Vascular endothelial cadherin phosphorylation modulates endothelial cell permeability and leukocyte transendothelial migration

Stephanie Hall Hart

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Approved by
Keith Burridge, Ph.D.
Klaus Hahn, Ph.D.
Channing Der, Ph.D.

Abstract

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(Under the direction of Keith Burridge, Ph.D.)

Leukocyte transendothelial migration (TEM) is a key step in many functions of the immune system such as immune surveillance, inflammation and wound repair. The controlled disassembly of endothelial adherens junctions (AJs) is a major component of TEM regulation. Studies have shown the tyrosine phosphorylation of VE-cadherin, a protein important in AJs, disrupts junctions and increases vascular permeability. We found that blocking the phosphorylation of VE-cadherin on residues Y658 and Y731 using non-phosphorylatable VE-cadherin mutants lead to increased barrier function and decreased endothelial cell monolayer permeability. This demonstrates that phosphorylation of VE-cadherin residues Y658 and Y731 is necessary for the disassembly of endothelial junctions. We demonstrated that blocking phosphorylation of these residues also inhibited neutrophil transmigration across endothelial cells. Together, these data demonstrate that phosphorylation of VE-cadherin residues, Y658 and Y731, are required for the regulation of endothelial cell junction permeability and also for effective neutrophil transendothelial migration through endothelial cell monolayers.

To my family, especially my parents, for their love and unfailing support through the ups and downs of graduate school. To my friends for your love, laughter and encouragement.
You have my gratitude and my love.

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LIST OF ABBREVIATIONS AND SYMBOLS

AJ	adherens junction
BSA	bovine serum albumin
DMEM	Dulbecco's modified eagles media
CD99	cluster differentiation 99
C-terminus	carboxyl-terminus
DM	double mutant
EC	endothelial cell
EDTA	ethylene diamine tetraacetate
E-selectin	endothelial selectin
F	phenylalanine
FBS	fetal bovine serum
GFP	green fluorescent protein
HEV	high endothelial venule
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
JAM	junctional adhesion molecule
LAD	leukocyte adhesion deficiency
LFA-1	lymphocyte function associated antigen-1
L-selectin	leukocyte selectin
Mac-1	macrophage integrin-1
ml	milliliter

mM	millimolar
mm	millimeter
mw	molecular weight
ng	nanogram
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PECAM	platelet endothelial cell adhesion molecule
P-selectin	platelet selectin
Pyk2	proline-rich tyrosine kinase 2
RT-CES	real-time cell electronic sensing
TEM	transendothelial migration
TNF-α	tumor necrosis factor alpha
VCAM	vascular cell adhesion molecule-1
VE-Cadherin	vascular endothelial cadherin
VE-PTP	vascular endothelial protein tyrosine phosphatase
VLA-4	very late antigen-4
Y	tyrosine
ZO-1	zonula occludens-1
°C	degrees Celsius
μm	micrometer, micron
μl	microliter

CHAPTER I

Introduction

The human immune system is a complex and intricate system of organs, tissues, cells and proteins that protects the body from infection, disease and other unwanted pathogens. An important part of both the innate and adaptive immune responses is the ability of leukocytes to leave the blood stream and enter the surrounding tissues. This migration of leukocytes out of the blood vessels is required for immune surveillance of pathogens and allows for rapid accumulation of leukocytes at sites of infection and injury. During immune surveillance, lymphocytes a specific type of leukocyte recirculate between the blood and lymphoid tissue. This occurs at specialized sites of the blood vessels, namely the high endothelial venules (HEVs). For immune defense, leukocytes enter tissues that are inflamed due to infection or injury. Leukocytes enter the inflamed tissue through blood vessels called postcapillary venules that have been activated by inflammatory stimuli (1). The lumen of blood vessels is lined with a sheet of cells called endothelial cells. This sheet or monolayer of endothelial cells forms a physical barrier to contain the blood and other cells within the blood vessels. Leukocytes must migrate through this barrier of cells in order to leave the blood stream. This migration of leukocytes across the endothelial cells is referred to as transendothelial migration (TEM). It is a key step in many functions of the immune system such as immune surveillance, inflammation and fighting infection. Because TEM is such an important, complex and on-going process, it is very tightly regulated. When TEM is misregulated, it can

lead to pathological events. Over-activation of TEM can lead to pathologies such as chronic inflammation, atherosclerosis, multiple sclerosis, rheumatoid arthritis, psoriasis, and ischemia reperfusion injury. In contrast, inefficient TEM can lead to chronic and fatal bacterial infections as seen in patients with Leukocyte Adhesion Deficiency (LAD) syndromes.

The development of atherosclerosis illustrates the ramifications that over-activation of TEM can have in disease. Atherosclerosis is a chronic inflammatory disease of the arterial wall, which begins with damage to the inner wall of the artery, namely the endothelium. It is thought that the initial damage is caused by the deposit of lipids from the blood into the endothelium, which then triggers an inflammatory response. Monocytes, a specific type of leukocyte, are then recruited from the bloodstream, transmigrate through the endothelium and take up residence in the arterial wall. The monocytes differentiate into macrophages which then phagocytose the lipid deposits. These macrophages turn into foam cells due to high lipid content in their internal vesicles and eventually die. The death of these macrophages further propagates the inflammatory response leading to more transmigration of monocytes into the arterial wall which eventually leads to calcification and stimulates proliferation of smooth muscle cells. This sequence of events leads to formation of atherosclerotic plaques which can then rupture to cause heart attacks and strokes.

Conversely, inefficient TEM leaves the body vulnerable to infection as leukocytes cannot leave the bloodstream in order to migrate to sites of injury and invasion of pathogens. One cause of inefficient TEM is a gene mutation in the integrin β_2 subunit (CD18) which is located on the long arm of chromosome 21 (2). This is known as Leukocyte Adhesion Deficiency (LAD) syndrome I and is a genetically inheritable syndrome. Patients with this

mutation in the β_2 subunit show absent or dramatically reduced surface levels of β_2 integrins, which are important molecules for the transmigration of leukocytes. The consequences of leukocytes not being able to transmigrate out of the bloodstream are frequently lethal and include recurrent infection, systemic sepsis, impaired immune surveillance and incomplete wound repair (2).

Leukocyte transendothelial migration

Transendothelial migration involves many complex interactions and signaling pathways between the leukocytes and the endothelial cells that line the lumen of the blood vessels. There are a number of obstacles that must be overcome for the efficient TEM of leukocytes. For one, the leukocytes are flowing through the blood vessels at a fast rate and must slow down in order to firmly attach to the endothelial cells and migrate through them. Another challenge is that in order to contain the blood and other macromolecules in the lumen of the blood vessel, the endothelial cells make an impermeable barrier which the leukocytes must penetrate. Endothelial cells make this impermeable barrier by forming a single monolayer of cells and attaching to the adjacent endothelial cells via multiple junctions such as tight and adherens junctions. It was once thought that the leukocytes actively bound to and crossed the endothelial cells and that the endothelial cells themselves were passive partners in this process. However, recent research has shown that the endothelial cells play an equally important role during transendothelial migration as do the leukocytes themselves.

Transendothelial migration can be divided into three stages in which the leukocytes adhere weakly to and roll across the endothelial cells, develop firm adhesions and finally transverse the endothelial cells (Figure 1). This is known as the multi-step paradigm (3, 4).

The activated endothelium in inflamed tissue and the HEVs of lymph nodes use several different molecules to achieve leukocyte TEM. The first step is the initial tethering of the leukocyte to the endothelium and reversible rolling. During this step the proteins endothelial selectin (E-selectin) and platelet selectin (P-selectin) on the endothelial cell binds to carbohydrates on the leukocytes and leukocyte selectin (L-selectin) binds to carbohydrate ligands on the endothelium (Figure 1) (5). The binding of these molecules between the leukocyte and the endothelial cell allows the leukocyte to slow down in the bloodstream and roll along the endothelial cells. Rolling leukocytes are then in contact with chemokines secreted by the endothelial cells. These chemokines activate leukocyte integrins which then initiate the next step in transmigration; firm adhesion (1). In firm adhesion, vascular endothelial cell adhesion molecule (VCAM) on the endothelium binds to activated integrins, α4β1 (VLA-4, very late antigen-4) and α4β7 on the leukocyte; while intercellular adhesion molecule (ICAM) on the endothelial cells binds to the activated β2 integrins, $\alpha_L\beta_2$ (LFA-1, lymphocyte function associated antigen-1) and $\alpha_M\beta_2$ (Mac-1, macrophage integrin-1), on the leukocyte (Figure 1) (5). This firm adhesion process enables the leukocyte to bind tightly enough to the endothelial cells to resist the shear force of blood flow and allows the leukocyte to begin the migration through the endothelium into the surrounding tissues. In addition to the molecules required for firm adhesion, two other molecules are needed for the third step; diapedesis. These are platelet endothelial cell adhesion molecule (PECAM) and cluster differentiation (CD99). These are found on both the leukocytes and the endothelial cells and bind through homophilic interactions (Figure 1) (6). In order for the leukocyte to cross the endothelial barrier, the endothelial cells must break their attachment to adjacent

endothelial cells to which they are bound in order to create a gap through which the leukocyte may pass.

This passing of the leukocyte between adjacent endothelial cells is known as paracellular migration. Another form of transmigration, namely transcellular migration, is also known to occur in which the leukocyte passes not in between adjacent endothelial cells but through a single endothelial cell. Only a minority of leukocytes transmigrate through endothelial cell monolayers using the transcellular route *in vitro*; 7% of monocytes, 5% of neutrophils, and 11% of lymphocytes (7). Consequently, paracellular migration is the most common form of leukocyte TEM (7-10) and thus this discussion will focus on paracellular TEM.

Figure 1. Leukocyte transendothelial migration: A three step paradigm

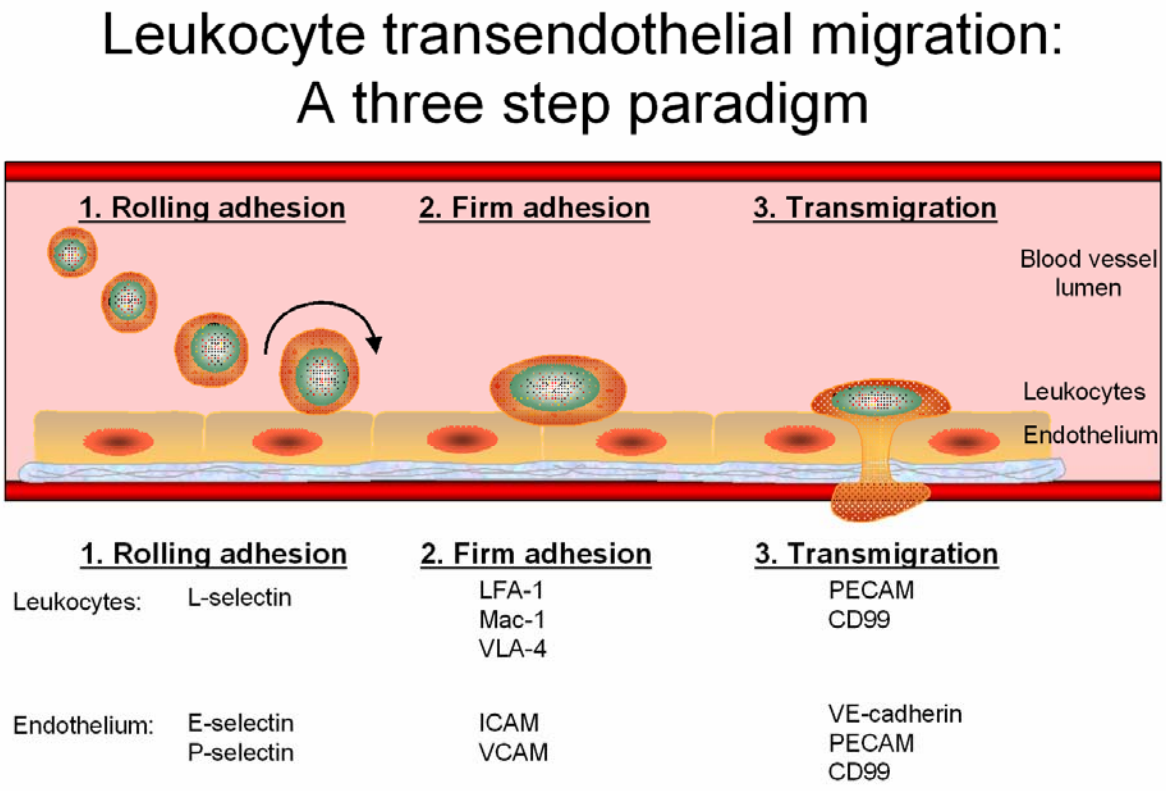


Figure 1. Leukocyte transendothelial migration: A three step paradigm. Schematic representation of the steps in leukocyte transendothelial migration and the adhesion molecules on leukocytes and endothelium involved in each step.

Endothelial Cell-cell junctions

The endothelial cells that line the blood vessels form an impermeable barrier to contain the blood, cells and other macromolecules within the blood vessels. A number of junctions between adjacent endothelial cells maintains the integrity of the endothelium and regulates vascular permeability. Despite this impermeable barrier, leukocytes must be able to leave the blood stream for immune surveillance and to fight infection. Thus adjacent endothelial cells must disassemble their junctions for the leukocyte to pass through the endothelium and allow this paracellular migration of leukocytes to occur. The controlled disassembly of these junctions is a major component of TEM regulation. There are two types of junctions important in endothelial cell-cell junctions, tight junctions and adherens junctions. Tight junctions are the most apical junctions and form between pairs of endothelial cells. These junctions are important in permeability of vasculature as they restrict the flux of fluid, solutes and other molecules across the endothelium especially across the blood brain barrier. Tight junctions are composed of three families of transmembrane proteins: occludin, claudins, and junctional adhesion molecules (JAMs), each of which has multiple members. Also important to tight junctions are many proteins found within the endothelial cells which serve as scaffolding and signaling partners. These include zonula occludens 1 (ZO-1) and cingulin (11). Tight junctions are not thought to be as important as adherens junctions in regulating TEM in HEVs and inflammation because the majority of leukocytes cross at tricellular junctions of endothelial cells where tight junctions are discontinuous. This has been confirmed in studies of proteins important in tight junction such as JAM-A. Antibodies against JAM-A inhibit recovery of cell contacts after disruption by calcium depletion but do not effect neutrophil migration through an endothelial monolayer (12,13).

Adherens junctions (AJs) are important junctions in regulating macromolecular permeability in microvascular endothelium and thus are important in regulating TEM (11). The major protein in adherens junctions is vascular endothelial-cadherin (VE-cadherin), a cell adhesion molecule essential for the maintenance of these interendothelial contacts. VE-cadherin has five homologous extracellular domains and a cytoplasmic tail (Figure 2) (14). A classical cadherin, VE-cadherin, links adjacent endothelial cells together through homophilic interactions of its extracellular domains and connects to the actin cytoskeleton through its intracellular domains (1). Of all the proteins that have been shown to be involved in leukocyte transmigration, the endothelial cell-specific cadherin, VE-cadherin, is unique because it is the only protein that blocks TEM rather than supporting it (1). Most of the proteins described so far in this discussion that are important in TEM serve as binding and signaling proteins between leukocytes and endothelial cells in order to recruit leukocytes to sites of inflammation; selectins bind carbohydrate moieties, ICAM-1 binds LFA-1, VCAM binds VLA-4, PECAM and CD99 are both homophilic adhesion molecules. Antibodies against these proteins disrupt leukocyte endothelial binding and thus inhibit TEM (15-20). In contrast, VE-cadherin forms a barrier against migrating leukocytes. Studies have shown that antibodies that bind VE-cadherin disrupt the homophilic VE-cadherin interactions that bind adjacent endothelial cells together thus creating gaps between endothelial cells which promotes leukocyte TEM (21-25).

VE-cadherin

These and other studies have shown that VE-cadherin is a crucial player in vascular permeability and leukocyte TEM. Knocking out VE-cadherin in mice leads to embryonic lethality at 9.5 days of gestation due to impairment of vascular maturation and remodeling

(26). *In vitro*, antibodies directed against the extracellular domain of VE-cadherin disrupt VE-cadherin adhesion and clustering and increases endothelial cell permeability (21). A similar finding was shown to hold true *in vivo*, as administration of anti-VE-cadherin antibodies in mice leads to a significant increase in vascular permeability, fragility and hemorrhages (27). Leukocyte and also hematopoietic stem cell TEM was increased *in vitro* in endothelial cells pretreated with blocking antibodies against VE-cadherin (23,24). Increased leukocyte TEM into inflamed peritoneum was shown *in vivo* in a mouse peritonitis model upon i.v. injection of anti-VE-cadherin antibodies (25). Allport *et al*., Shaw *et al*. and van Buul *et al*. have shown transient displacement of VE-cadherin at sites of transmigration upon leukocyte binding (10,24,28). Consequently, adherens junctions and specifically VE-cadherin is certainly an important regulator in vascular permeability and leukocyte paracellular transmigration. Therefore regulation of VE-cadherin within endothelial cells must also be an important part of regulation of leukocyte TEM.

Regulation of VE-cadherin occurs through many avenues, such as association with binding partners, level of protein expression, and phosphorylation. Homophilic binding of VE-cadherin is important for junctional integrity, while binding of other partners such as the catenin family regulates association of VE-cadherin and thus adherens junctions to the actin cytoskeleton. These regulatory pathways are not isolated events- each one affects the other. Binding of certain proteins affects VE-cadherin level of protein expression and stability at the plasma membrane, while phosphorylation of VE-cadherin affects the affinity to which it binds its intracellular partners.

Like other members of the cadherin family, VE-cadherin is linked through its cytoplasmic tail to other catenin binding proteins and also to the actin cytoskeleton. VE-

cadherin's cytoplasmic tail is bound by the adaptor proteins β-catenin and plakoglobin (γ-catenin) (Figure 2) (29). In turn, β-catenin and plakoglobin bind to α-catenin, which binds directly to the actin cytoskeleton and also binds to other actin binding proteins α-actinin, vinculin, AF6, ZO-1 and others (30,31). It should be mentioned that recent studies have shown that a stable actin filament, α-catenin, β-catenin, cadherin complex does not exist; that α-catenin cannot bind actin filaments and catenin-cadherin simultaneously (32,33). Though these studies were done with E-cadherin and not VE-cadherin, these two proteins are both classical cadherins that are calcium-dependent, homophilic adhesion molecules that bind similar adherens junction proteins (14,29,34). Another protein, p120 catenin, binds to the juxtamembrane domain in the C-terminal tail of VE-cadherin. This interaction with p120 catenin is important for VE-cadherin stability at the plasma membrane as well as abundance of cadherin protein levels within the endothelial cell (Figure 2) (35-37).

It has been shown previously that tyrosine phosphorylation of adherens junction proteins correlates with the disassembly of endothelial cell-cell junctions (38-40). Cadherin / catenin complexes have specifically been shown to be regulated by tyrosine phosphorylation (41,42). A decrease in tyrosine phosphorylation of adherens junctions and of VE-cadherin / catenin is observed with an increase in the confluence of endothelial cells (43). This makes sense, as endothelial cells make a confluent monolayer the more tight and stable cell-cell junctions they will make. It follows then that if leukocytes are to migrate through these endothelial cells, they need to initiate signaling within the endothelial cells that will increase the phosphorylation of VE-cadherin, which disrupts cell-cell junctions and creates gaps between adjacent endothelial cells through which the leukocytes may pass. This is exactly what has been shown to happen.

When the leukocyte binds firmly to the endothelial cells it initiates a cascade of signals within the endothelial cells that facilitates the weakening of the junctions with adjacent endothelial cells. This allows for paracellular migration of leukocytes between the endothelial cells (11). Both ICAM-1 and VCAM-1 initiate signaling within endothelial cells that is required for transmigration of leukocytes (44,45). When LFA-1 on the leukocyte binds to ICAM-1 on the endothelial cell, ICAM-1 clusters around the leukocyte and initiates signaling within the endothelial cell. Blocking ICAM clustering with antibodies inhibits the adhesion and migration of leukocytes across the endothelium (46). It has been shown previously that ICAM-1 engagement activates Src kinase (47,48). More recently, Allingham *et al.* showed that ICAM-1 engagement activates endothelial proline-rich tyrosine kinase 2 (Pyk2) in addition to Src. They demonstrated that both Src and Pyk2 are recruited to sites of ICAM-1 engagement and that inhibition of either kinase results in decreased tyrosine phosphorylation in response to ICAM-1 engagement (49). Potter *et al.* identified two critical residues in the C-terminal tail of VE-cadherin that become phosphorylated and lead to the disassembly of cell-cell junctions. These two tyrosine residues, Y658 and Y731, are located within the binding sites for two important VE-cadherin binding partners, p120-catenin and β-catenin, respectively. When these tyrosines, Y658 and Y731, become phosphorylated, p120- and β-catenin binding to the C-terminal tail of VE-cadherin is disrupted, which ultimately leads to a disruption of cell-cell junctions (Figure 2) (50). Loss of β-catenin binding to VE-cadherin uncouples VE-cadherin from the actin cytoskeleton, weakening cell-cell junctions and promoting their disassembly. Since p120-catenin is important for stable VE-cadherin at cell-cell junctions, loss of p120-catenin binding to VE-cadherin leads to clathrin-mediated endocytosis and thus disassembly of cell-cell junctions (37,51,52). VE-cadherin

phosphomimetic mutants Y658E and Y731E showed defects in endothelial cell barrier function and failed to bind to p120- and β-catenins, respectively. Phosphorylation of VE-cadherin and loss of barrier function were recently shown to be important specifically in leukocyte TEM. Incubation of endothelial cells with leukocytes caused an increase in tyrosine phosphorylation of VE-cadherin and engagement of ICAM-1 specifically caused a significant increase in phosphorylation of residues Y658 and Y731 on VE-cadherin (49). Allingham *et al.* demonstrated that ICAM-1 engagement initiates phosphorylation of VE-cadherin by activating Src and Pyk2 kinases. Inhibiting the activity of either Src or Pyk2 causes a significant decrease in the phosphorylation of VE-cadherin on both residues, Y658 and Y731, even below baseline levels (49). Inhibition of the activity of Src and Pyk2 also caused a significant decrease in leukocyte transmigration across endothelial cell monolayers (49). In summary, the two critical VE-cadherin residues are phosphorylated in response to ICAM-1 engagement and phosphorylation of these residues require the activity of Src and Pyk2 kinases. Inhibition of ICAM-1 engagement, Src kinase activity or Pyk2 kinase activity leads to a significant decrease in leukocyte TEM. These findings lead us to hypothesize that these two VE-cadherin tyrosine residues will also be important in leukocyte TEM.

In this thesis, we demonstrate that the tyrosine residues Y658 and Y731 in VE-cadherin are indeed important in leukocyte TEM. We also show that mutating these two tyrosine (Y) residues individually or both together to a non-phosphorylatable phenylalanine (F) residue inhibits the ability of leukocytes to transmigrate across endothelial cell monolayers. This inhibition of leukocyte TEM is due to increased barrier function and tightening of endothelial cell-cell junctions. This work was initiated by Michael Allingham as he generated the VE-cadherin Y to F mutants and performed the initial transmigration

experiment of leukocytes transmigrating across endothelial cell monolayers expressing the single VE-cadherin Y658F and Y731F mutants. My work focuses on characterizing the changes in barrier function of endothelial cells expressing the VE-cadherin single mutants, Y658F and Y731F, and the VE-cadherin double mutant, Y658F / Y731F. This project also investigates the changes in leukocyte TEM across endothelial cells expressing the VE-cadherin single mutants, Y658F and Y731F, and the VE-cadherin double mutant, Y658F / Y731F.

Figure 2. VE-cadherin structure and binding

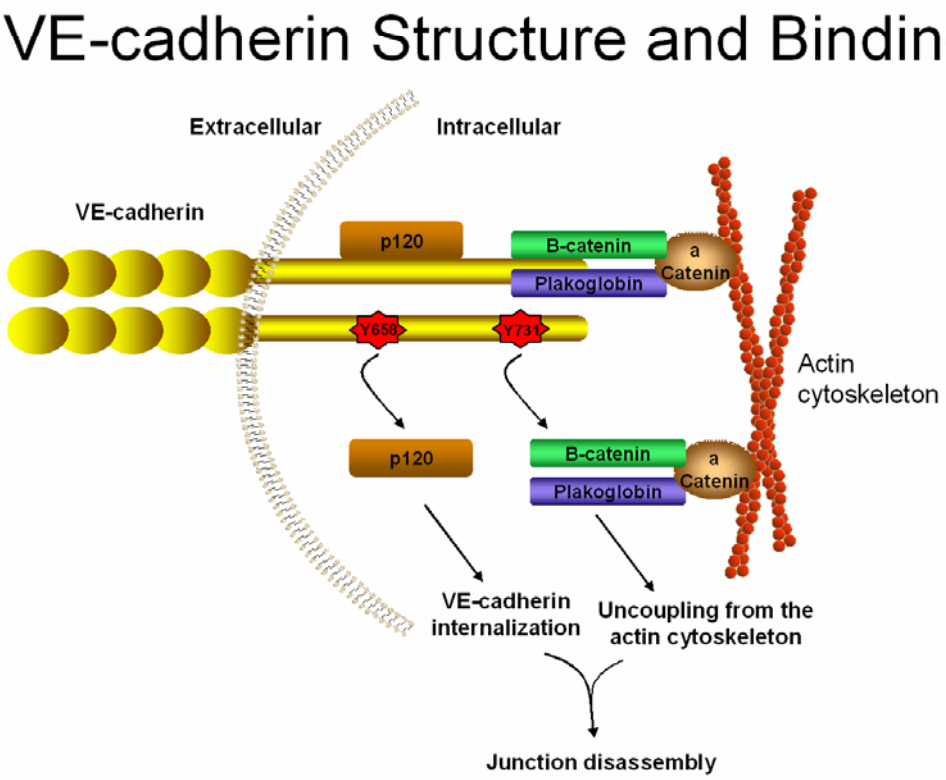


Figure 2. VE-cadherin structure and binding. VE-cadherin has five extracellular domains and a cytoplasmic tail. Adherens junction proteins such as p120-catenin, β-catenin and plakoglobin bind the cytoplasmic tail of VE-cadherin which keeps it stable at the plasma membrane and link it to the actin cytoskeleton.

CHAPTER II

Materials and Methods

Molecular Cloning and Virus Production

The wild type VE-Cadherin GFP adenovirus was a gift of Drs. F. Nwariaku and D. Nahari (Univeristy of Texas Southwestern Medical Center, Dallas, TX.) All other adenoviral constructs were generated using the Virapower Adenoviral Expression System (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The VE-cadherin-GFP wildtype construct (pEGFP, BD Biosciences Clontech, Mountain View, CA) was used as a template to create single point mutations in VE-cadherin. Residues Y658 and Y731 were individually mutated to phenylalanines using Stratagene's (La Jolla, CA) Quikchange kit according to manufacturer's instructions. The VE-cadherin GFP Y731F single mutant was then used as a template to create the double VE-cadherin mutant Y658F / Y731F. Primers were then used to create a 5' CACC site in the VE-cadherin GFP constructs in order to subclone them into Invitrogen's (Carlsbad, CA) pENTR/ D-Topo construct. The VE-cadherin GFP constructs with the 5' CACC site were then amplified using PCR, gel purified, and ligated into pENTR/ D-Topo. The VE-cadherin pENTR/ D-Topo contructs were then transformed into bacteria, plated on LB agar and allowed to grow overnight at 37°C. Single colonies were picked, grown in LB media and purified using Qiagen's (Valencia, CA) miniprep kit according to the manufacturer's protocol. The VE-cadherin pENTR/ D-TOPO constructs were sequenced to check for the appropriate mutations in VE-

cadherin and to assure no spontaneous mutations had occured. The VE-cadherin pENTR/ D-TOPO contructs were then recombined into the adenoviral expression construct, pAd/CMV/V5 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The VE-cadherin pAd/CMV/V5 constructs were then transformed into bacteria, plated on LB agar, and grown overnight at 37°C. Single colonies were picked, grown in LB media and purified using Qiagen's (Valencia, CA) midiprep kit according to the manufacturer's protocol. The purified VE-cadherin adenoviral constructs were then digested with Pac-I restriction enzyme (New England Biolabs, Ipswich, MA) and transfected into 293a cells with FuGene6 (Roche, Indianapolis, CA) according to the manufacturer's protocol. The infection of the 293a cells was allowed to proceed for 10-14 days until cytopathic effects were present. The cells were then harvested to produce a crude viral lysate. The crude viral lysate was then used to reinfect new 293a cells and amplify the VE-cadherin adenoviral stocks. The amplified VE-cadherin adenoviral stocks were then harvested and used to infect mammalian cells so that they expressed either VE-cadherin wildtype, Y658F, Y731F or the double mutant Y658F/Y731F.

Tissue Culture

The culturing of human derived cell lines was done according to standard laboratory procedure. All cells were incubated at 37°C at 5% CO_2. Stocks of cells were stored at -180°C in liquid nitrogen. Frozen cells were thawed as necessary to maintain low passage cells.

HUVECs

Human umbilical vein endothelial cells were obtained from Cambrex/ Clonetics (East Rutherford, NJ). HUVECs were grown in EGM-2 (Cambrex/Clonetics)

media replacing the media every other day and routinely used between passage 3-6. Monolayers of HUVECs were formed by seeding at near confluent density and culturing for 2-5 days. (53) Endothelial cells were activated with 10ng/ml TNF-α (R&D Systems, Minneapolis, MN) overnight as indicated to mimic inflammation for transendothelial migration assays.

Neutrophils

Primary human neutrophils were isolated from healthy volunteers according to Institutional Review Board-approved protocols as described previously (54). Briefly, 8ml of blood was added to a vacutainer cell preparation tube with sodium citrate (BD Vacutainer, Franklin Lakes, NJ). Monocytes and platelets were removed using density gradient centrifugation by spinning the vacutainers for 20 minutes at 1500rpm. Erythrocytes were lysed by resuspending the pellet fraction in ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1mM EDTA, pH 7.4). Remaining neutrophils were washed once with PBS and resuspended in EGM-2 without FBS or GA-1000 plus 0.25% delipidated BSA for transmigration assays.

293a Cells

293a cells were grown in DMEM (Invitrogen, Gibco, Carlsbad, CA) plus non-essential amino acids. Cells were split 1:10 apporximately every 4-5 days for maintenance. Cells were used for adenoviral production according to Invitrogen's (Carlsbad, CA) ViraPower Adenoviral Expression System.

Adenoviral VE-cadherin GFP Expression

HUVECs were seeded at near confluent density in 60mm dishes and grown to confluence in EGM-2 media (Cambrex/Clonetics, East Rutherford, NJ). They were then infected with VE-cadherin adenovirus by adding amplified adenoviral stock to the culture media. Infections were allowed to proceed for 24 hours. VE-cadherin GFP expression was analyzed by lysing HUVECs in hot sample buffer and boiled for 15 minutes. Cell lysates were run on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Millipore Immobilon, Billerica, MA). Lysates were blotted for VE-cadherin, GFP, and actin. Monoclonal antibodies against VE-cadherin were obtained from BD Transduction Laboratories (Lexington, KY). Monoclonal antibodies against GFP were obtained from Invitrogen (Carlsbad, CA). Monoclonal antibodies against actin were obtained from Millipore Chemicon (Billerica, MA).

Immunocytochemistry

HUVECs were cultured on glass coverslips coated with Matrigel (BD Matrigel, Bedford, MA) and infected with either VE-cadherin wildtype, Y658F, Y731F, or Y658F/Y731F adenovirus. Adenoviral infections were allowed to proceed for 24 hours then the cells were fixed with 4% formaldehyde for 10 minutes. Coverslips were mounted on glass slides using Mowiol (EMD Calbiochem, San Diego, CA). Images were recorded with a Zeiss Axiovert 200 inverted fluorescence microscope (Carl Zeiss, Gottingen, Germany) using Metamorph 7.1.7.0 (Molecular Devices, Downington, PA).

FITC-Dextran Passage Assay

FITC-Dextran passage assays were performed by culturing HUVECs on Transwell filters (Corning Inc., Corning, NY) of 12mm diameter and 0.4μm pore-size. The cells were seeded at near confluent density and allowed to form a monolayer for 2-3 days. The HUVECs were infected with either VE-cadherin wildtype, Y658F, Y731F, or Y658F/Y731F adenovirus. Adenoviral infections were allowed to proceed for 24 hours and then the FITC-Dextran passage assay was performed. The HUVECs were incubated with assay media (EGM-2, Cambrex/Clonetics) containing 400 ng/ml FITC-Dextran 10,000 mw (Invitrogen Molecular Probes, Eugene, OR). The lower chamber was filled with assay media. The cells were then incubated at 37°C, 5% CO2. 50μl samples were taken from the lower chamber every 30 minutes for 3 hours and transferred to a 96-well plate. The amount of FITC-Dextran that had passed through to the lower chamber was quantitated with a fluorescence plate reader.

RT-CES

HUVECs were seeded at confluent density (30,000 cells/well) on a 16X microplate, a specialized microplate integrated with sensor electrodes made specifically for the RT-CES system (ACEA Biosciences, San Diego, CA). Cells were infected with either VE-cadherin wildtype, Y658F, Y731F, or Y658F/Y731F adenovirus. Barrier function as a measure of cell index was recorded every 15 minutes for 24 hours. After 24 hours of adenoviral infection, HUVEC monolayers were then stimulated with 10 ng/ml thrombin (Sigma Aldrich, St. Louis, MO) and the barrier function as a measure of cell index was recorded every 2 minutes.

Transendothelial Migration Assay

Migration assays were performed by culturing HUVECs on Transwell filters (Corning Inc., Corning, NY) of 6.5mm diameter with 8μm pores and coated with Matrigel (BD Matrigel, Bedford, MA). The cells were grown to confluence and infected with either VE-cadherin wildtype, Y658F, Y731F, or Y658F/Y731F adenovirus. Adenoviral infections were allowed to proceed for 24 hours and then the assay was performed. HUVECs were activated by treating with 10 ng/ml TNFα overnight. A coverslip coated with Matrigel (BD Matrigel, Bedford, MA) was inserted into the lower chamber which was filled with assay media (EGM-2 minus FBS and GA-1000, plus 0.25% delipidated BSA [Sigma Aldrich, St.Louis, MO]) plus 5ng/ml IL-8 (R&D Systems, Minneapolis, MN) as a chemoattractant. HUVECs were washed twice with the assay media and then incubated with $1x10^5$ freshly isolated human neutrophils for 1 hour at 37° C, 5% CO_2. Transwells (Corning Inc.) were removed and the coverslips with attached transmigrated neutrophils were fixed with 4% formaldehyde for 10 minutes. Neutrophil nuclei were stained using Hoechst dye (Invitrogen Molecular Probes, Eugene, OR). Coverslips were mounted with Mowiol (EMD Calbiochem, San Diego, CA) and imaged using a Zeiss microscope. Neutrophil nuclei were quantitated using Metamorph's nuclei count application. To check for efficient expression of VE-cadherin GFP constructs, HUVECs were simultaneously grown on coverslips coated with Matrigel (BD Matrigel) in a 24-well dish and treated with an equivalent amount of VE-cadherin GFP adenovirus and analyzed by fluorescence microscopy.

CHAPTER III

Results

Characterization of VE-cadherin GFP wildtype, single and double mutant constructs

It has been shown previously that tyrosine phosphorylation of adherens junction proteins correlates with the disassembly of endothelial cell-cell junctions (38,39). Cadherin / catenin complexes have specifically been shown to be regulated by tyrosine phosphorylation (41). Potter *et al.* identified two critical residues in the C-terminal tail of a classical cadherin, VE-cadherin, which become phosphorylated and leads to the disassembly of cell-cell junctions. These two tyrosine residues, Y658 and Y731, are located within the binding sites for two important VE-cadherin binding partners, p120-catenin and β-catenin, respectively. When these tyrosines, Y658 and Y731, become phosphorylated, p120- and β-catenin binding to the C-terminal tail of VE-cadherin is disrupted which ultimately leads to a disruption of cell-cell junctions (50). Thus these two tyrosine residues are critical in stability of endothelial cell-cell junctions. Allingham *et al.* demonstrated that incubation of endothelial cells with leukocytes caused an increase in tyrosine phosphorylation of VE-cadherin and that engagement of ICAM-1 specifically caused a significant increase in phosphorylation of residues Y658 and Y731 on VE-cadherin (49). In order to determine whether the phosphorylation of tyrosine residues Y658 and Y731 in VE-cadherin are necessary in leukocyte TEM, we mutated each tyrosine residue individually or both together to a non-phosphorylatable phenylalanine (F) residue. These VE-cadherin GFP wildtype, single mutant

Y658F and Y731F and double mutant (DM) Y658F / Y731F constructs were cloned in an adenoviral expression system. To determine if these VE-cadherin GFP constructs were expressed properly primary endothelial cells, we infected HUVECs with either VE-cadherin GFP wildtype, Y658F, Y731F, or double mutant adenoviral particles. VE-cadherin GFP expression was analyzed by western blot using anti-VE-cadherin and anti-GFP antibodies. Equal loading was determined using an anti-actin antibody. We observed that all four VE-cadherin GFP construct expression was detectable in HUVECs 24 hours after adenoviral infection (Figure 3A). We next wanted to determine whether these VE-cadherin single (Y658F and Y731F) and double (Y658F / Y731F) mutant constructs localized properly to endothelial cell-cell junctions. HUVECs plated on glass coverslips coated with Matrigel were infected with either VE-cadherin GFP wildtype, Y658F, Y731F, or double mutant adenovirus. After 24 hours of infection, the HUVECs were then fixed and mounted on glass slides. VE-cadherin GFP localization was determined by fluorescence microscopy. We observed that all four VE-cadherin constructs, wildtype, Y658F, Y731F, and double mutant, localized properly to endothelial cell-cell junctions in HUVEC monolayers (Figure 3B).

Figure 3. VE-cadherin GFP constructs express and localize properly in endothelial cells.

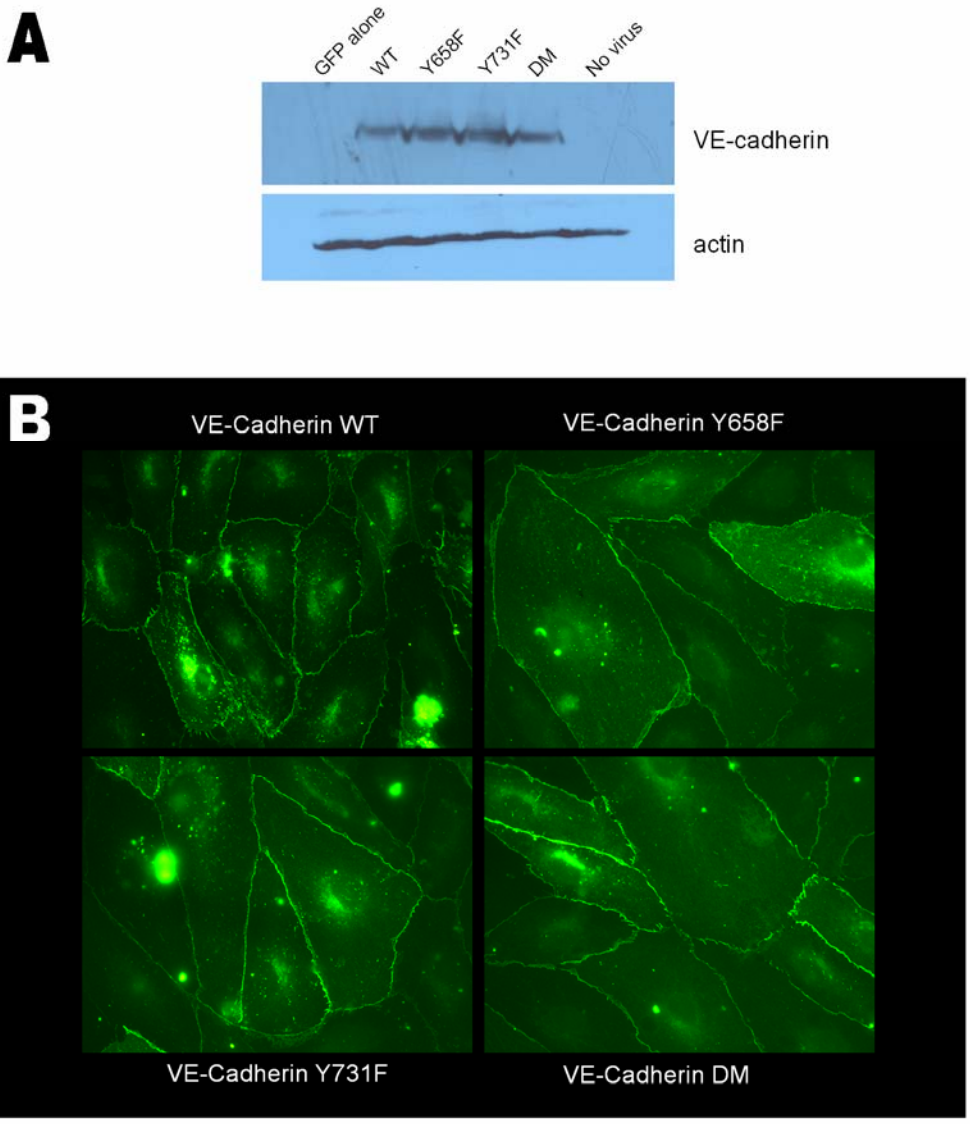


Figure 3. VE-cadherin GFP constructs are expressed and localize properly to cell-cell junctions in endothelial cells. (A) Endothelial cells express a C-terminally GFP-tagged VE-cadherin wildtype, single mutant Y658F and Y731F, or double mutant Y658F / Y731F using an adenoviral expression system. (B) Endothelial cells express a C-terminally GFP-tagged VE-cadherin wildtype (top left), single mutant Y658F (top right), single mutant Y731F (bottom left), or double mutant (DM) Y658F / Y731F (bottom right) adenovirus. All VE-cadherin constructs localize to cell-cell junctions properly.

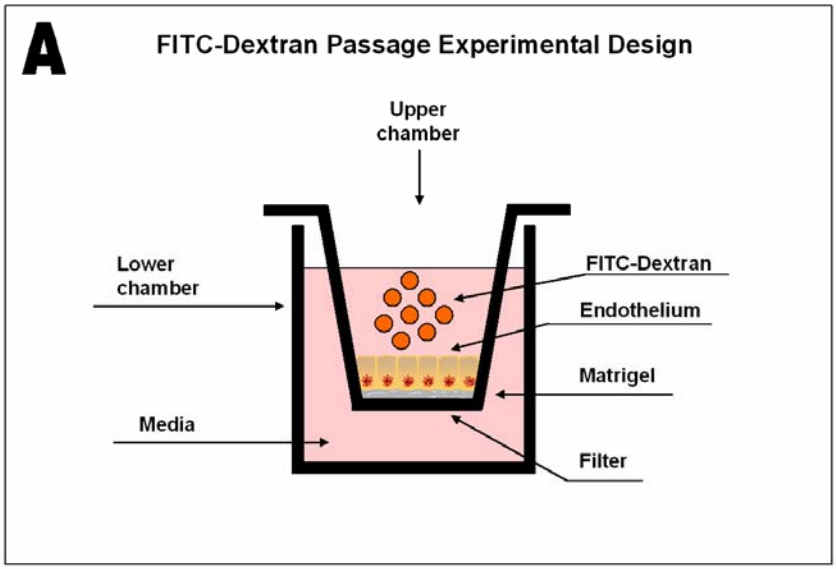
Phosphorylation of tyrosine residues Y658 and Y731 in VE-cadherin are important in barrier function of endothelial cell monolayers

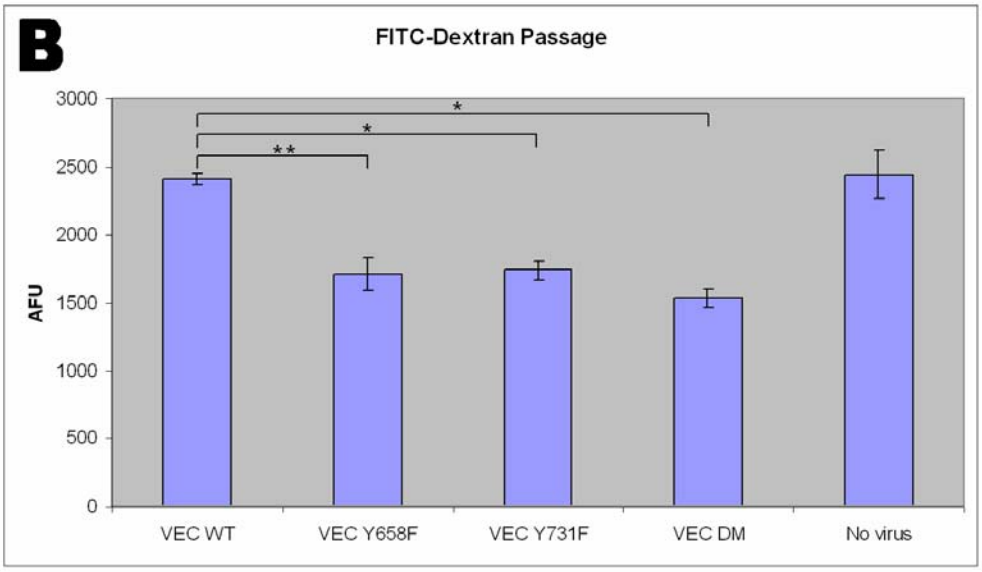
It has previously been shown that tyrosine residues Y658 and Y731 in VE-cadherin become phosphorylated in response to stimuli that increase vascular permeability (42,55). Potter *et al.* demonstrated that phosphorylation of tyrosine residues Y658 and Y731 in VE-cadherin is sufficient to abolish barrier function by use of phosphomimetic VE-cadherin single mutants, Y658E and Y731E (50). To determine whether phosphorylation of these tyrosine residues is necessary for regulating barrier function and junctional permeability, the characterized non-phosphorylatable VE-cadherin single mutants Y658F, Y731F and the double mutant Y658F / Y731F were expressed in primary endothelial cell monolayers and junctional permeability was assessed. Junctional permeability was assessed using a FITC-Dextran passage assay where HUVEC monolayers were grown on transwell filters coated with Matrigel. These transwell filters have a top and bottom chamber that are separated by a filter with 0.4μm pores (Figure 4A). While these pores allow the passage of small molecules they are too small to allow cells pass through to the bottom chamber. When the HUVECs form a monolayer over this filter the passage of molecules between the top and bottom chambers is dependent upon the junctional permeability of the HUVEC monolayer. In this assay, the lower chamber is filled with assay media while the top chamber is filled with assay media plus FITC-Dextran. The passage of FITC-Dextran through the HUVEC monolayer is then determined by taking media samples from the lower chamber and measuring the amount of FITC-Dextran fluorescence using a fluorescence plate reader. Compared to the HUVEC monlayer expressing VE-cadherin GFP wildtype, the HUVEC monolayers expressing VE-cadherin GFP Y658F, Y731F and the monolayer expressing the VE-cadherin GFP double

mutant all showed significantly* decreased FITC-Dextran passage to the lower chamber at steady state (*$p<0.05$) (Figure 4B) indicating an increased barrier function and decreased endothelial cell monolayer permeability. Similar results were obtained with another barrier function assay using the real-time cell electronic sensing (RT-CES) system. RT-CES measures electrical impedance of sensor electrodes integrated onto the bottom of specialized microplates to quantify barrier function in real time. Based on measured impedance, a cell index is derived and reported to provide quantitative information about the barrier function of the plated cells. HUVECs were seeded at confluent density on these specialized microplates and allowed to form a monolayer. These HUVEC monolayers were then infected with either VE-cadherin GFP wildtype, Y658F, Y731F or double mutant Y658F / Y731F adenovirus. Barrier function of the HUVEC monolayers at steady state was recorded as a measure of cell index every 15 minutes for 24 hours. Compared to the HUVEC monolayer expressing VE-cadherin GFP wildtype, the HUVEC monolayers expressing VE-cadherin GFP Y658F, Y731F and the monolayer expressing the VE-cadherin GFP double mutant all showed an increase in cell index at steady state (Figure 5A) indicating that the HUVEC monolayers expressing the VE-cadherin mutants have increased barrier function and thus tighter cell-cell junctions. The endothelial cell monolayers were then stimulated with thrombin (10 ng/ml) to determine whether they could still respond appropriately to vascular stimuli utilizing signaling pathways other than VE-cadherin. All endothelial cell monolayers expressing the VE-cadherin mutants responded appropriately to thrombin showing decreased cell index which reflects an increase in endothelial cell monolayer permeability (Figure 5B).

These data suggest that phosphorylation of tyrosine residues Y658 and Y731 are indeed necessary for the regulation of endothelial cell-cell junctions and increased endothelial cell monolayer permeability.

Figure 4. Phosphorylation of Y658 and Y731 in VE-cadherin are necessary for the increased endothelial cell monolayer permeability at steady state.





* p < 0.005 ** p = 0.005

Figure 4. Phosphorylation of Y658 and Y731 on VE-cadherin are necessary for the increased endothelial cell monolayer permeability at steady state. (A) FITC-Dextran passage assay experimental design. Endothelial cell monolayers were grown on transwell filters with 0.4μm pores. (B) Endothelial cell monolayers expressed either VE-cadherin GFP wildtype, Y658F, Y731F or double mutant Y658F / Y731F using an adenoviral expression system. Endothelial cells were incubated with 10,000mw FITC-Dextran to determine junctional permeability. Passage of FITC-Dextran was stopped at 120min and determined by a fluorescence plate reader. Both single non-phosphorylatable mutants and double non-phosphorylatable VE-cadherin mutants have significantly decreased junctional permeability at steady state (*p<0.05).

Figure 5. Endothelial cell monolayers expressing non-phosphorylatable mutants of VE-cadherin, Y658F, Y731F and Y658F / Y731F have increased barrier function.

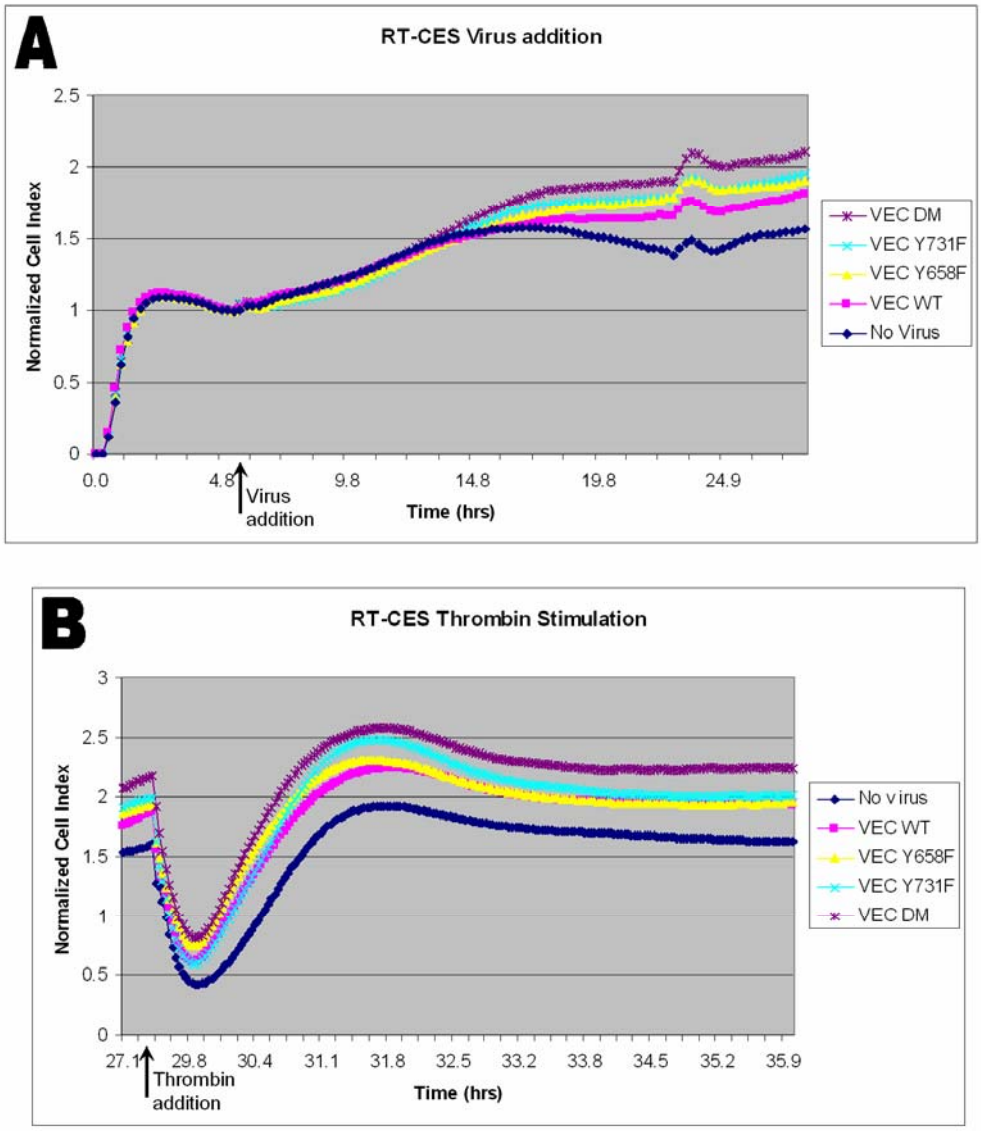


Figure 5. Endothelial cell monolayers expressing non-phosphorylatable mutants of VE-cadherin, Y658F, Y731F and Y658F / Y731F have increased barrier function. (A) Endothelial cells were grown on specialized microplates integrated with sensor electrodes made for the RT-CES system. The cell index readout assesses changes in cell permeability by electrical impedance. GFP-tagged VE-cadherin wildtype, Y658F, Y731F, or double mutant Y658F / Y731F was expressed using an adenoviral expression system. Endothelial cell monolayers expressing non-phosphorylatable mutants of VE-cadherin, Y658F, Y731F and Y658F / Y731F have increased barrier function at steady state. (B) Endothelial cell monolayers expressing the VE-cadherin mutants were then stimulated with thrombin (10 ng/ml). All endothelial cell monolayers expressing the VE-cadherin mutants responded appropriately to thrombin showing decreased cell index which reflects an increase in endothelial cell monolayer permeability.

Phosphorylation of tyrosine residues Y658 and Y731 in VE-cadherin are important in leukocyte transendothelial migration

Considering that the phosphorylation of the two tyrosine residues Y658 and Y731 in the C-terminal tail of VE-cadherin have been shown to be both necessary and sufficient for loss of barrier function in endothelial cell monolayers, we hypothesized that these two tyrosine resides would also be important in regulating leukocyte TEM. To test this hypothesis, we utilized transendothelial migration assays in which endothelial cell monolayers are grown in transwell chambers. These transwell chambers have a top and bottom chamber that are separated by a filter with 8μm pores. The filters are coated with Matrigel on which the HUVECs form monolayers. The HUVECs are treated with TNFα overnight to activate the cells simulating an inflammatory response. The leukocytes are placed above the HUVEC monolayer and must transmigrate through the HUVEC monolayer and the pores in the filter in order to reach the bottom chamber which is filled with assay media plus the chemoattractant IL-8 (Figure 6A). Once the activated leukocytes reach the bottom chamber they attach to a coverslip coated with Matrigel at the bottom of the chamber. The leukocytes were allowed to transmigrate for 1 hour then the coverslips were fixed and stained. For these assays, primary human neutrophils were used. Compared to the endothelial cell monolayers expressing VE-cadherin GFP wildtype, the endothelial cell monolayers expressing the Y658F, Y731F or Y658F / Y731F mutants had a decreased amount of neutrophil transmigration (Figure 6B). This data demonstrates that VE-cadherin phosphorylation of tyrosine residues Y658 and Y731 is important in regulating neutrophil TEM.

Figure 6. Phosphorylation of Y658 and Y731 on VE-cadherin are essential for effective neutrophil transendothelial migration.

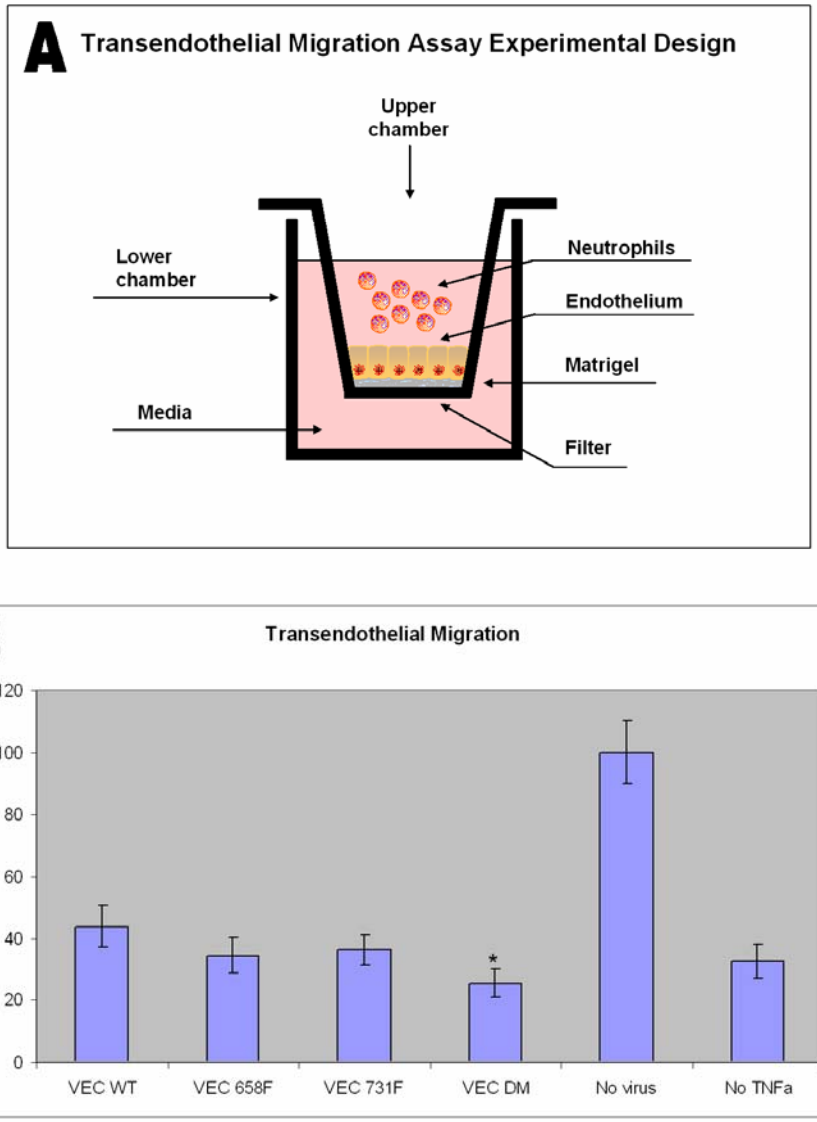


Figure 6. Phosphorylation of Y658 and Y731 on VE-cadherin are essential for effective neutrophil transendothelial migration. (A) Transendothelial migration assay experimental design. Endothelial cell monolayers were grown on transwell filters with 8μm pores. (B) Endothelial cell monolayers expressed either VE-cadherin wildtype, Y658F, Y731F, or double mutant Y658F / Y731F using an adenoviral expression system. Endothelial cells were activated overnight with TNF-α and then incubated with freshly isolated human neutrophils for 1 hour at 37°C. Data is representative of at least 3 independent experiments with each condition performed in triplicate. Non-phosphorylatable VE-cadherin mutants inhibit neutrophil TEM. Thus, phosphorylation of Y658 and Y731 on VE-cadherin are essential for effective neutrophil transendothelial migration.

CHAPTER IV

Discussion and Future Directions

Leukocyte transendothelial migration is a key step in many functions of the immune system such as immune surveillance, inflammation, fighting infection, and wound repair. Transendothelial migration involves many complex interactions and signaling pathways between the leukocytes and the endothelial cells that line the lumen of the blood vessels. It used to be thought that the leukocytes actively bound and crossed the endothelial cells, while the endothelial cells themselves were passive partners in this process. However, it has become apparent that the endothelial cells play just as important a role during transendothelial migration as the leukocytes themselves. Endothelial cells make an impermeable barrier by forming a single monolayer of cells and attaching to the adjacent endothelial cells via multiple junctions such as adherens junctions. Adjacent endothelial cells must disassemble their junctions to allow for the leukocyte to pass through the endothelium in order for paracellular migration of leukocytes to occur. The controlled disassembly of these junctions is a major component of TEM regulation. Many studies have shown a correlation between tyrosine phosphorylation and disassembly of adherens junctions (31,38-41,56). In particular, the tyrosine phosphorylation of VE-cadherin, a protein important in adherens junctions, has been shown to disrupt cell-cell junctions and increase vascular permeability; while decreased tyrosine phosphorylation of VE-cadherin correlates with confluent endothelial monolayer and increased barrier function (42,43,49,50,55). Recently,

Potter *et al.* identified two critical tyrosine residues in the C-terminal tail of VE-cadherin that are important in regulation of barrier function in endothelial cells. These two residues, Y658 and Y731, correlate to binding sites for p120- and β-catenin, respectively. Phosphorylation of residue Y658 causes the uncoupling of p120-catenin binding to the C-terminal of VE-cadherin (50). Since binding of p120-catenin is important for the stability of VE-cadherin at the plasma membrane and also level of protein expression of VE-cadherin, loss of p120-catenin binding leads to clathrin-mediated endocytosis of VE-cadherin and thus disassembly of cell-cell junctions (37,50-52). Phosphorylation of residue Y731 causes the loss of β-catenin binding to the tail of VE-cadherin which uncouples VE-cadherin from the actin cytoskeleton which also weakens cell-cell junctions and promotes their disassembly (50).

Our findings support the importance of VE-cadherin phosphorylation in regulating junctional permeability in endothelial cells. We found that blocking the phosphorylation of VE-cadherin on residues Y658 and Y731 using non-phosphorylatable VE-cadherin mutants, tyrosine to phenylalanine mutations specifically, lead to increased barrier function and decreased endothelial cell monolayer permeability. This demonstrates that phosphorylation of residues Y658 and Y731 in the C-tail of VE-cadherin is necessary for the regulation of endothelial cell junction permeability. Phosphorylation of VE-cadherin and loss of barrier function was recently shown to be important specifically in leukocyte TEM. Allingham *et al.* demonstrated that VE-cadherin Y658 and Y731 becomes phosphorylated downstream of Src and Pyk2 following leukocyte binding to ICAM-1 (49). Here we demonstrate that mutating tyrosine residues Y658 and Y731 to non-phosphorylatable phenylalanine residues, inhibits neutrophil transmigration across endothelial cell monolayers. Together, these data demonstrate that phosphorylation of the two VE-cadherin tyrosine residues, Y658 and Y731,

are required for increasing endothelial cell junctional permeability and thus for effective neutrophil transendothelial migration through endothelial cell monolayers.

Though VE-cadherin has been shown by this work and others to be important in endothelial cell signaling in leukocyte TEM, there is still much to be discovered in the active role that endothelial cells play in leukocyte TEM. For instance, which kinase directly phosphorylates the tyrosine residues Y658 and Y731 in the C-terminal of VE-cadherin is not known. Allingham *et al.* showed that Src and Pyk2 kinase activity are required for the phosphorylation of these residues but it is unclear if this is a direct or indirect interaction with VE-cadherin (49). Also, phosphatases must play a role in the degree of VE-cadherin phosphorylation in the endothelium. Vascular endothelial protein tyrosine phosphatase (VE-PTP) co-precipitates with VE-cadherin and reverses the phosphorylation of VE-cadherin in response to VEGFR2 (42). Other phosphatases such as density-enhanced phosphatase-1 (DEP-1), protein tyrosine phosphatase μ (PTPμ), and SH2-containing phosphotyrosine phosphatase (SHP2) have also been shown to associate with VE-cadherin and might play a role in its degree of phosphorylation (14,57-59).

While the role of VE-cadherin has been shown in paracellular TEM, there is much debate whether the paracellular or the transcellular pathways are both used as a means of leukocyte TEM. *In vivo* studies show that leukocytes utilize both the transcellular and paracellular routes of TEM (60,61). Most *in vitro* studies have found that leukocytes transmigrate across primary endothelial monolayer preferentially using the paracellular route (7,9,62). Though some studies have shown the route taken can be influenced by the type of transmigrating cell, the type of blood vessel or the leukocyte recruiting stimulus (1,9,61). For example, leukocytes may take different transmigratory routes when they are migrating across

high endothelial venules verses the blood-brain barrier. At present, whether leukocyte transmigration occurs primarily through a transcellular or paracellular route or both equally is a major question still to be determined.

In conclusion, we have demonstrated the phosphorylation of tyrosine residues Y658 and Y731 in VE-cadherin are necessary for increasing endothelial cell junction permeability and efficient leukocyte transendothelial migration. Misregulation of leukocyte TEM can lead to pathologies such as chronic inflammation, atherosclerosis, multiple sclerosis, rheumatoid arthritis, psoriasis, and ischemia reperfusion injury. Anti-adhesion therapies have the potential of being some of the most specific and advanced in the treatment of chronic inflammatory disease. Thus solving the mystery of transendothelial migration may provide new targets for the treatment of inflammatory diseases.

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