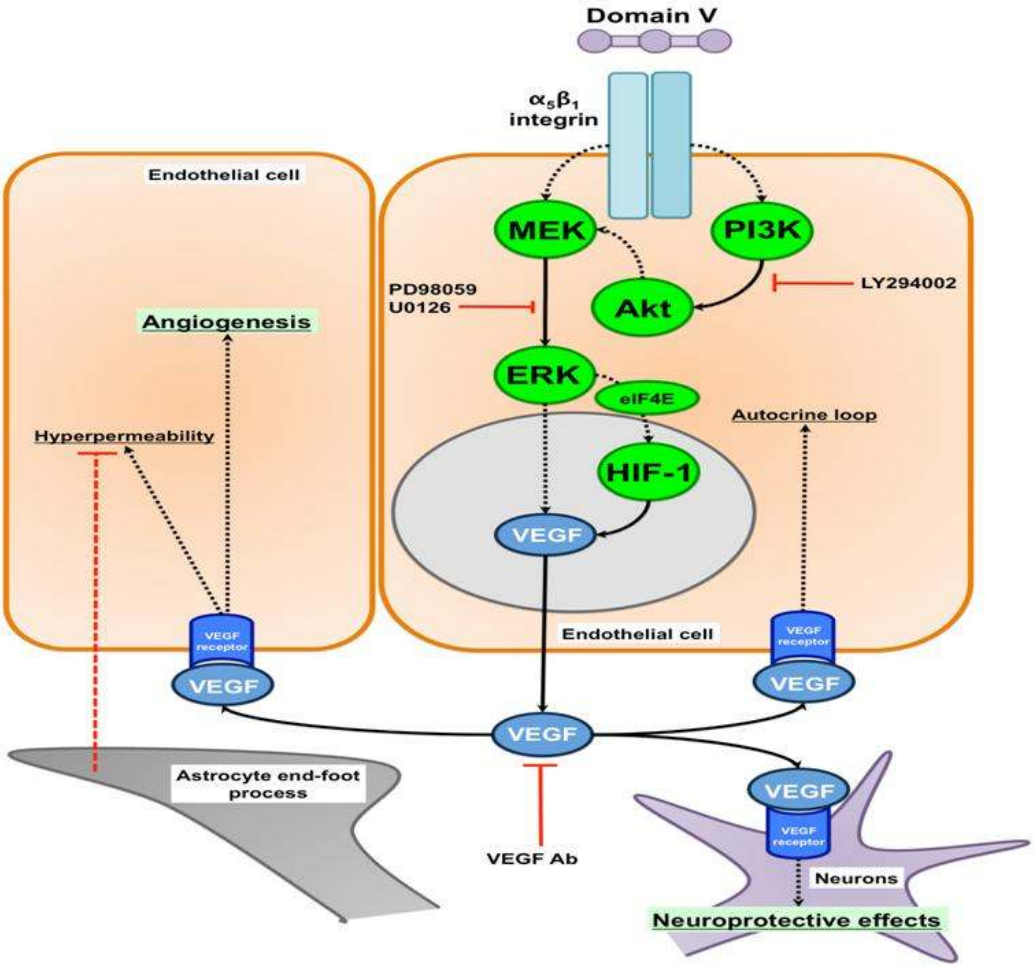

Figure 5.1. DV mechanism for inducing a pro-angiogenic and neuroprotective effect. DV is released from perlecan due to up-regulation of proteases and this free DV is capable of interacting with a different receptor the $\alpha_5\beta_1$ integrin. Interaction with the α5β1 integrin leads to phosphorylation of MAPK and HIF-1 stabilization and the subsequent induction of VEGF expression. VEGF is now capable of affecting the surrounding neurovascular unit where it can modulate angiogenesis during brain repair.

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