MODULATION OF ENDOTHELIAL ACTIVATION AND CEREBRAL ANGIOGENESIS BY TNF FAMILY LIGANDS AND RESVERATROL: AN *IN VITRO* STUDY

by

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Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
December 2010

DALHOUSIE UNIVERSITY

DEPARTMENT OF PATHOLOGY

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "MODULATION OF ENDOTHELIAL ACTIVATION AND CEREBRAL ANGIOGENESIS BY TNF FAMILY LIGANDS AND RESVERATROL: AN *IN VITRO* STUDY" by Pei-Lin Chen in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DALHOUSIE UNIVERSITY

DATE: December 10, 2010

AUTHOR: Pei-Lin Chen

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DEPARTMENT OR SCHOOL: DEPARTMENT OF PATHOLOGY

DEGREE: PhD CONVOCATION: May YEAR: 2011

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Signature of Author

For my dear parents —

who I love and respect; and

who give me the life of a princess, and the strength and spirit of a phoenix.

Dr Ming-Tsun Chen & Shu-Chin Chen Wu

and

Dr Hong Dao Tze & Mrs. Hong
(my Kungfu Shifu/Master & Shimu)

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ABSTRACT

Vascular endothelial cell activation and apoptosis (programmed cell death) are critical in inflammation and angiogenesis (the formation of new blood vessels). Tumor necrosis factor (TNF) is a pro-inflammatory cytokine known for its ability to induce endothelial cell activation and apoptosis. However, the ability of two death ligands in the TNF superfamily: TRAIL (TNF-Related Apoptosis-Inducing Ligand) and Fas ligand (FasL), to activate vascular endothelium is less well defined, and forms the basis of this work. We find that in the human endothelial cell line EA.hy926, TRAIL induces endothelial cell activation (activation of the transcription factor NF-κB with increased expression of the adhesion protein ICAM-1 and adhesion of human neutrophils) when it concurrently induces apoptosis. In addition, angiogenesis is implicated in diseases of the central nervous system, and its modulation represents an attractive therapeutic strategy. We investigated the modulatory potential of the two endogenous molecules TRAIL and FasL as well as an exogenous molecule resveratrol, a phytochemical present in red wine, in angiogenesis. We modeled cerebral angiogenesis with the human brain endothelial cell line hCMEC/D3 and primary bovine brain endothelial cells. Resveratrol inhibited several parameters of angiogenesis (proliferation, migration and tube formation) in human umbilical vein endothelial cells, however, neither TRAIL nor FasL had an effect on this model. By contrast, in hCMEC/D3 cells both resveratrol and TRAIL inhibited all parameters while FasL had minimal effects. Resveratrol did not induce apoptosis in hCMEC/D3 but arrested cell cycle progression to G2/M phase and inhibited phosphorylation of Akt/PKB, a key cell survival protein kinase. This leads to a reduction in cell growth, endothelial migration and tube formation, hence, inhibition of *in vitro* angiogenesis. TRAIL induced anti-angiogenic effects in hCMEC/D3 due to apoptosis. The data suggests that TRAIL primarily influences angiogenesis through induction of vascular endothelial apoptosis while resveratrol induces cell cycle arrest, both of which may lead to vessel regression. These are the first studies to report the modulation of different aspects of endothelial cell activation by TRAIL and resveratrol in several endothelial cell culture models, with a particular focus on the central nervous system.

LIST OF ABBREVIATIONS AND SYMBOLS USED

^{o}C	degree Celsius
AM	acetoxymethyl ester
Ang	angiopoietin(s)
ANOVA	analysis of variance
Apaf	apoptotic protease activating factor
BBB	blood-brain-barrier
BBMEC	bovine brain microvascular endothelial cells
Bcl-2	B-cell lymphoma 2
bFGF	basic fibroblast growth factor
BrdU	5-bromo-2deoxyuridine
BSA	bovine serum albumin
CAM	chick chorioallantoic membrane
caspase	cysteinyl aspartic acid-protease
CCL	chemokine ligand(s)
CD	cluster of differentiation molecule(s)
Cdc	cell-division cycle
c-FLIP	cellular FLICE inhibitory protein
CI	caspase-inhibitor
CNS	central nervous system
CO_2	carbon dioxide
DD	death domain
ddH_2O	double-distilled or distilled-deionized water
DED	death effector domain
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPBS	Dulbecco Phosphate Buffered Saline
DTT	dithiothreitol
ECM	extracellular matrix
ECGS	endothelial cell growth supplement
EDTA	ethylenediaminetetraacectic acid
EGF	epidermal growth factor
EM	emission
ERK	extracellularsignal-regulated kinase(s)

EX	excitation
FACS	fluorescence-activated cell sorting
FADD	Fas-associated death domain
FasL	Fas ligand (CD95L)
FasR	Fas receptor (CD95)
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FLICE	FADD-like IL-1β-converting enzyme
FLT	Fms-related tyrosine kinase
xg	acceleration due to Earth's gravity (9.8m/s2)
g	gram(s)
GM-CSF	granulocyte macrophage colony-stimulating factor
h	hour(s)
H_2O	water
HCl	hydrochloride acid
hCMEC/D3	human cerebral microvascular endothelial cell/D3 cell line
HIF	hypoxia-inducible factor
HMVEC	human dermal microvascular endothelial cells
HRP	horseradish peroxidase
HSPG	heparin sulphate proteoglycan
HT	hypoxanthine and thymidine
HUVEC	human umbilical vein endothelial cells
ICAM	intercellular adhesion molecule
IGF	insulin-like growth factor
IgG	immunoglobulin G
IκB	inhibitor of kappa B
IKK	inhibitors of kappa B kinase
IL	interleukin
JNK/SAPK	c-jun N-terminal kinase/stress-activated protein kinase
kDa	kilodalton
KDR	kinase insert domain receptor(CD309)
L (l)	litre(s)
LDH	lactate dehydrogenase
LFA	lymphocyte function-associated antigen
LPS	lipopolysaccharide (endotoxin)
M	molar; moles per liter

MAPK	mitogen-activated protein kinase
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar
MMP	matrix metalloproteinase
MS	multiple sclerosis
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa B
nM	nanomolar(s)
NO	nitric oxide
O_2	molecular oxygen
OD	optical density
PAK	platelet-activating factor
p-Akt	phosphorylated Akt
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PE	phycoerythrin
PECAM	platelet/endothelial cell adhesion molecule (CD31)
pH	logarithmic unit measuring acidity
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PIGF	phosphatidylinositol-glycan biosynthesis class F protein
PIP	phosphatidylinositol phosphate
PMN	polymorphonuclear neutrophils
p-value	probability (of incorrectly rejecting the null hypothesis)
PVDF	polyvinylidene fluoride
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
s or sec	second(s)
SEM	standard error of the mean
TBST	Tris buffered saline with Tween-20
TEMED	N,N,N’,N’-tetramethylethylenediamine

TERT	telomerase reverse transcriptase
TNF	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TRAIL receptor
Tris-HCl	tris[hydroxymethyl]-amino methane hydrochloride
TSP	thrombospondin
TWEAK	TNF-related weak inducer of apoptosis
µL	microliter(s)
µM	micromolar
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLA	very late antigen, an integrin
XIAP	X-linked inhibitor of apoptosis proteins

ACKNOWLEDGEMENTS

What a great experience I've had at the Easton Lab! Many people have assisted me directly or indirectly with this thesis work. I cannot thank everyone enough for helping me grow as a person and gain the necessary knowledge to survive as a graduate student.

Firstly, I would like to thank my supervisor, Dr. Alex Easton, for the opportunity you gave me to pursue a degree that I never thought I had the courage to take on. I especially appreciate your encouragement, patience, and support over the past six years. Your amazing talent at hiring the most wonderful group of people truly made my PhD life one sweet memory. As well, thank you for allowing me to personalize my bench space, so I never want to leave this pleasant lab environment after work.

Many thanks to the Department of Pathology for giving me the opportunity to study and making the time spent here so enjoyable. I am grateful to my supervisory committee: Drs. David Hoskin, Andrew Issekutz, and Brent Johnston for helpful discussions and invaluable guidance on my studies. I could not complete my thesis research without your mentorship. Your devotion to research set the best example for me and all the students around. Also, thank you to Dr. Wenda Greer for walking me through the tough times and always having your office doors open. Of course, I must also thank Eileen Kaiser for working very hard behind the scene and I love that we share the same passion.

Thanks to Dr. A. Issekutz for providing us the human endothelial cells, HUVEC and EA.hy926 cell lines. This research would not be possible without expert technical assistance from many people, especially Pat Colp, Derek Rowter, Mary-Ann Trevors, and Stephen Whitefield. I would also like to acknowledge those labs which kindly offered to share their valuable equipment: Guernsey's, Hoskin's, Johnston's, and Marshell's labs.

My heartfelt appreciation goes to the fellow pathology graduate students and the 11th floor Tupper Building community for their support, their friendship, and their patience in enduring my laughter as well as some grunts and screams, when experiments did not work. In particular, thank you Duane for your interest in my research and always being there to encourage students to take the next step. I am especially grateful for the support from you and C.J. ("Siege") in the months leading to my thesis defense. Of course, I will never forget the "benefits of resveratrol experiments" we conducted after work.

I would like to express my most sincere gratitude to the past and present members of the Easton lab: fellow graduate students, Shannon, Krystyna, C.J., Firdaus, Sam, and Nadine (an honorary member of the Easton's); past technicians, Antar, Yaping, Emily, Jing, and Erin; and previous summer students, Brent, Aishwarya, Jahnavi, and Ryan. All are friends both inside and outside of the lab. You guys made the lab the best place to be. Thanks for introducing me to the true Canadian culture, taking my hands and showing me what Canadian University life is like. Thanks to the "Easton Girls", my English vocabulary has expanded dramatically in the past several years. It was an honour to work with everyone! Also, I promised to mention in this thesis the multicultural nickname you guys proudly gave me. Who knows what it means, but Wynnwaa McChen is pure fun!

I appreciate Dr. Zhongmin Dong at Saint Mary's University in Halifax, NS, for initiating me into the field of research. I also feel blessed to have many caring friends, both in Canada and in Taiwan. I am especially grateful to share a friendship with Patricia Irvine, Shannon Joice, Susan McCurdy, Erin Mentink, and Ron Patterson. They have

supported me in many ways beyond what a friend can do. They really are my guardian angels.

I would like to express my warmest acknowledgments to my Chinese Kungfu Shifu (like teacher and father; Master), Tai Ji Men Zheng-men-ren, the director of Tai Ji Men Qigong Academy and the founder of the Federation Of World Peace And LOVE (FOWPAL), Dr. Hong Tao Tze, and Shimu, his wife, Mrs. Hong. I owe a great deal of gratitude for Dr. Hong, if he had not been saved my life when I was 17. Shifu, the Kungfu and Philosophy of Yin & Yang what you taught me cannot be found in books. However, they can be easily applied to understanding book knowledge, most importantly, to real life situations which are not described on the page. I learned to stand back and view the big picture with a smile before dealing with any frustrating moments both in research and in life. I will follow your steps to give, to care, and to love the world!

Last, but certainly not the least, I appreciate my Mom and Dad for their everlasting love. Mom and Dad, no words can describe my thankfulness for your trust in and support of me. You have not only provided me with a sweet and carefree home, but have also given me the courage and opportunity to explore the world without worries. I have benefited so much from the integrity and moral values you taught me. And finally, a big thank to all my family who have always made me laugh and are just a phone call away.

CHAPTER 1- INTRODUCTION

1.0. Overview

There has been an increasing awareness of the link between cancer and chronic inflammatory diseases, such as atherosclerosis, ulcerative colitis, and rheumatoid arthritis (1-5). In this respect, angiogenesis is of interest. Angiogenesis refers to the formation of new blood vessels from pre-existing vasculature. Its involvement in cancer has been well delineated. For instance, angiogenesis promotes the growth and metastasis of cancer to distant sites. Conversely, the link between angiogenesis and inflammation is not as clearly outlined. The detailed physiology of these events, particularly in the central nervous system (CNS), has not been adequately described. Many investigators are describing how therapies interfere with angiogenesis, without describing the basic mechanisms that underlie this process. Finding treatment options with less toxic side effects are desirable and in high demand. Nevertheless, it is also important to understand that both inflammatory and angiogenic processes are not always pathological, they are also essential in keeping physiological homeostasis in a healthy individual.

Endothelial activation and apoptosis are important aspects of vascular physiology. The ability of members of the tumor necrosis factor (TNF) family of inflammatory cytokines to induce apoptosis in tumors and inflammatory cells is well documented (6, 7). Nonetheless, their role in endothelial activation and apoptosis in endothelium is not as clearly understood. The first portion of work described in this thesis (chapter 3) will investigate the link between endothelial apoptosis and activation in response to two

TNF family cytokines known as “death ligands”, namely TRAIL (TNF-related apoptosis inducing ligand) and FasL (Fas ligand).

The second body of work (chapter 4) aims to describe how these inflammatory cytokines affect endothelial processes linked to angiogenesis. Recent reports demonstrate that some patients with multiple sclerosis (MS) have an increased density of blood vessels in disease foci (8, 9). In addition, a study has found that serum concentration of TNF family cytokines are elevated in MS patients (10-12). This raises interesting questions about the role of TNF family members in blood-brain-barrier inflammation and cerebral angiogenesis. Therefore, one of my goals was to establish a cerebral angiogenesis model in order to examine the effects of TRAIL and FasL on this process.

In addition to the interest shown in the role of pro-inflammatory cytokines in angiogenesis, growing attention has been paid to dietary compounds or supplements and the potential of these exogenous chemicals to prevent and ameliorate disease. Resveratrol is a phytochemical derived from multiple plant sources including grapes (red wine), whose effects on the cardiovascular system have been extensively studied (13-19). However, little is known of its corresponding effects on the cerebrovascular system. Therefore, the third set of studies (chapter 5) describes the effect of resveratrol on mechanisms linked to angiogenesis in endothelium derived both from the CNS and from peripheral vessels.

1.1. Endothelial Dysfunction, Activation, Injury and Death

The normal cell has defined structures and functions that maintain a steady state called homeostasis. Alterations of physical, chemical, or biological status or the

deprivation of vital substrates triggers cellular responses. The cellular response to a mild injurious stimulus consists of adaptations that allow the cell to survive and continue to function. If the stimulus persists or is severe enough, reversible or irreversible injury, or even cell death may occur (20). This thesis examines endothelial responses in particular. However, the concept of endothelial cell dysfunction, adaptation, injury, and death, in general, could also be applied to other cell types.

1.1.1. Physiological Functions of Endothelial Cells

Vascular endothelium located at the interface between blood and tissue was once thought to be an inert structure but is now recognized to perform many critical homeostatic functions. These include serving as a barrier to control the passage of materials or cells between blood and tissue, maintenance of vascular tone, control of blood pressure and flow through vasoconstriction and vasodilation, regulation of blood coagulation, participation in angiogenesis, inflammatory responses, and immune surveillance(20, 21).

Endothelial cells form a single layer of cells that line the entire circulatory system including both cardiovascular and lymphatic systems (22). While some common features are shared (i.e. flat elongated shape and Weibel palade bodies that store von Willebrand (vW) factor and P-selectin), endothelial cells from different anatomic locations exhibit a wide diversity of unique characteristics and functions (21, 23). Endothelium from an organ system such as lung or kidney possesses a distinct phenotype compared to endothelial cells in the CNS. In addition, endothelial cells found in a large vessel (artery or vein) also differ in morphology and functionality from those originating from microvessels (arteriole, capillary, or venule) (20). This heterogeneity is largely programmed by the tissue microenvironment, surrounding cells, extracellular

matrix components, shear stress, and biological factors/mediators (24). Furthermore, alterations in the microenvironment may cause the endothelium to respond differently to the same set of mediators (21).

Endothelium in the CNS that separates the bloodstream from the neuronal milieu is perhaps the most unique class of endothelium in the body. Part of this unique function is defined by the term 'blood brain barrier' (BBB). The BBB refers to the very low permeability of water soluble substances such as ions, peptides, and proteins between blood and brain (25, 26). This is several orders of magnitude lower than across vascular beds in the periphery (27-29). The highly specialized cerebral endothelial cells are sealed by complex tight junctions, have reduced density of vesicles, and possess a unique pattern of cell surface receptors, transporters, and intracellular enzymes (25, 26, 30). All of these traits are crucial in regulating microvascular permeability and maintaining CNS homeostasis. However, in order for cerebral endothelium to achieve its functions, it needs to act in concert with astrocytes, pericytes, smooth muscle cells, neurons, and the extracellular matrix of the basement membrane, collectively known as the neurovascular unit (25, 31-34).

1.1.2. Endothelial Responses to Injury and Endothelial Activation

1.1.2.1. Causes of Endothelial Injury and Cell Death

Cell injury, and death results when cells are under severe stress or sustain irreversible injury that overrides adaptive responses. Depending on the cause, injury type, duration, and severity, cell injury could result in acute or chronic responses and these may be reversible or irreversible. If adaptive changes are unable to restore cellular functions and homeostasis, a variety of degradation processes are initiated that lead to eventual cell death.

The causes of endothelial cell injury may be grouped into the following categories (20, 35): 1. physical in nature such as ionizing radiation, temperature, mechanical trauma; 2. chemical agents such as drugs and poisons; 3. biological causes such as enzyme dysfunction, viral or other microbial infections, cytokines, cell-mediated immunological reactions and inflammation; 4. deprivation of vital substrates, for example oxygen and glucose. These injurious stimuli may exert different types of stress such as oxidative stress, metabolic stress, endoplasmic reticulum stress, and genotoxic stress (DNA damage) in endothelial cells (21). Moreover, oxidative stress caused by accumulation of reactive oxygen-derived free radicals has been implicated in a wide variety of pathologic conditions and processes, such as chemical and radiation injury, hypoxia, ischemia-reperfusion injury, cancer, aging and inflammatory responses (20, 21). Therefore, oxidative stress is perhaps one of the major stresses causing endothelial injury and death.

1.1.2.2. Endothelial Cell Activation

There is no 'gold standard' definition for endothelial cell activation (36). Many researchers restrict the definition strictly to the expression of adhesion and signaling molecules that promote leukocyte interactions with endothelium in response to inflammatory cytokines, such as TNF and Interleukin (IL)-1 (37-39). A broader definition for endothelial cell activation is an alteration in endothelial cell phenotype or function in response to environmental stimuli (36). This definition includes a role for endothelial cell activation in many physiological or pathological conditions, such as inflammation, immune responses, coagulation, and angiogenesis, as well as in atherosclerosis, autoimmune disease, and sepsis (21). Nevertheless, endothelial cell activation involves significant changes in endothelial cell protein expression, production of cytokines and

chemokines, alterations in cytoskeletal organization, cell shape, and permeability (36, 38). Many of the stimuli recognized to induce endothelial cell injury are also known to activate the endothelium. These include (but are not limited to) cytokines, pleotrophic signaling factors such as, thrombin, LPS, bacterial endotoxin and other microbial products, hemodynamic perturbation, oxidants and radiation (20, 21).

1.1.3. Mechanisms of Cell Death – Injury Beyond Repair

1.1.3.1. Types of Cell Death

Homeostasis in multicellular organisms results from the balance between the generation of new cells by cell proliferation and the removal of unwanted or damaged cells by cell death. Apoptosis and necrosis are two major mechanisms of cell death (40, 41). Necrosis is a form of accidental or uncontrolled cell lysis in which noxious contents of cells are released into surrounding tissues and induce inflammatory responses. In contrast to necrosis, apoptosis is programmed cell death. Cell suicide by programmed cell death is a finely regulated process that is occurring constantly in an otherwise healthy individual. Cell deletion by apoptosis is vital to the health of multicellular organisms, especially in embryogenesis and tissue remodeling (42, 43), cell and tissue homeostasis (44), function of the immune system (45) and resolution of inflammation (46). Defective apoptotic processes have been implicated in a variety of diseases. Insufficient apoptosis causes uncontrolled cell proliferation or failure to eliminate autoreactive lymphocytes and infected cells that can lead to cancer, autoimmunity, and persistent infections (47, 48). Excessive apoptosis can result in atrophy, such as seen in neurodegenerative diseases and ischemic damage (49, 50). Although apoptosis is a physiological process to remove unwanted cells, apoptosis can also result from non-physiological mechanisms that cause DNA damage, such as ultraviolet radiation and

many chemotherapeutic drugs (51, 52).

Apoptosis is distinguished from necrosis by several morphological features, such as nuclear fragmentation, chromatin condensation, DNA fragmentation, cell shrinkage, and membrane blebbing (40, 41). The key distinction between apoptosis and necrosis is that plasma membrane integrity is usually maintained in apoptotic cells (20). These dying cells express molecules, such as phosphatidylserine, on their cell surface which signals them to be engulfed by phagocytes so that potentially harmful cellular components are not released into surrounding tissues (53-55). Therefore, ridding the body of unwanted or damaged cells by apoptotic mechanisms avoids an inflammatory response which causes tissue swelling, damage and scar formation, as would occur in necrosis (53-55). In the case of insufficient removal of apoptotic cells, post-apoptotic cytolysis, or so called secondary necrosis, can follow with potential pro-inflammatory and pro-immunogenic consequences (56). In addition, there is a subtype of programmed cell death termed anoikis that plays a significant role in death of anchoring cell types, such as endothelial cells (57, 58)(59). Anoikis is a form of apoptosis that is triggered when anchorage dependent cells lose contact with the extracellular matrix. Besides the aforementioned types of programmed cell death, autophagic cell death (autophagocytosis) is another type of programmed cell death in endothelial cells that has been recognized in more recent years (60). Autophagocytosis is a tightly regulated catabolic process that involves the degradation of intracellular components through the lysosomal machinery. Several antiangiogenic agents, such as endostatin (61)and Bortezomib (62), have been shown to stimulate endothelial cell autophagy.

1.1.3.2. Pathways of Apoptosis

Processes of apoptosis involve numerous signaling proteins and the detailed

signaling pathways that trigger apoptosis are not fully understood. Apoptosis can be initiated by diverse stimuli from outside or inside the cell, e.g. death ligands, DNA damage, a lack of survival signals, growth factor withdrawal, paradoxical cell cycle signalling, or by developmental death signals (20). Nevertheless, apoptosis is known to occur through two key pathways: the extrinsic (or cytoplasmic) pathway and the intrinsic (or mitochondrial) pathway, see Figure 1.1. (63).

a. Extrinsic Pathway

The extrinsic pathway begins outside the cell through activation of the so called "death receptors" on the cell surface by ligation specific molecules known as "death ligands". Death ligands and receptors belong to the tumor necrosis factor (TNF) protein superfamily, including TNF-TNF receptors (TNF-R1), FasL-Fas (Fas ligand and receptor), and TRAIL-TRAIL receptors (death receptors-4, 5; DR-4, DR-5) (64-66). Death receptors contain an essential cytoplasmic domain called the death domain (DD) which engages in protein-protein interactions that transmit apoptotic signals (67). Formation of the Death Inducing Signaling Complex (DISC) is a key step in initiation of extrinsic death signaling (68). The DISC recruits DD containing adapter molecules like FADD (Fas-associated death domain) or TRADD (TNF receptor associated death domain) to the DDs of the activated death receptor as well as enrollment of initiator caspases 8 or 10 to the complex (69, 70). Other than DDs, both adaptor protein FADD and pro-caspase-8 contain a death effector domain (DED) and through DED-DED interaction, elevated concentration of procaspase-8 molecules at the DISC leads to their proximity-induced autocatalytic activation and production of active caspase-8 (69, 71, 72). Active caspase-8 molecules then further activate downstream effector caspases which in turn cleave specific substrates that result in cell death. In some cell types, death ligands also trigger intrinsic

mitochondria-dependent apoptotic pathways (73). The link between the caspase signaling cascade and mitochondria is through Bid, a Bcl-2 family protein (73, 74). Bid is cleaved by caspase-8 in the cytosol and the active form of truncated Bid (tBID) translocates to the mitochondria where it interacts with pro-apoptotic Bcl-2 family members (Bax and Bak) to induce the release of cytochrome c and other pro-apoptotic factors from mitochondria into the cytosol (73-75).

b. Intrinsic Pathway

The mitochondrial or intrinsic pathway is initiated by intracellular stress signals that direct pro-apoptotic Bcl-2 family proteins to the mitochondria where they interact and compete with anti-apoptotic Bcl-2 proteins (76). Pro-apoptotic Bcl-2 proteins trigger the release of cytochrome c and other mitochondrial membrane components (76). Cytochrome c binds to apoptotic peptidase activating factor-1 (Apaf-1) and ATP/dATP in the cytosol assembling to form a large multiprotein structure known as the apoptosome (77). Once the apoptosome is formed, pro-caspase 9 is recruited and activated (77). This results in cleavage and activation of executioner pro-caspases, which then further cleave downstream targets, leading to chromatin condensation, nuclear degradation, DNA fragmentation and apoptosis (77).

c. Caspase Cascade

Both extrinsic and intrinsic pathways converge to activate caspases which mediate the final phase of programmed cell death (63). Caspases are cysteine proteases that are synthesized as inactive pro-caspases (zymogens). Pro-apoptotic caspases can be split into two functional distinct groups, caspase-2, -8, -9, and -10 are initiator caspases, while caspases-3, -6, and -7 are executioner caspases. The active form of executioner caspases proceeds to shut down DNA repair mechanisms, to break down nuclear structural

protein, and cleave DNA into nucleosomal units by inhibition of enzymes involved in DNA repair such as PARP (Poly-ADP-Ribose-Polymerase), degradation of intra-nuclear proteins such as lamins, and activation of DNases such as CAD (Caspase Activated DNase) (63).

Although caspase-induced apoptosis is most likely the predominant form of programmed cell death, other evidence suggests that apoptosis may also be mediated by caspase-independent mechanisms (78, 79). Several mitochondrial proteins are able to translocate to the nucleus when released into the cytosol and induce DNA fragmentation and apoptosis, without activating caspases. Factors such as AIF (apoptosis-inducing factors), EndoG (endonuclease G), HtrA2/Omi (serine protease high temperature requirement protein A2), and Bnip3 are examples of proteins involved in caspase-independent apoptosis (80, 81). Some phytochemicals are also known to induce apoptosis in a caspase-independent manner (82-84).

1.1.3.3. Regulatory Mechanisms of Apoptosis Signaling

Apoptosis signaling requires a delicate balance between death-inducing signals and cell survival signals. Molecules that activate cell growth and survival pathways such as growth factors with downstream activation of PI-3K/Akt and the NF-kB signaling pathways can counteract apoptosis. In viable cells, pro-apoptotic molecules are repressed and kept in an inactive state. Anti-apoptotic regulatory factors also assist in inhibiting these signals. Many anti-apoptotic regulatory factors interfere with many steps of the extrinsic and intrinsic pathways (20). The Bcl-2 family proteins include more than 20 members and are known for their ability to regulate intrinsic apoptosis. Bcl-2, Bcl-x, and A1 are examples of Bcl-2 family proteins that play anti-apoptotic roles (76) while Bax and Bak proteins are known for their pro-apoptotic function (75, 76). In addition, cellular

FLICE (Fas-associated death domain protein- like interleukin-1β- converting enzyme)-like inhibitor protein, c-FLIP, is an example of a molecule that negatively regulates the extrinsic pathway (85). c-FLIP is a catalytically inactive homologue of caspase-8/-10 that can bind to pro-caspase-8/-10 but cannot cleave and activate the caspase because it lacks a protease domain (72, 73). Another important group of proteins called IAP (inhibitors of apoptosis proteins) are key anti-apoptotic regulators that inhibit caspase activation (86). Furthermore, a mitochondrial protein with the arcane name Smac/DIABLO can neutralize IAP in the cytoplasm and initiate a caspase cascade (87), which demonstrates the complicated cross talk between regulatory molecules that influence apoptosis.

1.2. Inflammation and Angiogenesis

1.2.1. Inflammation

Inflammation is the physiological process whereby tissues of the body respond to injury (i.e. physical injury, chemical irritants, toxins, infections). In normal circumstances, inflammation results in clearance of infections/toxins and the restoration of normal cell function and structure. The typical signs of inflammation include: rubor (redness), tumor (swelling), calor (heat), dolor (pain), and loss of function (20). There are two forms of inflammation, acute and chronic. Acute inflammation is an immediate and early innate (i.e. intrinsic and not antigen triggered) immune response to tissue injury. It involves an increase in blood flow, structural changes in the microvasculature that permit the rapid influx of blood leukocytes and plasma proteins into the tissue; typically neutrophils enter followed by monocytes (20). These monocytes are then quickly transformed to macrophages that affect the functions of resident tissue macrophages (20). These

leukocytes ingest and eliminate foreign substances and they also produce growth factors that facilitate in repair. During the resolution phase of inflammation, a large number of leukocytes are eliminated by undergoing apoptosis. These apoptotic leukocytes are also gradually removed by phagocytes. Failure to remove apoptotic leukocytes and the injurious stimulus can trigger persistent inflammation or adaptive (i.e. antigen-specific) immune responses that result in the development of autoimmune diseases. Termination of inflammation involves activation of anti-inflammatory mechanisms that prevents further damage to the host. The usual outcome of acute inflammation is successful resolution and repair of tissue damage. In contrast, chronic or persistent inflammatory responses can lead to proliferation of blood vessels, fibrosis, and further tissue destruction (20).

1.2.1.1. Leukocyte (Neutrophil) Recruitment During Inflammation

As mentioned, a key role of inflammation is to bring leukocytes to the site of injury to assist in removal of the offending agents and dead tissue. The passage of leukocytes from the vessel lumen to the interstitial tissue is called extravasation. This process can be divided into the following steps (Figure 1.2., (20)): 1. *Activation of vascular endothelial cells*. Under physiological conditions, circulating platelets and leukocytes do not adhere to quiescent vascular endothelium, which has a low expression of adhesion molecules, or adhesion molecules existing in a non-adherent state. However, during inflammation, endothelial cells become activated by cytokines (i.e. TNF, IL-1, and chemokines) and other mediators (histamine, thrombin, and PAF; platelet-activating factor). These mediators stimulate the redistribution of P-selectin from Weibel-Palade bodies (intracellular storage in endothelial cell granules) to the cell surface. Also, within 1-2 hours, endothelial cells begin to express adhesion molecules such as E-selectin and

ligands for L-selectin that promote the binding and transmigration of circulating leukocytes into the tissues, including neutrophils early in inflammation, and lymphocytes and macrophages at later stages. 2. *Leukocyte margination and rolling*; rows of leukocytes bind transiently to the endothelium, detach, and adhere again. Sialyl-Lewis X-modified proteins (and L-selectins) on leukocytes as well as P- and E-selectins on endothelium mediate the rolling process. 3. *Activation of leukocytes*. To promote stable adhesion, integrin (at its low-affinity state) on the surface of rolling leukocytes undergoes a conformational change to a high-affinity state. Integrin is activated through leucocyte binding to chemokines (i.e. IL-8) presented on the proteoglycan surface of endothelial cells. 4. *Firm adhesion of leukocytes*. High avidity integrins on the leukocyte (i.e. VLA-4, LFA-1, and Mac-1) can stably attach to cell adhesion molecules on endothelium (i.e. vascular cell adhesion molecule-1; VCAM-1, and intercellular adhesion molecule-1; ICAM-1). 5. *Transmigration called diapedesis*. Several adhesion molecules that are present in the intercellular junctions between endothelial cells (i.e. CD31/ PECAM-1; platelet endothelial cell adhesion molecules and junctional adhesion molecules) are involved in the migration of leukocytes through the endothelium. This process occurs mainly in post-capillary venules. 6. *Chemotaxis of leukocytes*. After passing through the endothelium, leukocytes may secrete collagenases (endothelial cells may also secrete various proteases) to degrade the basement membrane to help leukocytes enter the tissue. Leukocytes then migrate toward the chemotactic gradient and accumulate at their site of action. (20)

1.2.2. Angiogenesis

Angiogenesis is an important process in both health and in disease. In health,

physiological angiogenesis is significant in embryonic development, resolving inflammation, wound healing, and the normal menstrual cycle. In disease, pathological angiogenesis plays a critical role in tumor growth and metastasis, diabetic retinopathy, rheumatoid arthritis, multiple sclerosis, Alzheimer's disease, and more (1, 5, 20).

1.2.2.1. Processes of Angiogenesis

By definition, angiogenesis is a multistep process whereby new blood vessels develop from pre-existing vasculature in response to pro-angiogenic factors and inhibitors. Angiogenesis includes a cascade of events (Figure 1.3.): 1. production and diffusion of angiogenic factors by tumor or injured tissues to nearby endothelial cells; 2. ligand-receptor binding leading to detachment of pericytes and activation of endothelial cells; 3. endothelial production of proteases (e.g. matrix metalloproteinases, MMPs) leading to degradation of the vascular basement membrane; 4. proliferation and migration of endothelial cells; 5. remodeling of extracellular matrix; 6. formation of capillaries and blood vessel loops, and 7. stabilization of newly formed vessels by specialized supporting cells (i.e. smooth muscle cells and pericytes) before initiating blood flow. (1, 20)

1.2.2.2. Modulation of Angiogenesis

A delicate balance of pro-angiogenic and anti-angiogenic factors controls the growth of blood vessels. Modulation of angiogenesis by using anti-angiogenic approaches can be a promising strategy in treating diseases such as cancer, where excessive growth of vessels exacerbates the condition. On the other hand, for disorders characterized by inadequate blood supply, such as myocardial ischemia, pro-angiogenic therapies may be an option. Endogenous pro-angiogenic factors such as growth factors (i.e. VEGF, bFGF, PDGF), angiopoietins, MMPs (MMP-9), cytokines/chemokines (IL-8),

and transcription factors (HIF), promote endothelial proliferation and formation of vessels (1, 2, 20, 88). Endogenous anti-angiogenic factors, such as angiostatin, endostatin, and thrombospondin-1 (TSP-1), negatively modulate angiogenesis and prevent excessive vessel growth (89).

Pro-angiogenic factors:

Vascular Endothelial Growth Factor (VEGF)/VEGF receptors

Among all the diverse factors that participate in angiogenesis, VEGF and its receptor (VEGF-R) signaling plays a central role in adult physiological angiogenesis as well as in chronic inflammation and tumor growth (39, 90). VEGF is secreted by many mesenchymal and stromal cells, including tumor cells and activated macrophages (1). In a healthy individual, VEGF is expressed at low levels in a variety of tissues. VEGF receptors are tyrosine kinase receptors and expressed by endothelial cells, their precursors, and by many tumor cells (2).

VEGF is a family of growth factors that include VEGF-A, -B, -C, -D, and PIGF (placenta growth factor) in humans (91). Each of the subtypes has its unique function: VEGF(-A) signaling through VEGF-R2 (Flk-1/KDR) is the key angiogenic pathway; it is important in stimulating endothelial cell proliferation, migration, and production of proteolytic enzymes, has chemotactic properties to attract macrophages and granulocytes, and is also a vasodilator that promotes endothelial permeability (91). In addition, VEGF-A has 6 different splice variants (isoforms) whose expression varies with tissue environment, ambient conditions and tissue type (91). Among all the isoforms, $VEGF_{165}$ and $VEGF_{121}$ are the predominant variants (91). $VEGF_{165}$ has protein domains that interact with heparan sulfate proteoglycans (HSPGs) and co-receptors (neuropilins) on the cell surface (92, 93). It has an enhanced ability to bind and activate VEGF-R2 (92). The role of

VEGF-R1 (Flt-1) signaling is less clear with respect to VEGF-A; it is thought to be a decoy receptor (91). However, both PIGF and VEGF-B also bind to VEGF-R1 which plays a significant role in embryonic angiogenesis and in angiogenesis during ischemia, inflammation, wound healing, and cancer (94). Therefore, VEGF-R1 is believed to be critical in pathological angiogenesis. In addition, VEGF-C and -D induce signaling by binding to VEGF-R3 (Flt-4) which induces lymphangiogenesis (91, 94).

Other pro-angiogenic regulators:

Platelet-derived growth factor (PDGF) and angiopoietins (Ang) that bind to endothelial-specific Tie2 receptors have an established role in recruitment of pericytes and maturation of the microvasculature (95, 96). Stabilization and maturation of the vessel wall is a vital step in angiogenesis. Compared to the leaky immature vessel, it has been shown that endothelial cells in the vessel wall, surrounded by pericytes and extracelluar matrix are more resistant to apoptosis induced by VEGF withdrawal or anti-angiogenic factors (97).

In addition to signaling by growth factors, a class of zinc-dependent endopeptidases known as matrix metalloproteinases (MMPs) are major modulators of angiogenesis and have a critical role in tissue remodelling (98). These lytic enzymes are expressed by proliferating endothelial cells to promote cellular migration in the early stages of angiogenesis, also by malignant tumors to increase tumor invasion and metastatic potential (99). Together, MMPs are capable of degrading all components of extracellular matrix proteins (i.e. collagen, gelatin), as well as regulating a number of bioactive molecules (i.e. cleavage of cell surface receptors, activation or inactivation of chemokines/cytokines, release of apoptotic (Fas) ligands) (98, 99). Since MMPs are important in numerous physiological and pathological processes, their bioactivity is also

very tightly regulated. They are synthesized in the inactive form as a zymogen, and they are also regulated by a family of endogenous protease inhibitors called tissue inhibitor of metalloproteinases (TIMPs) (98). These factors form part of a complex interacting network of regulators in angiogenesis.

Negative regulators of angiogenesis:

A number of endogenous factors are known inhibitors of angiogenesis (reviewed in (89)). Angiostatin, an internal fragment of plasminogen, is produced by proteolytic cleavage of this circulating plasma protein (100); fragments of type XVIII collagen, endostatin and tumstatin, are also known to have anti-angiogenic capacities (101, 102). Unlike mitogenic factors such as VEGF, that act on endothelial cells through specific receptors and pathways to induce cell proliferation and angiogenesis, anti-angiogenic factors, such as angiostatin and endostatin, seem to have an impact through diverse mechanisms, and not necessarily through specific cell surface receptors. For instance, angiostatin can reduce endothelial cell proliferation; it can induce apoptosis through intrinsic and extrinsic pathways (103, 104) as well as anoikis (105) and also inhibit cell migration through $\alpha_V\beta_3$ integrin and angiomotin binding (106). Moreover, endostatin has been shown to modulate VEGF-R2, MAPK (mitogen-activated protein kinases), PI3K/PKB signaling pathways and to regulate the activity of MMPs (107-109) leading to decreased cell proliferation, survival, and migration. In fact, many exogenous compounds, such as chemotherapeutic drugs and phytochemicals, which inhibit angiogenesis also exert their angiostatic actions through multiple pathways similar to endogenous inhibitory molecules.

Key molecular signaling pathways

A number of signaling pathways play an important role in coordinating the multiple

steps involved in angiogenesis. Among all of these, PI3K/Akt and MAPK signaling are major pathways that transduce survival and proliferation signals in endothelial cells.

PI3K/Akt signaling

Akt/protein kinase B (PKB) is a protein-serine/threonine kinase that phosphorylates a wide range of target proteins upon activation, including cell survival proteins, transcription factors, and metabolic proteins (110). The starting point of this signaling pathway is PIP2 (phosphatidylinositol biphosphate) where the enzyme PI3K (phosphatidylinositol 3 kinase) phosphorylates at the third position of inositol to form the second messenger PIP3 (phosphatidylinositol 3,4,5-triphosphate) (110). PIP3 then interacts with Akt/PKB via pleckstrin homology (PH) domain of Akt at the plasma membrane (110). One downstream action of Akt is activation of NF-κB which regulates the expression of VEGF, IL-8, and MMP-9, which can alter angiogenesis (111). It also promotes endothelial cell proliferation by affecting proteins that regulate the cell cycle and apoptosis, including mTOR and cyclin D and it inhibits pro-apoptotic proteins such as Bad (111, 112).

p38 MAPK, ERK1/2, and JNK/SAPK pathways

The mitogen-activated protein kinase (MAPK) superfamily is a group of highly conserved serine/threonine-specific protein kinases that respond to extracellular stimuli (i.e. mitogens and proinflammatory cytokines) (113). The MAPK signaling pathway modulates various cellular activities such as mitosis, proliferation and cell survival/apoptosis (113). The extracellular signal-regulated kinases (ERK1, ERK2) are the most characterized family members which regulate cell proliferation and differentiation in response to growth factors (113). Other MAPK signaling pathways, such as those triggered by p38 and JNK/SAPK (c-Jun N-terminal kinases/stress activated protein

kinases) modulate cell differentiation and apoptosis in response to stress stimuli (113). While ERK1/2 and JNK/SAPK signaling are important in modulating endothelial proliferation, p38 signaling plays a crucial role in cell migration (114, 115). Collectively, all of these MAPKs contribute to modulation of angiogenesis.

1.2.3. Cross-talk Between Angiogenesis and Inflammation

The outcome of angiogenesis can either be beneficial or detrimental, an effect that is highly dependent on context. In the case of stroke recovery, increasing blood flow is beneficial, and can promote neurogenesis (116). On the other hand, angiogenesis in chronic inflammation may promote cancer development and lead to a positive feedback cycle in which further angiogenesis supports cancer growth and metastasis. Although angiogenesis is a necessary part of inflammation (20), it must also be limited to avoid detrimental effects. This requires precise coordination of signals that both promote and inhibit angiogenesis.

Many angiogenic factors are expressed in chronic inflammatory conditions and hypoxic tissues (i.e. cancer; stroke). In theory, angiogenesis will improve blood supply and reverse tissue hypoxia; it also increases the influx of leucocytes, such as phagocytes, that promotes the clearance of necrotic tissue and induce wound repair (20). However, the recruitment of inflammatory and immune cells, such as macrophages, dendritic cells and lymphocytes, also leads to generation of more inflammatory cytokines. These can exacerbate inflammation and induce the expression of angiogenesis modulators. This in turns causes activation of endothelial cells, increasing number of endothelial cells in the tissue and an excessive influx of pathogenic leucocytes as has been suggested in chronic inflammation (i.e. rheumatoid arthritis and multiple sclerosis) (117). Activated endothelial cells around inflamed tissues also increase the amount of proinflammatory

mediators released into the underlying tissues that modulate other stromal cells (i.e. fibroblasts; macrophages; pericytes; dendritic cells; inflammatory cells) (117) to produce various angiogenic mediators as well as angiostatic factors. Through molecular cross-talk involving inflammatory cytokines and angiogenic modulators, cells of the immune system and vasculature define a delicately regulated relationship between inflammatory processes and angiogenesis.

Among all the modulators produced by stromal cells that are involved in inflammation and cancer, the VEGF growth factor and receptor family plays the principal role in angiogenesis (117-119). Besides VEGF, other growth factors, various cytokines, chemokines, proteases, and transcription factors (i.e. HIF-α; NF-kB) are also known to have pivotal roles in this process (90, 118, 120, 121). Growth factors, such as bFGF, PDGF, epidermal growth factor (EGF) and transforming growth factor (TGF)-β, are also released into chronic inflammatory tissues (90, 118, 120, 121). Depending on the concentration, TGF-β can act as an angiogenic mediator or inhibitor (120, 122). In addition, numerous chemokines have been shown to modulate angiogenesis, for example: IL-8/CXCL8 is chemotactic and mitogenic for vascular endothelial cells *in vitro* and is a key regulator of angiogenesis in hypoxic conditions (123). A chemokine called monocyte chemoattractant protein-1 (MCP-1) is one of the main contributors to post-ischemic neovascularization (124). Proinflammatory cytokines, such as TNF and IL-6, account for a significant portion of stromal cell activation resulting in the release of chemokines and potent angiogenic mediators. However, depending on concentration and the timing of cytokine production, cytokines can exert a dual function in angiogenesis. For instance, TNF has been demonstrated to stimulate endothelial cells and upregulate MMP expression (99); TNF can also induce IL-10 synthesis in microvascular endothelium at the early phase (1-24

hours) of ischemic reperfusion injury that inhibits angiogenesis (88). However, after the first 24 hours of reperfusion injury, IL-10 production is downregulated by TGF-β so the balance shifts toward angiogenesis (88). This example gives us a hint of this intricately coordinated cascade of inflammation and angiogenesis.

1.3. Tumor Necrosis Factor (TNF) Superfamily

The tumor necrosis factor family of cytokines has effects on diverse functions including inflammation, cell proliferation, differentiation and apoptosis. There are more than 20 ligands in the TNF family and close to 30 TNF family receptors identified (7). TNF-α is the most well known and prototypic member of this family while another member called TNF-β is known as lymphotoxin (LT). The nomenclature was changed several years ago: TNF-α is referred to as TNF and TNF-β is referred to as LT.

TNF family ligands can act as secreted cytokines or membrane bound molecules by binding to specific receptors (7). All members of the TNF receptor family have cysteine rich extracellular sub-domains which mediate ligand-receptor binding; this leads to trimerization of the ligand-receptor complex, recruitment of several adapter proteins and activation of multiple signal transduction pathways (i.e. apoptosis, JNK, p38, ERK, PI3K and NF-kB) (125). There are three different types of receptor. Death receptors have a DD (death domain) in the cytoplasmic tail that signals death signaling upon activation through the extrinsic (caspase dependent) apoptotic pathway described earlier (7). Another subtype of receptor lacks a cytoplasmic death domain; these activate inflammatory cascades through the NF-κB pathway (described below) but their role in mediating apoptosis is not as well known. The third subtype of receptor is known as a decoy receptor that lacks functional intracellular motifs so that receptor binding does

not induce downstream signaling (7). By binding ligand, it also impairs the effect of binding to functional receptors. To add another layer of complexity, the extracellular portion of membrane bound receptors can also be cleaved and secreted into the circulation in soluble forms that also function as a decoy receptor to neutralize the action of ligands (7). Cleavage of cytokines and receptors can be carried out by MMPs or by modulatory enzymes such as TACE (TNF-alpha converting enzymes; ADAM-17) (126).

NF-kB Signaling Pathway

As mentioned, stimulation of TNF family receptors may lead to inflammatory responses through activation of NF-κB signaling. NF-κB is a family of transcription factors (i.e. RelA/p65, RelB, c-Rel, p50 and p52) that control many genes involved in inflammation (127). Dysregulation of NF-κB has been linked to inflammatory and autoimmune disease, as well as cancer (128). In quiescent cells, NF-κB exists as dimers that are sequestered in an inactive state in the cytoplasm by a family of inhibitors, called inhibitor of κB (i.e. IκBα, IκBβ, IκBε) (127). Upon stimulation, a kinase called IKK (IκB kinase) phosphorylates the inhibitory IκBα proteins (127). Following phosphorylation, IκBα proteins are ubiquitinated which targets them for degradation by the proteasome (127). With the degradation of IκBα proteins, the NF-κB dimers are then freed to enter the nucleus where they bind to specific DNA binding sites to modulate the expression of certain genes (127). The activation of these target genes results in cell survival, proliferation, inflammatory, and/or immune responses (128, 129).

1.3.1. TNF

TNF, the prototype of the family, was first discovered as a soluble factor in blood that can cause necrosis of tumors. Since then, it has been identified as a critical regulator of inflammatory responses. TNF is mainly produced by activated macrophages

and T lymphocytes but a wide range of cells can produce TNF, including endothelial cells, neutrophils, smooth muscle cells and fibroblasts (130). Two membrane-bound receptors TNF-R1 and -R2 are differentially expressed on cells and tissues. TNF-R1 is better characterized and known to mediate cellular responses related to tissue injury, while TNF-R2 has been linked to signals that promote tissue repair and angiogenesis. TNF mediated responses are highly regulated by the recruitment of various adapter proteins and inhibitors. Despite the differential expression of TNF receptors in diverse cell types, the proinflammatory actions of TNF are mainly carried out on vascular endothelium and through endothelial-leukocyte interactions (130). Endothelial cells activated by TNF express different combinations of adhesion molecules on the cell surface to attract leukocytes, increase permeability and trans-endothelial passage of fluid and also, express pro-coagulant proteins (21). These inflammatory responses are largely regulated by activation of NF-κB (127).

In a healthy individual, TNF is not usually detectable in serum. However, TNF is a necessary component of effective immune surveillance and is required for immune cells to proliferate and respond. The level of TNF is elevated in several pathological conditions, such as autoimmune diseases (i.e. rheumatoid arthritis, multiple sclerosis), infections and cancer (10-12). Extensive research has shown that TNF plays a central role in cancer associated inflammation and angiogenesis (131). TNF and its receptors are produced by many types of tumor cells, including glioblastoma and neuroblastoma, and are known to mediate proliferation by modulating the expression of growth factor receptors (132, 133). As well, TNF can induce angiogenesis by upregulation of angiogenic factors as seen in malignant glioma cells (134). With many diseases associated with TNF expression and signaling, anti-TNF therapies have been developed, including anti-TNF antibodies (i.e.

infliximab), soluble TNF receptors (i.e. etanercept), inhibitors to impede TNF expression, to suppress ligand trimerization, or to block the downstream signaling pathways (131). Many of these inhibitors have side effects and are costly, therefore it is important to find safer, less expensive alternatives.

1.3.2. TRAIL

TNF-Related Apoptosis Inducing Ligand (TRAIL) is also a member of the TNF family of ligands and is normally expressed in multiple tissues, including endothelial cells and predominantly by various immune cells (135). It was once thought that TRAIL only induces cell death in tumor cells but not normal tissues. This lead to much research focused on using TRAIL as anti-tumor therapy (136, 137). Later, it was shown that TRAIL mediated apoptosis can occur in normal hepatocytes, neurons, vascular endothelial cells and transformed non-tumor cell lines (reviewed in (138)). In healthy brain, TRAIL is expressed at very low to almost undetectable levels (139). However, under pathological conditions such as multiple sclerosis, TRAIL and TRAIL receptor concentrations are elevated (140). Moreover, TRAIL receptors are widely distributed in normal brains, on neurons, astrocytes , and oligodendrocytes (140). This may contribute to TRAIL mediated parenchymal cell death in multiple sclerosis (140). In fact, intracerebral injection of a soluble TRAIL receptor in an animal model of multiple sclerosis (experimental autoimmune encephalomyelitis; EAE) shows less apoptosis and myelin loss that prevents neurological disability (141).

In humans, TRAIL acts on five known receptors, four of which are membrane bound. Two are death receptors, TRAIL-R1 and TRAIL-R2 (also called death receptor DR3 and DR4), two are decoy receptors (DcR), TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), as well as one soluble receptor osteoprotegerin (OPG) (142). All four cell surface TRAIL receptors

are expressed in almost all tissues but the expression level may be altered in disease (138). Ligation of TRAIL to TRAIL-R1 and R2 triggers the formation of the death inducing signaling complex (DISC) or ligand-receptor complex (LRC). Depending on the type of adapter proteins recruited to the DISC/LRC, TRAIL-R1 and R2 can induce the extrinsic apoptosis pathway, activate the mitochondria dependent intrinsic pathway, and initiate survival signals via NF-κB (143). The survival signaling is mediated by recruitment of TRADD (TNF-R1 associate death domain protein), RIP (receptor interacting protein) and TRAF2 (TNF-receptor associated factor 2) to the DISC/LRC (143). Moreover, decoy receptor TRAIL-R3 lacks an intracellular domain while TRAIL-R4 has a truncated but non-functional death domain (143). OPG has a role in the regulation of osteoclast activity; however, along with TRAIL-R3 and R4, these decoy receptors compete for the binding of TRAIL (143). While TRAIL-R4 is incapable of transducing apoptosis signals, overexpression of TRAIL-R4 demonstrates its ability to activate NF-κB (144). TRAIL does not induce apoptosis in human umbilical vein endothelial cell (HUVEC) under normal culture conditions, with the exception of one study (145). However, the complicated TRAIL/TRAIL-R network means that the cellular effects of TRAIL are diverse and cell-type, context dependent. The effect of TRAIL on endothelial cell activation and angiogenesis is less well understood, and so forms the basis for this study.

1.3.3. FasL

Fas ligand is another death ligand in the TNF superfamily; similar to other members of the family, FasL is synthesized as a transmembrane protein or a secreted form. It also plays a critical role in regulating the immune system (146). Unlike TRAIL, which is expressed in many tissues and cell types, FasL expression is limited to leukocytes including neutrophils, macrophages and T cells, as well as cells in sites of 'immune

privilege' such as brain, eye, and testis where immune responses are suppressed (64, 147). In healthy brains, FasL is constitutively expressed in microglia, astrocytes, and neurons at low levels (148, 149). The level of FasL increases in many pathological conditions in the CNS, including infections and autoimmune diseases.

Two cognate receptors of FasL has been identified; one is membrane bound called Fas receptor (Fas) and the other is a decoy receptor, DcR3 (150). Similar to the other TNF family members, the FasL/Fas system can induce apoptosis and NF-κB signaling upon ligand-receptor ligation while binding of FasL to DcR3 neutralizes Fas mediated actions (150, 151). Though FasL expression is limited to cells of the immune system, Fas is ubiquitiously expressed by multiple cell types throughout the body (146). The basal expression of Fas in normal brain, however, remains low, and when Fas is upregulated in the CNS as a result of disease it can result in direct or indirect damage to neurons and microglia. Astrocytes resist Fas-mediated apoptosis and engagement of FasL to Fas on astrocytes promotes proliferation (152) and inflammatory responses such as increasing chemokine MCP-1 production (153). In the periphery, the FasL-Fas reaction induces cell-mediated cytotoxicity as part of the peripheral immune response (146). It was shown that HUVEC resist FasL mediated apoptosis (154, 155), however, FasL induces apoptosis in endothelial cells from coronary artery (156) and liver (157).

The role of Fas/FasL in immune system regulation and inflammatory responses is well characterized. However, the role of Fas/FasL in angiogenesis has been studied to a lesser extent and mostly in peripheral models. In an *in vitro* experiment using peripheral endothelial cells (HUVEC, human aortic endothelial cell/HAEC, and HMVEC/dermal microvascular endothelial cells), it was shown that TNF downregulates FasL expression by the endothelial cells, thus reducing their cytotoxicity (158). Also, FasL expression on

endothelial cells induces monocyte apoptosis and decreases the extent of TNF induced monocyte infiltration. This in turn may reduce the angiogenic growth factors that can be brought into the tissue by leukocytes. Therefore, it was speculated that endothelial FasL may act as a negative modulator of angiogenesis (159). This was supported by an *in vivo* experiment. Biancone *et al.* showed that administration of anti-Fas antibody to murine Matrigel™ implants resulted in increased neovascularization and infiltration of inflammatory cells (160). However the role of FasL on endothelial cells and its angiostatic modulatory role in physiological conditions is still not completely understood.

1.4. Resveratrol

There is of considerable interest for the therapeutic potential of dietary supplements because of their low toxicity and minor to no side effects. Growing evidence supports the potential for clinical application of various plant derived polyphenolic compounds (161-167). Due to their antioxidant properties, polyphenols have been linked to an extensive list of health benefits, such as prevention and amelioration of cancer (162), cardiovascular diseases (164, 168, 169), inflammatory diseases (161, 170), and slowing of the aging process (171). In addition to these antioxidant effects, polyphenols act on multiple signaling pathways and therefore have diverse cellular effects. One of the most widely studied polyphenols is resveratrol (trans-3,5,4'-trihydroxystilbene) that has been identified in a variety of plants, including Japanese knotweed, peanuts, berries and pine. Since it is abundant in the skin of grapes, it also is a constituent of red wine (19).

1.4.1. Background and Sources of Resveratrol

Resveratrol was first isolated in the 1940s from the roots of white hellebore (*Veratrum grandiflorum*), but the history of using grape extracts for human health can be traced back over hundreds of years (172). Resveratrol is produced naturally by many plants in response to stress, injury and microbial infections, and it is a phytoalexin compound (19). Fresh grapes contain about 50-100mg/g of resveratrol (173), and the concentration in wine ranges from 0.2-7.7mg/L (172). *Trans*-resveratrol is commercially available and sterically more stable than *cis*-resveratrol (174). When consumed by humans, resveratrol is absorbed mainly in the duodenum and is subsequently metabolized to glucuronated and sulfated forms (175). Clinical and *in vivo* reports demonstrate that free *trans*-resveratrol is short-lived (< 30mins) and present in low (nanomolar) concentrations in plasma (176). Metabolites of resveratrol remain in plasma much longer (72h) than the unconverted form (177). Studies in mice indicate that resveratrol is absorbed by the duodenum and can be detected in brain 3 hours after administration and in liver, kidney, and duodenum from 1.5 hours up to 6 hours post-administration (178). In addition, resveratrol is a lipophilic compound which can cross plasma membrane freely (179). It has been demonstrated that resveratrol is able to pass through the BBB and exert protective effects against cerebral ischemic injury in an animal model (180).

1.4.2. Cellular Effects of Resveratrol

Several studies report that the effects of resveratrol are tissue, and/or species specific as well as dose dependent (172, 181-184). It seems that many phytochemicals target various signaling pathways and have a diverse effect on cells. Resveratrol is no exception; it interacts with diverse cell surface and intracellular receptors, modulates

signaling molecules, DNA repair mechanisms, oxidative systems, and transcription factors. As a result, resveratrol affects numerous cellular processes, including cell proliferation, cell cycle arrest, and cell death, reviewed in (19, 172, 173).

Resveratrol, like most phytochemicals, lacks a specific cell surface receptor. Nonetheless, due to its structural similarity to estrogens, resveratrol binds to estrogen receptors and acts as an estrogen receptor modulator (172, 185). The effect of the estrogenic signaling induced by resveratrol is dose dependent. At lower doses (<10μM), resveratrol activates phosphoinositide 3-kinase (PI3K) through estrogen receptor-α leading to the promotion of cell growth in breast adenocarcinoma cells (MCF-7)(186); however exposure to greater than 50μM has an opposite effect that downregulates Bcl-2 and NF-κB and triggers cell death signaling pathways (186-188).

In addition, the dose dependent effects of resveratrol have also been seen in other growth factor receptor induced MAPK signaling pathways (189). For instance, resveratrol appears to modulate EGF signaling in highly metastatic breast cancer cells (MDA-MB-231) (190); at 5μM, the effect of resveratrol is to enhance cell migration and invasion while higher concentrations (≥ 50μM) inhibit these processes.

Resveratrol is also reported to modulate cell cycle progression in many types of cells, tumor cells (191), endothelial cells (184, 192), and fibroblasts (193). Cell cycle progression is tightly regulated by regulatory proteins at several control points and checkpoints (20). Dysregulation of cell cycle progression leads to uncontrolled cell growth and cancer will ensue. A quiescent cell in G_0 phase is non-dividing and may progress through four phases of the cycle upon stimulation: G_1, S, G_2, (interphase) and M (mitosis phase) (20). Cells in the G_1 phase have increasing cellular activity and are preparing for DNA synthesis. The G_1/S transition is a critical cell cycle checkpoint where

the integrity of the cell is assessed and the decision to repair or eliminate damaged cells is made. This is based on factors such as DNA integrity. Cells that have progressed through this transition point enter the S phase in which DNA synthesis and chromosomal replication occurs. Once the S phase is complete, cells enter the G_2 phase and will continue to synthesize proteins in this last stage of interphase before entering mitosis (M). The G_2/M checkpoint ensures no damaged DNA and the genome is completely replicated once the cell is ready to enter the M phase where cell growth stops and cell division occurs (20). Resveratrol mediates cell cycle regulation by several mechanisms such as induction of cell-cycle inhibitor protein expression (i.e. p21), downregulation or inhibition of activator proteins (i.e. cyclins and cyclin-dependent kinases) (193, 194). Together, these cause cell arrest at the G1/S-, S-, or G2/M- phase and confer the anti-proliferation activity of resveratrol. It was reported that resveratrol inhibits proliferation and regulates cell cycle control in human aortic and pulmonary artery endothelial cell cultures (184). Most studies have found anti-proliferative actions of resveratrol in various cell types (195-202); however this inhibition is cell type and dose specific, as resveratrol (50μM) has been reported to promote proliferation of human peripheral blood endothelial progenitor cells and angiogenesis in *vitro* by Wang *et al.*(203).

Other than modulation of cell cycle progression, resveratrol can induce cell death by various mechanisms. First, induction of cell growth arrest by resveratrol may lead to apoptosis through modulation of apoptotic and survival pathway genes. In various human malignant cells, resveratrol has been shown to mediate cell death by inducing pro-apoptotic protein expression and suppression of anti-apoptotic Bcl-2 family proteins (194, 204). Second, resveratrol possibly induces the death receptor dependent extrinsic

apoptotic pathway through redistribution of death receptors (i.e. FasL) in lipid rafts that sensitize cells to death receptor agonists (205). Another mechanism by which resveratrol can mediate cell death is sensitization of cells to other inducers of apoptosis. For example, it has been shown that resveratrol sensitizes cancer cells, but not normal human fibroblasts, to TRAIL-induced apoptosis (193, 205).

In addition to modulation of cell proliferation, cell cycle control, and apoptosis, resveratrol induces many other effects on human endothelium. In human umbilical cord endothelial cells (HUVEC), resveratrol (0.1μM) significantly increased the expression of endothelial nitric oxide (NO) synthase (eNOS) and synthesis of NO (a vasodilator); it also decreased the expression of the vasoconstrictor endothelin-1 (ET-1) (183). This may contribute to the beneficial effects of resveratrol (red wine) at lowering blood pressure and reducing the risk of cardiovascular diseases, also termed 'the French Paradox' (206). The anti-inflammatory actions of resveratrol have also been demonstrated. In human coronary artery endothelial cells, resveratrol (40μM) significantly inhibits IL-1β induced inflammatory responses (182). Rius *et al.* showed that *trans*- (but not *cis*-) resveratrol mediates anti-inflammatory effects *in vivo* through modulation of NF-κB signaling and reduction of adhesion molecule expression on both endothelial cells and monocytes (207).

1.4.3. Health Benefits of Resveratrol

Evidence supporting anti-cancer properties of resveratrol has been generated from both *in vitro* cell culture studies and *in vivo* animal model experiments. Overall, studies strongly support the use of this molecule in the treatment of cancer. Resveratrol modulates signal pathways that control cell division, growth, apoptosis, inflammation, angiogenesis, and metastasis (173). In animal studies (mostly mice and rat), it has been

shown that resveratrol is effective in suppressing tumor initiation, promotion, and progression in cancers from skin, breast, prostate, lung, gastrointestinal tract, as well as brain, review in (173, 208).

Cardiovascular protective effects of resveratrol have been demonstrated *in vivo*; this plant molecule increases expression of both eNOS and iNOS (inducible nitric oxide synthase) that may effectively promote vasorelaxation (209). Studies also demonstrate that resveratrol protects rat hearts against ischemia-reperfusion injury when animals are treated with resveratrol (210-213). However, most of the cardioprotective effects mediated by resveratrol are achieved through pre-conditioning (209, 213-215). This suggests a potential usage of resveratrol in preventive health management.

In the brain, resveratrol differentially modulates the inflammatory responses of microglia and astrocytes (216). It has been shown that resveratrol prevents CNS neuron death and degeneration both in vitro and in vivo. A number of neurodegenerative diseases, including Alzheimer's disease (217, 218), Parkinson's disease (219), as well as stroke (220-222), could benefit from including resveratrol in the therapeutic management. However, there is a continuing debate surrounding the mechanisms of neuroprotection (223, 224). Possible factors include antioxidant effects or the longevity factor Sir2/Sirt1 (225-227). Despite the uncertainty of mechanisms of resveratrol action in the CNS, Tseng *et al* demonstrated that resveratrol inhibits glioma-induced neovascularization in rats (228). Moreover, resveratrol has been shown to protect against brain damage following cerebral ischemia (180, 229, 230). In an *in vivo* stroke model, rats given resveratrol for 21 days prior to cerebral ischemia showed less motor impairment and decreased infarct volume comparing to untreated animals (220). Evidence that resveratrol could be a potential therapy in neurological disorders, makes it

interesting to understand the effects of resveratrol in the CNS and on cerebral endothelial cells.

1.5. Rationale and Objectives:

Rationale

Angiogenesis is the balance between vessel formation and regression (1, 5, 20). Many studies emphasize on the process linked to vessel formation. However, vessel regression and the factors that regulate this process are not as well studied. We hypothesized that vessel regression during angiogenesis is modulated by the immune system. Specifically, that vessel regression is linked to increased interactions between vascular endothelial cells and immune effector cells. In addition, that immune effector cells might regulate vessel regression through apoptosis by death ligands. This underlies the objectives A and B of the study. Finally, vessel regression might also be induced by exogenous factors, such as the phytochemical resveratrol which underlies objective C of this study.

Objectives

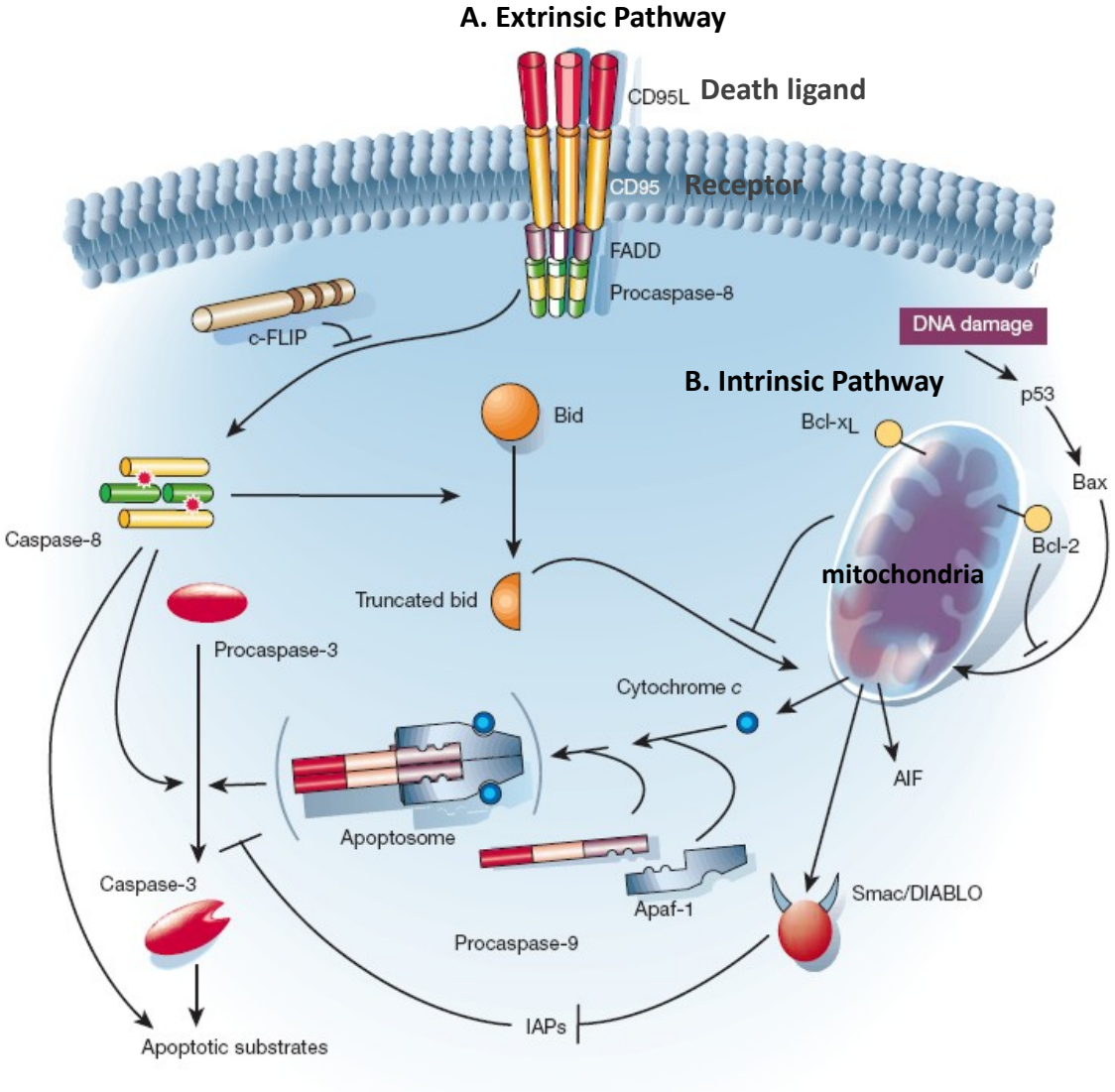
A. To assess the effects of two death ligands from the TNF superfamily, TRAIL and FasL, on vascular endothelial cell activation, neutrophil adhesion, and apoptosis (Chapter 3, Manuscript 1).

B. To set up an *in vitro* cerebral angiogenesis model using a newly developed human cerebral microvascular endothelial cell line, hCMEC/D3. Then, using this model, to evaluate the role of the inflammatory cytokines (endogenous molecules) TRAIL and FasL, on processes of angiogenesis. (Chapter 4, Manuscript 2).

C. To investigate the effects of an exogenous molecule, resveratrol, on the *in vitro* model of cerebral angiogenesis. (Chapter 5, Manuscript 3).

Figure 1.1. Extrinsic and Intrinsic Apoptosis Pathways

The extrinsic (death-receptor) pathway (A) is activated upon death ligand-receptor trimerization and ligation, while the intrinsic (mitochondrial) pathway (B) can be triggered by DNA damage and a variety of stress inducing stimuli. Both pathways can trigger initiator caspase (i.e. caspase 8) activation, which subsequently activate downstream executioner caspases (i.e. caspase-3, 9), leading to apoptosis. Cross talk between extrinsic and intrinsic pathways is linked by Bid.

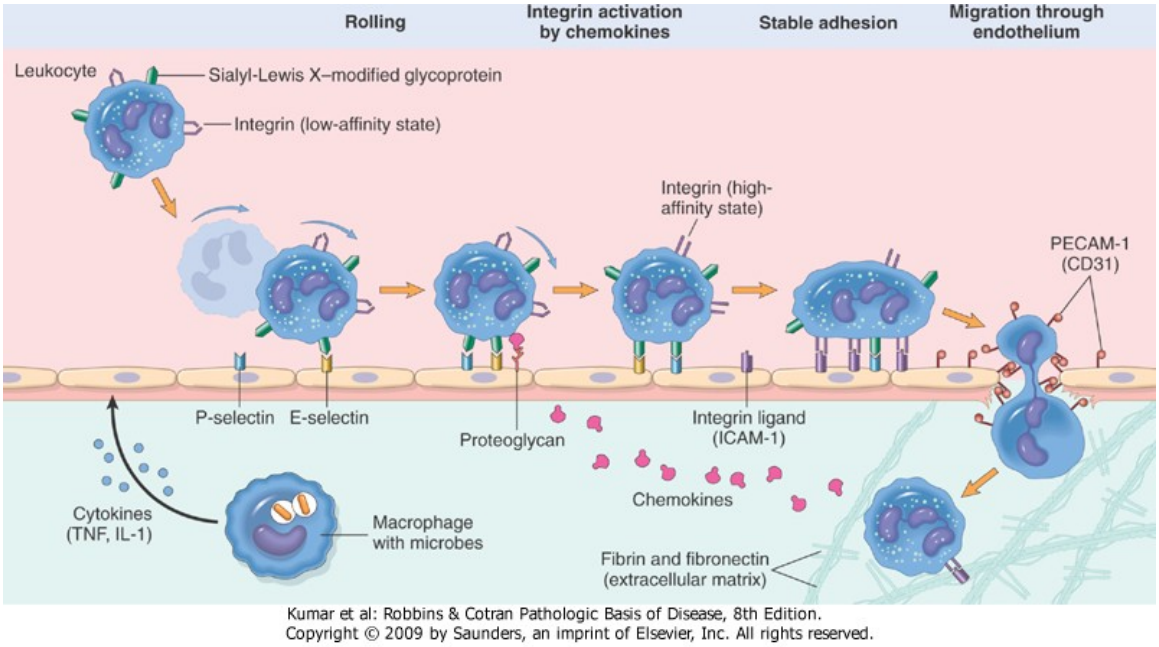


(adapted by permission from Macmillan Publishers Ltd: Hengartner *et al.* (231), see Appendix I-1 for the copyright permission)

Figure 1.1.

Figure 1.2. Cascade of Events During Leukocyte Recruitment

See text for detail.



(adapted from Kumar *et al.* (20))

Figure 1.2.

Figure 1.3. Multistep Processes of Angiogenesis

See text for detail.

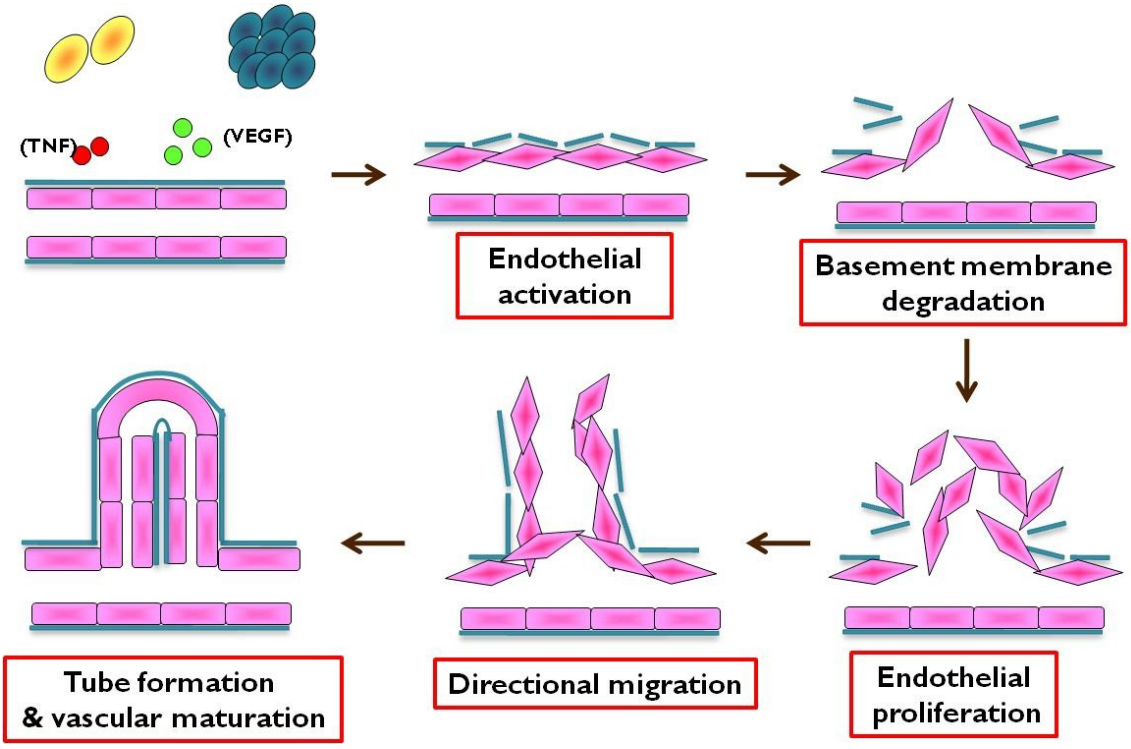


Figure 1.3.

CHAPTER 2- METHODS AND MATERIALS

2.1 Cell Cultures

2.1.1. HUVEC (Human Umbilical Vein Endothelial Cells)

Primary cultures of HUVEC used in Chapter 3 were a kind gift from the laboratory of Dr. Andrew Issekutz (Dalhousie University, Halifax, NS, Canada) and were used in experiments at passage 3-4. Unless otherwise specified, cells used in Chapter 3 were cultured on plastic flasks and multiwell plates coated with 0.1% gelatin, in M199 (Sigma-Aldrich Canada, Oakville, ON) supplemented with 20% (v/v) fetal bovine serum (FBS; heated inactivated at 56^{o}C for 30 minutes, Hyclone laboratories, Thermo Fisher Scientific Inc., Ottawa, ON), 2mM Glutamax (GIBCO-Invitrogen Life Technologies, Burlington, ON), 1x penicillin/streptomycin (Sigma-Aldrich), 100µg/ml heparin (Sigma-Aldrich) and 100µg/ml endothelial cell growth supplement (ECGS; from bovine neural tissue, Sigma-Aldrich). HUVEC results presented in Chapters 4 & 5 were generated using primary cultures (from a single donor) purchased from the Clonetics® Endothelial Cell Systems (Lonza Walkersville, Inc., Walkersville, MD) and used at passage 3-5. Clonetics® HUVEC are characterized by morphological observation throughout serial passage. These cells were cultured on plastic flasks or multi-well plates coated with Type I collagen from rat tails (BD Biosciences, Mississauga, ON) in endothelial basal medium (EBM)-2 supplemented with EGM-2 SingleQuotes (hydrocortisone, hEGF, FBS, VEGF, hFGF-B, Ra3-IGF-1, ascorbic acid, heparin and gentamicin/amphotericin-B; Lonza Walkersville Inc.). The medium was replaced every other day.

2.1.2. EA.hy926

The human umbilical vein immortalized cell line, EA.hy926, established by Dr. Cora-Jean S. Edgell (232) was derived from a fusion of HUVEC with the human lung

carcinoma cell line A549. The cell line was provided by Dr. Andrew Issekutz's laboratory (Dalhousie University) with the agreement of Dr. Edgell. Cells were used at passage 4 after subcultivation, and grown on uncoated plastic in DMEM (Dulbecco's Modified Eagle Medium; Sigma-Aldrich) supplemented with 10% FBS (Hyclone laboratories, Thermo Fisher Scientific Inc.), 1x penicillin/streptomycin (Sigma-Aldrich) and 1x HT (hypoxanthine and thymidine) supplement (Life Technologies). The medium was replaced every other day.

2.1.3. HMVEC (Human Microvascular Vein Endothelial Cells)

Primary cultures of HMVEC from a single donor were purchased from Clonetics® (Lonza) and maintained on Type I collagen coated culture flasks in the EGM-2MV medium (Lonza) as recommended by the manufacturer; and used at passage 3 & 4. The EGM-2MV medium consisted of the EBM-2 basal medium supplemented with EGM-2MV SingleQuotes (hydrocortisone, hEGF, 5% FBS (v/v), VEGF, hFGF-B, R3-IGF-1, ascorbic acid and gentamicin/amphotercin-B). The Conetics® endothelial cells test positive for von Willebrand Factor VIII, and acetylated LDL uptake and test negative for alpha smooth muscle actin according to the Certificate of Analysis provided by Clonetics®/Lonza. The medium was replaced every 2-3 days.

2.1.4. hCMEC/D3 (Human Cerebral Microvascular Endothelial Cells/D3)

The immortalized human adult brain endothelial cell line hCMEC/D3 was generated as described (233) and kindly provided by Dr. Weksler (Weill Medical College of Cornell University, NY). This cell line was derived from a primary cell culture through coexpression of hTERT and the SV40 large T antigen and retains most of the morphological and functional characteristics of brain endothelial cells. The hCMEC/D3 cells were recovered from liquid nitrogen at the 4^{th} passage after subcultivation and

plated onto Type I collagen coated plastic tissue culture flasks in EGM-2MV medium. The medium was replaced every 2-3 days.

2.1.5. BBMEC (Bovine Brain Microvascular Endothelial Cells)

The bovine brain microvascular endothelial cell system was purchased from Clonetics® (bMVEC-B; Lonza) and cultured on Type I collagen coated tissue cultureware in an optimized medium system (EMVB medium) recommended by the manufacturer. EMVB medium contains EBM-2 basal medium supplemented with ascorbic acid, b-ECGF, horse serum (platelet poor), heparin, and penicillin/streptomycin/fungizone. Cells were used in experiments at passage 2-4.

All cell cultures were maintained at 37^{o}C in a 5% CO_2 humidified atmosphere. Cells were subcultured by trypsiniziation using 0.5x trypsin-EDTA (Sigma) for primary cell cultures (HUVEC, HMVEC, and BBMEC) and 1x trypsin-EDTA for immortalized cell lines (EA.hy926 and hCMEC/D3). Unless otherwise specified, seeding density was between 15,000 cells to 20,000 cells/cm^2 for all cell types when subculturing.

2.2. Reagents and Antibodies

Reagents were obtained from the following sources: Tumor necrosis factor (TNFα, human recombinant, expressed in yeast), bFGF (human recombinant fibroblast growth factor-basic expressed in *E. coli)*, EGF (human recombinant epidermal growth factor expressed in *E. coli*) are purchased from Sigma-Aldrich; recombinant human VEGF 165 from R&D Systems (Minneapolis, MN); FasL and enhancer (1ug/ml) from Alexis Biochemicals (San Diego, CA); non-tagged soluble recombinant human TRAIL (amino acids 114-281) was from Peprotech Inc (Rocky Hill, NJ). All of the above reagents were purchased as lyophilized-powder (with the exception of TNF, which was pre-diluted),

diluted in either sterile PBS or ddH_2O as recommended by manufacturer, and stored at -80^{o}C. Resveratrol, (3,4',5-Trihydroxy-trans-stilbene ($C_{14}H_{12}O_3$), >99% (GC)), was purchased from Sigma-Aldrich and a 100mM stock solution was prepared and aliquot in dimethylsulfoxide (DMSO), and stored in the dark at -80^{o}C.

Caspase inhibitors, Z-VAD-FMK (pan), Z-IETD-FMK (caspase-8), and Z-DEVD-FMK (caspase-3) were obtained from R&D Systems and a 20mM stock solution was prepared in DMSO and aliquots were stored at -20^{o}C. PI3K inhibitor, LY294002 (IC_{50}=10μM), was purchased from Sigma-Aldrich, prepared in DMSO, and stored at -20^{o}C.

Blocking monoclonal antibodies to TRAIL-R1 (clone HS101) and TRAIL-R2 (clone HS201, both IgG1, mouse anti-human) were obtained from Alexis. A blocking monoclonal mouse anti-human CD18 antibody (IgG2a, cloneIB4) was obtained from Ancell Corp. (Bayport, MN). Isotype control antibodies (IgG subtype specific) were obtained from Cedarlane Laboratories Limited (Burlington, ON).

Flow-cytometric antibodies (all IgG1 phycoerythrin (PE)-conjugated monoclonal mouse anti-human) against TRAIL-R1 (clone DJR1), R2 (clone DJR2-4), R3 (clone DJR3), R4 (clone DJR4-1), TRAIL (clone RIK-2), and FasL (clone NOK-1) were obtained from eBioscience (San Diego, CA), Fas receptor (clone DX2), E-selectin (clone 68-5H11), P-selectin (clone AK-4), ICAM-1 (clone HA-58), and VCAM-1 (clone 51-10C9) were obtained from BD Biosciences. IgG1 isotype control antibodies (clone MOPC-21) were obtained from eBioscience and BD Bioscience. All antibodies were purchased as pre-diluted solutions and stored in the dark at 4^{o}C.

Antibodies for Western blotting were all purchased from Cellular Signaling Technology Inc. (Danver, MA): β-actin rabbit mAb (HRP conjugate; clone 13E5), phospho-Akt (Ser473) XP^{TM} rabbit mAb (clone D9E), Akt (pan) rabbit mAb (clone C67E7),

p42/p44 MAP kinase (ERK1/2) rabbit mAb (clone 137F5), phospho-p44/p42 MAPK (Thr202/Tyr204) rabbit mAb (clone D13.14.4E), NF-κB p65 rabbit mAb (clone C22B4), phospho-NF-κB p65 (Ser536) rabbit mAb (clone 93H1), HRP-linked goat anti-rabbit IgG (H&L) antibody.

2.3. Neutrophil Isolation and Neutrophil Adhesion Assay

2.3.1. Neutrophil Isolation and Labeling

Neutrophils were isolated from venous blood of healthy human donors using Lympholyte®-poly (Cedarlane) following the manufacturer's instructions. Briefly, venous blood was collected into K2-EDTA coated BD Vaccutainer® tubes. Anticoagulated whole blood (5.0ml) was carefully layered on top of an equal volume of Lympholyte-poly® in a 15ml centrifuge tube. It was centrifuged at 500 x g for 40 minutes in a swing-out rotor at room temperature (RT). After centrifugation, the neutrophil band was carefully harvested using a glass Pasteur pipette, then diluted with 3 volumes of PBS. The extracted cells were then washed by centrifugation at RT, 400 x g for 10 minutes. If red blood cells were present in the neutrophil sample, they were lysed by gently resuspending the cell pellet in 0.2% of sterile NaCl (hypoosmotic) solution (500µL/ 5ml of whole blood) for 30 seconds. An equal volume of 0.16% NaCl (hyperosmotic) solution was then added for an additional 30 seconds to restore normal osmolarity, followed by a washing step with PBS (2ml/ 5ml of whole blood). Red blood cell lysis steps were repeated until the pellet consisted almost exclusively of neutrophils (generally, 1-3 times). Then, the cell pellet was suspended in a serum free medium. Viability and purity of neutrophils were assessed and routinely exceeded 95% as judged by Trypan Blue and 2% crystal violet/acetic acid staining. On average, we were able to extract 1.5-2 x 10^6

neutrophils from each ml of whole blood, however, it was varied from 0.8-4.0 x 10^6 neutrophils/ml, depending on the donor. Freshly isolated neutrophils were labelled with the cell permeant fluorescent label Calcein-AM (Life Technologies; 10uM diluted in serum free medium M199, 60 minutes, 37^{o}C). Then, Calcein-AM labelled neutrophils were washed three times with 10%FBS-M199 buffer and used within 3 hours of isolation.

2.3.2. Neutrophil Adhesion Assay

Labelled neutrophils were applied to confluent wells of EA.hy926 or HUVEC in 96-well plates. Some were pre-treated with ligands (TNF, FasL, and TRAIL) as detailed in the Results. The stimulus were removed by washing (10%FBS in M199 medium) prior to the assay ensuring the applied neutrophils were not exposed to the ligands. Labelled neutrophils were applied (5x10^5/well) and incubated for 30 minutes at 37^{o}C, and each well washed gently four times to remove non-adherent neutrophils. Fluorescence was measured with a plate reader (Fluorskan FL, Thermo Electron) using a fluorescein filter set (EM: 485nm; EX: 538nm). A separate calibration was carried out during each assay, which obtained a linear relationship between neutrophil numbers and fluorescence, used to convert fluorescence to neutrophil counts. Data were normalized to the neutrophils in untreated control wells, and expressed as a percentage.

2.4. Cell Number Assessment/Cytotoxicity Assay

2.4.1. Acid Phosphatase Assay

In Chapter 3, cell numbers were assessed by the acid phosphatase assay, according to a previously established method (234). HUVEC or EA.hy926 were seeded in 96-well tissue culture plates, and grown to 60-70% confluence. Cells were treatmented with

different concentrations of ligands TNF, FasL, TRAIL for 24 hours. Cells were washed with PBS three times and then resuspended in 100µL assay buffer (1M sodium acetate, pH5.5, 0.1% Triton X-100, and 4mg/ml phosphatase substrate, all from Sigma-Aldrich). Following incubation at 37^{o}C in 5% CO_2 humidified atmosphere for 90 minutes, 10µL of 1N NaOH was added to each well and the absorbance at 405nm was measured with a microplate reader (Bio Tek ELx 800 with KC Junior 2 software, Bio Tek Instruments, Inc., Winooski, VT). For each experiment, a calibration plate with known numbers of untreated cells (0-12,500 cells/well) were set up to ensure the linear relationship of standard curve was obtained between cell numbers and absorbance. After subtracting background absorbance, cell numbers in each well were calculated using the standard curve obtained on each day of experiment, and expressed as percentage of cell numbers in the control wells.

2.4.2. MTT Assay

In Chapter 4 and 5, endothelial cell viability, cell numbers, and growth *in vitro* were assessed with the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide) assay (Cell proliferation kit I, Roche Applied Science, Laval, QC). This assay relates cell viability to the metabolic reduction of the tetrazolium salt MTT into a purple reaction product. Briefly, 5x10^3 cells were added to each well of a 96 well plate and allowed to attach in complete medium for 24 hours. In some experiments with hCMEC/D3, cells were serum starved in basal medium containing 2% bovine serum albumin (BSA) overnight (14-16 hours) to render the cells quiescent. All other experiments, cells were not serum starved prior to treatments with TNF family ligands or resveratrol at 37^{o}C. At the end of treatments (24 hours), the MTT labeling reagent (final concentration at 0.5mg/ml) was added to each well for an additional 4 hours at 37^{o}C.

Thereafter, 100µL of solubilizing solution was added and incubated overnight. The absorbance at 570nm was measured with a microplate reader (Bio Tek ELx 800). For each experiment, a calibration plate with known numbers of untreated cells (0-12,500 cells/well) was included to ensure the linear relationship of the standard curve was obtained between cell numbers and absorbance. After subtracting background absorbance, cell numbers in each well were calculated using the stand curve obtained on each day of experiment, and expressed as percentage to cell numbers of the control wells.

2.4.3. Lactate Dehydrogenase (LDH)-Release Assay

Cell lysis (necrosis) and cytotoxicity in resveratrol treated cultures of hCMEC/D3, BBMEC, and HUVEC were assessed by an LDH-release assay. LDH released from the cytosol of damaged cells into the supernatant was quantified with the Cytotoxicity Detection Kit^{plus} (LDH; Roche Applied Science) as per the manufacturer's instructions. Briefly, 5x10^3 cells were added to each well of a 96-well Type I collagen coated tissue culture plate and left to attach in complete medium for 24 hours. Cells were then placed in basal medium containing 5% FBS with different doses of resveratrol or vehicle alone for an additional 24 hours. In each experiment, a background control (wells containing no cells) was included to determine the LDH activity in the serum containing assay medium. At the end of treatment period, 5µL of lysis buffer was added to those wells set up to assess maximum LDH release and incubated for additional 15 minutes in a 37^{o}C incubator. Then, a freshly prepared reaction mixture (catalyst and dye solutions) was added to each well and incubated for 20 minutes at the RT before applying the stop solution. Absorbance was determined by a microplate reader (Bio Tek ELx 800) at 490nm and percent LDH release was calculated. The results are plotted in reference to positive

control wells (100% lysed cells). Each sample was set up in quadruplicate and each experiment was repeated at least three times on different experimental days.

2.5. Cell Proliferation/BrdU Assay

Proliferation was assessed from the measurement of bromodeoxyridine (BrdU) incorporation during DNA synthesis in replicating (cycling) cells using a colorimetric BrdU cell proliferation ELISA kit (Roche Applied Science). Briefly, cells were seeded and manipulated as described for the MTT assay. Cells were then treated with various compounds of interest for 24 hours with BrdU labeling in the final 4 hours (10uM) at 37^{O}C. This was followed by the addition of a fixation and denaturing solution (60 minutes, RT) followed by peroxidase conjugated Fab fragments against BrdU (2 hours, RT), and removal of excess antibody with three washes (PBS). The sample was then reacted with tetramethyl-benzidine substrate (10-30 minutes, RT) to develop color. The reaction was stopped by addition of 1M H_2SO_4 (sulphuric acid; 25µL/well), and measured as absorbance at 450nm with a plate reader (Bio Tek ELx 800). For each experiment, a calibration plate with known numbers (0-12,500 cells/well) of untreated cells was included to ensure the linear relationship was obtained between cell numbers and absorbance. After subtracting background absorbance, cell numbers in each well were calculated using the standard curve obtained on each day of experiment, and expressed as percentage of cell numbers in the control wells.

2.6. Flow Cytometry Analysis

Flow cytometric analysis was used to detect expression of various cell surface proteins (FasR, FasL, TRAIL-R1, R2, R3, R4, TRAIL, VEGF-R1, R2, ICAM-1, VCAM-1,

P-selectin, and E-selectin). Cells ($1x10^6$ cells/ treatment) were collected by trypsinization and, washed twice with cold immunofluorescence (IF) buffer (1%BSA, 0.2% sodium azide in PBS) and incubated for 1 hour at 4^oC with an appropriate dilution of PE-conjugated antibodies or isotype control antibodies (typically 20µL for 10^6 cells sample as recommended by manufacturer). After incubation, cells were then washed three times with IF buffer and then resuspended in 1% cold paraformaldehyde in PBS. Cells were kept at 4^oC for up to 3 days until ready to analyze. Analysis was performed on a FACSCalibur (BD Biosciences) and ten thousand events were collected from each sample. Data acquisition was obtained by Cell Quest computer software and analyzed or plotted with the WinMDI version 2.9 (The Scripps Research Institute).

2.6.1. Annexin V-FITC Apoptosis Assay

To detect surface expression of phophatidylserine as a marker of apoptosis (235), we used an annexin V-FITC apoptosis detection kit (BD Biosciences), according to manufacturer's instructions. Propidium iodide (PI) was added as a vital marker. Briefly, EA.hy926 cells grown to 90% confluency were exposed to medium alone or treated with TNF, TRAIL, or FasL for 6 hours, then both supernatant (with floaters) and the attached cells were collected and washed. Thereafter, $1x10^5$ cells were resuspended in 100µL of 1x Binding Buffer (provided with the detection kit) and annexin V-FITC and PI (5uL each) were added, then incubated for 15 minutes at room temperature in the dark. Each tube was then diluted with 400uL of Binding Buffer and analyzed by flow cytometry within 1 hour.

2.6.2. PI (Propidium Iodide) Cell Cycle Analysis by Flow Cytometry

PI staining was used to determine the proportion of cells in G_0/G_1, S, and G_2/M phases of the cell cycle based on their cellular DNA content. The hCMEC/D3 cells were

grown to 70% confluency and then treated with resveratrol or DMSO vehicle control at various doses for 6 or 24 hours at 37^{o}C. After treatment, 1x10^{6} cells were collected, washed once with ice-cold PBS, and then resuspended in 0.5ml ice-cold PBS until a monodispersed cell suspension was achieved. Then, 4.5ml of ice-cold 70% ethanol was mixed in to the cell suspension drop by drop while gently vortexing the sample. The samples were placed at -20^{o}C freezer for at least 24 hours. On the day of flow cytometric analysis, the ethanol in the samples was removed by centrifugation for 10 minutes at 300 x*g* and the cell pellets were washed twice in 5ml PBS. Then, the pellets were suspended in 0.5ml PI/RNase staining buffer solution (BD Biosciences). The samples were incubated for 15 minutes in the dark at RT and were kept on ice until analysis by flow cytometry. A control sample containing cells treated with resveratrol but no PI/RNase staining buffer solution was included to ensure the phytochemical resveratrol did not produce background fluorescent that might interfere with data analysis. The list mode data were analyzed to determine the proportion of cells in each phase of the cell cycle using ModFit LT software (Verity Software House, Topsham, ME).

2.7. NF-κB Activation Assay

EA.hy926 cells (3-5x10^{6} cells) were treated with TNF (100U/ml), FasL (10ng/ml) or TRAIL (100ng/ml) for 1, 3, 6, and 24hr. Nuclear extracts were then obtained using a nuclear extraction kit (Panomics Inc., Redwood City, CA). Protein concentration of nuclear extracts was determined by Bio-Rad Protein Bradford based assay and NF-κB activation was determined using the TransBinding NF-κB assay kit, according to manufacturer's instructions (Panomics Inc.). Briefly, the kit consists of 96-well plates coated with an oligonucleotide containing the NF-κB consensus binding site for the

activated NF-κB p50 subunit. Antibody directed against bound p50 was added and detected with a secondary HRP-conjugated antibody. The HRP reaction signal was read at 450nm with a microplate reader (Bio Tek ELx 800).

2.8. Caspase 3 Activation Assay

Caspase-3 activity was detected using a flurometric immunosorbent enzyme assay kit (Caspase 3 Activity Assay, Roche Applied Science) following the manufacturer's instructions. Briefly, hCMEC/D3 were grown to confluency in 6 well plates and treated with molecules of interest (TNF ligands, growth factors, and resveratrol at different doses), or vehicle control (DMSO), for 1, 3, 6, and 24 hours. 2 x 10^6 cells per treatment condition were lysed, and cell supernatants (100uL) added to wells coated with a monoclonal antibody to caspase 3. A reagent was then added that undergoes proteolytic cleavage in proportion to the amount of activated caspase 3 to generate a fluorescent product whose level was read at 505nm with a plate reader (Fluorskan FL, Thermo Electron). The release of AFC (amido-trifluoromethyl-coumarin) was monitored for 2 hours at 37^{o}C on a fluorescence plate reader (λ excitation = 400nm, λ emission = 505nm). The fluorescent reading from the blank wells (substrate only) were averaged and subtracted from that of the sample wells.

2.9. Microscopy, Image Acquisition, and Processing

For bright field microscopy, images were captured with a digital camera (Cannon PowerShot A630) attached to an inverted Leica DM-IL microscope (Richmond Hill, ON). The images were analyzed using the NIH Image J (version 1.41) image analysis program.

2.10. Cell Migration/ Scratch Wound Healing Assay

The scratch wound healing assay was based on a previously described protocol (236). 2.5 x 10^5 cells per well were plated in a 6 well plate and grown to ~90% confluency (2-3 days). A 200uL plastic pipette tip was drawn across the monolayer to create two linear regions denuded of cells. Wells were washed twice with PBS to remove cell debris, and molecules of interest (TNF ligands, growth factors, and resveratrol at different doses), or vehicle control (DMSO) were added. Initial images of the denuded zones (two from each line) were collected. Plates were then incubated at 37^{O}C for 20-24 hours (hCMEC/D3) or 16-18 hours (HUVEC), just before the gap between the denuded zone is closed by the migrating cells in the positive control (growth factor treated) well. Post treatment images of the same fields were then captured and analyzed using NIH Image J program to count the number of cells that migrated into the scratched region.

2.11. Tube Formation/ *in vitro* Matrigel™ Assay

Wells of a 48 well plate were coated with 100µL of Matrigel™ (growth factor reduced; BD Biosciences). To ensure an even polymerization of Matrigel™ , the coated plate was kept at RT for 1 hour and then 37^{O}C for 30 minutes. Molecules of interest (TNF ligands, growth factors, and resveratrol at different doses), or vehicle control (DMSO) mixed with the serum free EBM-2 medium were then added to the wells and kept at 37^{O}C for 40 minutes in order to saturate the Matrigel™ before adding 4 x 10^4 cells suspended in serum free EBM-2 medium containing 2% BSA. Cells and ligands were then incubated together for varying periods as indicated in the Results. At each time point, 4 random fields from each well were photographed (x20 magnification). The total length, area, and number of tubular structures formed were determined by image analysis (NIH

Image J). To determine the total number of tubular structures and the pixel numbers of a total area covered in a digital image, a color photograph was first transformed to an 8-bit image, and then a binary image was made prior to the area analysis. To determine the the total number of tubular structures and total tubular length in an image, the binary image was skeletonized prior to the length analysis. The area and length analysis was set to exclude cell debris or those individual cells that did not migrate into the tubular structures. The average area and average length were calculated by the pixel numbers of area or length divided by the total number counted. Data are expressed as a mean value derived from independent experiments performed on at least three different days with duplicate or triplicate samples in each experiment.

2.12. *Ex vivo* Rat Aorta Angiogenesis Assay

An established *ex vivo* angiogenesis assay involving rat aortic rings was previously described in detail (237). This assay was performed to assess the anti-angiogenic effects of resveratrol on an *ex vivo* angiogenesis model. Briefly, the rat aortas were obtained from adult Wistar rats with the technical assistance of another student, C.J. MacMillan. The animals used in this study were kept at the Carleton Animal Care Facility, Dalhousie University, and sacrificed with sodium pentobarbital following the University guidelines for the care and use of laboratory animals. These experiments used the left over tissues from control (untreated) animals that were used in C.J. MacMillan's study. Aortas were surgically excised, the fibro-adipose tissues were removed and the excised aortas were rinsed in serum free culture medium RPMI-1640 (Sigma-Aldrich). The aorta was then flipped inside out to expose endothelial cells and cut into ~1mm long fragments. The cleaned aortic segments were then carefully placed in the bottom wells of a 12-well

plate. Subsequently, 500µL of ice cold collagen gel matrix solution (Collagen Gel Culturing Kit, Wako Chemicals USA, Richmond, VA) was added to each well and allowed to polymerize at 37^{o}C for 30 minutes. After polymerization, resveratrol (10, 25, 50, 100µM) or DMSO vehicle control were added to the culture medium in 0.5ml of serum free medium RPMI-1640 supplemented with penicillin/streptomycin. The cultures of rat aorta were maintained at 37^{o}C, in 5% CO_2 humidified atmosphere for 7 days. Digital images were taken daily to monitor the migration of cells and new microtubular structure formation from aorta fragments. The area covered with migrated cells was measured using Image J analysis. At least 5 measurements per group were recorded.

2.13. Western Blotting Analysis

Western blot analysis was performed according to a standard procedure. Briefly, hCMEC/D3 cells were plated in 6-well tissue culture plates until reaching 70%-90% confluency. Cells were treated with resveratrol (10, 25, 50, 100µM) or vehicle (DMSO); (monolayers started at ~70% confluency for cells that underwent 24 hours resveratrol treatment and 90% for cultures treated with resveratrol for less than 6 hours). Protein extractions were performed using the following procedure: cells were washed twice in ice-cold PBS, and then the ice-cold lysis buffer was added and incubated at 4^{o}C for 5 minutes on an orbital shaker with a gentle agitation. The lysis buffer was purchased from Pierce-Thermo Fisher Scientific Inc. (Waltham, MA) and contained M-PER® Mammalian Protein Extraction Reagent with an addition of 1x Halt™ Protease and Phosphatase Inhibitor Cocktail (Aprotinin, Bestatin, E-64, Leupeptin, Sodium Fluoride, Sodium Orthovanadate, Sodium Pyrophosphate, β-glycerophosphate, and EDTA). Total cellular extracts were then centrifuged at 12, 000xg for 10 minutes at 4^{o}C and the supernatants

collected, aliquoted, and stored at a -80^{o}C freezer. Total protein concentration of cell lysate was determined for each sample by Bradford based assay using Coomassie Plus (Bradford) Assay Reagent (Pierce-Thermo Fisher). Cell lysates were boiled in sodium dodecyl sulfate (SDS) sample buffer and 20µg of total protein from each sample was resolved by SDS-polyacrylamide gel electrophoresis and were electro transferred onto PVDF membranes. Protein molecular weight standards from Cell Signaling Technology (CST prestained protein marker, broad range, and CST biotinylated protein ladder detection pack) were run alongside of the samples to provide accurate molecular weight estimation. The resulting blots were blocked overnight at 4^{o}C on an orbital shaker with the blocking buffer (Tris bufferd saline-Tween-20 (TBS-T), 5% (w/v) BSA and 0.1% Tween-20 (v/v)), and incubated with primary antibody (1:2500 in the blocking buffer) overnight at 4^{o}C. After washing with TBS-T buffer, blots were probed for 1 hour at RT with the appropriate secondary antibodies (1:10,000 in the blocking buffer). Blots were further washed in TBS-T and protein bands were visualized using an enhanced chemiluminescence (ECL) plus detection system (GE-Healthcare., Baie-d'Urfe, QC) and scanned using the Typhoon 9410 system from GE-Amersham Bioscience. To confirm equal protein loading, blots were stripped (30 minutes incubation at 37^{o}C in a Restore^{TM} Plus Western Blot Stripping Buffer (Pierce-Thermo Fisher) and probed for β-actin. Protein expression relative to steady state actin expression was quantified by densitometric analysis using NIH Image J software.

2.14. Statistical Analysis

Differences between mean values were analyzed by one-way analysis of variance with the Tukey-Kramer or the Bonferroni multiple comparisons post test, using statistical

software Graph Pad Prism 5.02. For all comparison, *p* values of less than 0.05 were considered as significant.

CHAPTER 3 – Apoptotic Phenotype Alters the Capacity of Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) to Induce Human Vascular Endothelial Activation

Pei-Lin Chen and Alexander Easton, Journal of Vascular Research (2008); 45:111-122. (Published)

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Copyright permission for this manuscript to be reproduced in the candidate's thesis has been granted from the publisher, S. Karger AG Basel, see Appendix I-2. All experimental studies were completed by the candidate. Data analysis and the manuscript were prepared by the candidate with editorial guidance from Dr Easton. Note: Methods and Materials as well as Reference sections of the manuscript were removed from the chapter to avoid repetition, those contents are included in Chapter 2 and the Reference section of the thesis.

3.1. Abstract

Background/Aims: The ability of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to activate vascular endothelium is unclear. This study investigates the link between endothelial apoptosis and activation in response to TRAIL. *Methods and Results*: Endothelial cell apoptosis was modeled with the immortalized human endothelial cell line EA.hy926, and with primary human umbilical vein endothelial cells (HUVEC) sensitized with the phosphatidylinositol 3-kinase inhibitor LY294002 in 1% serum. EA.hy926 expressed greatest levels of TRAIL receptors R1 and R2, and HUVEC of R2 and R3, determined by flow cytometry. Recombinant human TRAIL induced apoptosis in both models as confirmed by annexin V staining. Reduction in cell numbers were prevented by caspase inhibitors. In EA.hy926, TRAIL activated NF-κB (1 h) with increased ICAM-1 expression (24 h). TRAIL also increased adhesion of human neutrophils, blocked with an antibody to neutrophil CD18 and with antibodies to TRAIL and TRAIL-R1 and R2. TRAIL increased neutrophil adhesion to sensitized HUVEC, without effect on unmodified HUVEC. TRAIL did not increase surface labeling of ICAM-1 or E-selectin in sensitized

HUVEC. *Conclusions*: TRAIL increases neutrophil adhesion when it concurrently induces apoptosis both in EA.hy926 and in sensitized HUVEC. TRAIL may therefore induce endothelial activation in concert with endothelial apoptosis.

3.2. Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family of ligands, with the ability to bind to cell surface death receptors, which trigger programmed cell death (apoptosis) through caspase activation. TRAIL binds to four cell surface TRAIL receptors, two death receptors, R1 and R2 (which can transduce apoptosis) and two further receptors, R3 (which does not transduce intracellular signals) and R4 (which can activate the transcription factor nuclear factor κB; NF-κB) (144, 238). *In vitro* studies examining TRAIL function in vascular endothelium have employed human umbilical vein endothelial cells (HUVEC), and in most of these studies TRAIL does not induce apoptosis (239-244). However, HUVEC will undergo apoptosis upon exposure to TRAIL following inhibition of phosphatidylinositol 3-kinase, which regulates cell survival through protein kinase B/Akt (242, 244). This process of sensitizing endothelial cells to undergo apoptosis is widely employed experimentally, and may model particular states in which endothelial apoptosis occurs, such as angiogenesis and atherosclerosis (245, 246).

Vascular endothelial activation is critical in inflammation, converting endothelial cells from a quiescent barrier to one that promotes thrombosis and leukocyte adhesion. Treatment with TNF and other cytokines activates cultured endothelium, resulting in activation of NF-κB and *de novo* synthesis and surface expression of cell surface adhesion molecules that facilitate increases in leukocyte adhesion (247). The ability of

TRAIL to activate vascular endothelium is uncertain. One report found no evidence of NF-κB activation or of altered surface expression of cell adhesion molecules when HUVEC were treated with 100 ng/ml TRAIL (242). The same group found that TRAIL inhibits leukocyte adhesion by downregulating expression of chemokines induced by co-treatment with TNF (248). By contrast, another report found that TRAIL (50 ng/ml) activates NF-κB and increases surface expression of the cell adhesion molecules endothelial (E)-selectin and intercellular adhesion molecule (ICAM)-1 and the chemokine IL-8, promoting adhesion of HL-60 cells (145). The main difference between these reports was the ability of TRAIL to induce apoptosis. Activation appeared to accompany apoptosis (145), but interpretation is hampered because TRAIL does not cause apoptosis in most other studies of unmodified HUVEC (239-244). The aim of this study was therefore to clarify the link between endothelial activation and apoptosis induced by TRAIL, by specifically examining this question in endothelial models of apoptosis.

We were able to model endothelial apoptosis in the immortalized human endothelial cell line, EA.hy926, generated by fusion of HUVEC with A549 lung carcinoma cells (232). We report here that these cells undergo apoptosis without further modification on exposure to recombinant human TRAIL, presumably sensitized through immortalization. This cell line has been used for over 20 years, and is well characterized, exhibiting a number of defining endothelial characteristics, including traits pertinent to endothelial cell activation such as upregulation of cell adhesion molecules when treated with TNF (249, 250), and modification of leukocyte interactions (251-253). We complimented this model by reference to the primary cells themselves, using HUVEC treated with LY294002 in reduced (1%) serum medium, since this had previously been shown by two independent groups to sensitize the cells to TRAIL (242-244). TRAIL

resulted in apoptosis of EA.hy926 cells, with concurrent activation of NF-κB, increased surface expression of ICAM-1 and increased human neutrophil adhesion in a CD18-dependent manner. TRAIL did not induce apoptosis in unmodified HUVEC or cause endothelial cell activation as assessed from neutrophil adhesion. However, HUVEC treated with LY294002 in low serum conditions underwent TRAIL induced apoptosis, and also showed increased neutrophil adhesion, but this was not related to increased surface expression of the adhesion molecules E-selectin or ICAM-1.

3.3. Results

3.3.1. Characterization of TRAIL and FasL Responses in EA.hy926 Cells and HUVEC

Surface expression of the ligands TRAIL and FasL and their cognate receptors TRAIL-R1, TRAIL-R2, TRAILR3, TRAIL-R4 and Fas were examined by flow cytometry. HUVEC expressed TRAIL-R2, TRAIL-R3, and Fas, with lower expression of TRAIL-R1 and no TRAIL-R4 (Figure 3.1.A). EA.hy926 cells showed TRAIL-R1, TRAIL-R2, and Fas, with lower expression of TRAIL-R4 and undetectable TRAIL-R3 (Figure 3.1.B). Surface expression of TRAIL and FasL was not detected (Figure 3.1). Having detected receptors to TRAIL and FasL, their impact on cell number was determined (Figure 3.2). Twenty-four-hour treatments with TRAIL (10, 100, 300, and 1,000 ng/ml) resulted in a dose-related reduction in the EA.hy926 cell number. The cell number was not reduced by lower doses of FasL (1, 10, and 30 ng/ml), with a reduction at the highest dose (100 ng/ml, Figure 3.2.A). By contrast, these treatments had no effect on the HUVEC number (Figure 3.2.B). The effect of TRAIL (10, 100, and 300 ng/ml) on the EA.hy926 cell number was further investigated by combining TRAIL with caspase inhibitors (24 h treatments, Figure 3.2.C). Once TRAIL has bound to its death receptor (R1/R2), apoptosis is initiated by cleavage of

caspase-8 followed by downstream cleavage of caspase-3 (71, 254). The dose-related reduction in cell numbers was partially inhibited by the caspase-3 inhibitor Z-DEVD-FMK, and completely blocked by the caspase 8 inhibitor Z-IETD-FMK (both 50 μM). To confirm apoptosis in EA.hy926 cells, we double stained cells with annexin V-FITC and PI. Annexin V binds to phosphatidylserine, which is exposed on the external cell membrane surface of apoptotic cells, while PI labels permeable cells, both necrotic cells and cells in the later stages of apoptosis. Flow-cytometric data were assigned quadrant markers using separate annexin V-FITC and PI staining of untreated cells, to define treated cells with increased annexin V-FITC staining but no increase in PI staining (Figure 3.3.B). This group was taken to measure the early stages of apoptosis, since only cells at later stages of apoptosis as well as necrotic cells will label with PI (255). TRAIL (100 ng/ml) significantly increased early apoptosis above controls (treated with vehicle alone) in cells treated for 1, 3, 6, and 24 h. FasL (10 ng/ml) and TNF (100 U/ml) had minimal effects (Figure 3.3.A). Representative scatter plots of the annexin V-PI data are shown (Figure 3.3.B), comparing untreated (control) cells with cells treated with TRAIL for 6 h. Together, these data indicate that TRAIL induces apoptosis in EA.hy926 cells.

3.3.2. Regulation of TRAIL-Induced Increases in Neutrophil Adhesion to EA.hy926 Cells

The effect of TRAIL and FasL on neutrophil adhesion to EA.hy926 cells, and regulation of the TRAIL responses was then determined (Figure 3.4). FasL (100 ng/ml, 24 h) caused a small but significant increase in neutrophil adhesion, while 10 ng/ml of FasL had no effect. TRAIL (100 ng/ml) caused a significant increase in neutrophil adhesion, and 6- and 24-hour treatments with TNF (100 U/ml) induced an expected increase in neutrophil adhesion, being greater at 24 h than at 6 h. The effect of TNF was significantly greater than TRAIL at both 6 h and 24 h (Figure 3.4.A). Since TRAIL treatments had also

induced apoptosis, we assessed whether the increase in neutrophil adhesion reflected adhesion to EA.hy926 cells rather than exposed substrate. Neutrophil adhesion to cell-free wells was lower than adhesion to untreated (control) wells coated with confluent monolayers of EA.hy926 cells (2.46 ± 0.34% of control, n = 16) or HUVEC (8.86 ± 1.23% of control, n = 16). This indicates that neutrophil adhesion would not increase from substrate exposure due to apoptosis and cell detachment. We also observed that apoptosis caused EA.hy926 cells to detach leaving small gaps between them, such that large contiguous areas of substrate were not exposed. Neutrophils were observed to adhere to residual cells rather than the gaps.

The increase in neutrophil adhesion induced by TRAIL (100 ng/ml) was investigated by co-application of caspase inhibitors (24 h, Figure 3.4.B). The increase was partially inhibited by the caspase-3 inhibitor Z-DEVD-FMK (50 μM), and completely prevented by the pan-caspase inhibitor Z-VAD-FMK and caspase-8 inhibitor Z-IETD-FMK (both 50 μM). The action of TRAIL (100 ng/ml) was also blocked by co-treatment with a blocking monoclonal antibody against TRAIL, and combined blocking antibodies against TRAIL receptors TRAIL-R1 and TRAIL-R2 (Figure 3.4.C). Neutrophil adhesion to endothelial ICAM-1 is mediated in part by binding of the β_2-integrin subunit CD18 (256). When neutrophils were co-applied with a blocking monoclonal antibody against CD18, adhesion was significantly inhibited. CD18 blockade reduced neutrophil adhesion to untreated cells, and prevented increases in neutrophil adhesion after TRAIL treatment (100 ng/ml). TNF treatment in the presence of the antibody induced a small increase (100 U/ml, $p < 0.001$), which was significantly lower than the response to TNF alone. Treatments with isotype control antibodies were not significantly different from control.

3.3.3. TRAIL Treatment of EA.hy926 Cells Results in NF-κB Activation and Increased Surface Expression of ICAM-1

Many aspects of endothelial activation are regulated by NF-κB, dimeric transcription factors with a Rel homology domain (247, 257, 258). Among these dimers are the p50/p65 heterodimer and the p50/p50 homodimer. To measure NF-κB activation in EA.hy926 cells, we measured p50 from nuclear extracts, using an assay which quantifies its binding to an oligonucleotide containing the NF-κB consensus binding site. The concentration of bound p50 increased sharply 1 h after treatment with TNF (100 U/ml) or TRAIL (100 ng/ml), diminishing at 3, 6, and 24 h, while FasL (10 ng/ml) had no effect (Figure 3.5). We then determined the surface expression of adhesion molecules implicated in neutrophil adhesion. Surface expression of ICAM-1 was measured by flow cytometry (Figure 3.6). Untreated EA.hy926 cells expressed ICAM-1, and increased expression following 24-hour treatment with TNF (100 U/ml) or TRAIL (100 ng/ml). This increase was greater for TNF than for TRAIL. FasL (10 ng/ml) had no effect. In keeping with another report in EA.hy926 cells (254), E- or P-selectin were not detected in untreated or ligand-treated cells.

3.3.4. TRAIL Induces Apoptosis in HUVEC Treated with LY294002 in 1% Serum

Previous reports, including our own (242-244), have shown that TRAIL induces apoptosis in HUVEC in the presence of LY294002 and reduced (1%) serum medium. We confirmed this observation (Figure 3.7). Cell numbers reduced in the presence of 1% serum alone (71.08 ± 1.45% of 20% serum control, $n = 24$, $p < 0.001$). All treatments were then applied in 1% serum for 6 h (244). Compared to treatment with 1% serum alone, additional reductions were noted with LY294002 (5 or 20 μM). The reduction caused by 20 μM LY294002 was partially inhibited by the caspase-3 inhibitor Z-DEVD-FMK, but not

the caspase-8 inhibitor Z-IETD-FMK (both 50 μM). When TRAIL (100 ng/ml) was co-applied with LY294002, further reductions in cell number occurred. The further reduction induced by TRAIL was blocked by Z-DEVD-FMK and Z-IETD-FMK (both 50 μM). This indicated that the reduction in cell number induced both by LY294002 and its combination with TRAIL was caspase dependent, consistent with apoptosis (Figure 3.7-A). To confirm these data, we double stained cells with annexin V-FITC and PI. Quadrant markers were assigned to define the percentage of treated cells with increased annexin V-FITC staining but no increase in PI staining, as a measure of early apoptosis. Early apoptosis increased significantly above the control (1% serum) following treatment with LY294002 at both 5 and 20 μM. The effect of 20 μM was significantly greater than that of 5 μM ($p< 0.001$). At both concentrations, the addition of TRAIL (100 ng/ml) induced a further increase in early apoptosis, significantly greater at both doses than LY294002 alone (Figure 3.7-B)

3.3.5. TRAIL Treatment, in the Presence of LY294002/1% Serum, Increases Neutrophil Adhesion to HUVEC

In standard medium, 24-hour treatments with TRAIL (100 ng/ml) and FasL (10 ng/ml), alone or in combination, did not increase neutrophil adhesion to HUVEC. TNF (100 U/ml) induced an expected increase in neutrophil adhesion, which was unchanged in combination with TRAIL (Figure 3.8.A). When cells were treated in 1% serum with TRAIL in the presence of 20 μM LY294002 for 6 h, this increased neutrophil adhesion. Treatment with the higher dose of LY294002 alone increased neutrophil adhesion, but this was augmented by co-application of TRAIL (Figure 3.8.B). ICAM-1 expression was assessed in HUVEC following these treatments for 6 h, expressed as percent cell labeling above isotype control (see Figure 3.6). There was no significant difference in ICAM-1

labeling when comparing cells treated with 20% serum (30.66 ± 0.26%, mean ± SEM, n = 3) and 1% serum (31.05 ± 0.26%, n = 3). Treatment with 20 μM LY294002 in 1% serum induced a small but significant reduction in labeling (28.13 ± 0.25%, n = 3, $p < 0.01$ compared to 1% serum alone). The addition of TRAIL (100 ng/ml) to 20 μM LY294002 in 1% serum induced a further significant reduction in labeling (25.59 ± 0.29%, n = 3, $p < 0.01$ compared to 20 μM LY294002 alone). Labeling in 1% serum was unaffected when TRAIL (100 ng/ml) was applied alone (31.88 ± 0.23%, n = 3). E-selectin expression was also assessed upon TRAIL treatment. E-selectin expression (% labeling above isotype control, 6-hour treatments) was significantly increased by TNF (100 U/ml) from very low control levels (20% serum, 0.45 ± 0.08%, n = 3) to 48.11 ± 0.13% (n = 3, $p < 0.001$). Expression was unaffected by 1% serum (0.72 ± 0.05, n = 3), the application in 1% serum of 20 uM LY294002 (0.87 ± 0.08, n = 3), 100 ng/ml TRAIL alone (0.74 ± 0.02, n = 3), or these concentrations of TRAIL and LY294002 combined (0.75 ± 0.02, n = 3).

3.4. Discussion

This study demonstrates that TRAIL induces activation of human vascular endothelial cells sensitized to undergo apoptosis. Unlike HUVEC in standard culture medium (Figure 3.2), TRAIL induces apoptosis in the HUVEC-derived immortalized endothelial cell line EA.hy926 (Figure 3.2, 3.3) consistent with the presence of TRAIL death receptors (TRAIL-R1/R2) on the cell surface (Figure 3.1). TRAIL also activates EA.hy926 cells, with activation of NF-κB (Figure 3.5) and increased surface expression of ICAM-1 (Figure 3.6), as well as increased adhesion of human neutrophils (Figure 3.4). To a lesser extent than TRAIL, FasL (100 ng/ml) also induced apoptosis in EA.hy926 cells, and caused a smaller increase in neutrophil adhesion (Figures 3.2, 3.4). The increase in

neutrophil adhesion induced by TRAIL in EA.hy926 cells was dependent on its death receptors TRAIL-R1/R2, caspase activation and binding to CD18, the β_2-integrin subunit on neutrophils that binds to endothelial ICAM-1 (Figure 3.4). Although HUVEC in standard medium did not undergo activation (Figure 3.8) or apoptosis (Figure 3.2) upon exposure to TRAIL, the cells were sensitized by treatment with LY294002 in 1% serum (Figure 3.7). These cells also demonstrated increased neutrophil adhesion when treated with TRAIL (Figure 3.8). TRAIL treatment of sensitized HUVEC did not increase surface expression of E-selectin, and was associated with a small but significant reduction in ICAM-1 labeling.

The ability of TRAIL to activate vascular endothelium is unclear. A number of studies examined HUVEC cultured in standard medium, containing 10–20% serum, but these differ in their results. TRAIL was reported to have no effect on NF-κB activation or expression of cell surface adhesion molecules (242), but another study reported that TRAIL induces NF-κB activation and cell adhesion molecule expression, with increased adhesion to leukocyte-derived HL-60 cells (145). Indeed, TRAIL was reported to downregulate expression of the CCL8 and CXCL10 chemokines induced by treatment with TNF, and to thereby abrogate activation and reduce leukocyte adhesion (248). One striking difference between these studies was in the capacity of TRAIL to induce endothelial apoptosis. In the study that reported endothelial activation by TRAIL (145), apoptosis was concurrently present. However, TRAIL has often not been found to induce apoptosis in unmodified HUVEC (239-244), and it is difficult to support a concept of TRAIL-induced apoptosis in quiescent endothelium, given the widespread expression of TRAIL both by vascular smooth muscle and hematological cells in close proximity to the endothelium (239, 259). Despite this caveat, the cited work (145) did raise the possibility

that TRAIL might activate endothelial cells when their phenotype is altered, such that they undergo apoptosis. Such an alteration is considered to be relevant in vascular biology because endothelial cell apoptosis can contribute to states as diverse as angiogenesis and atherosclerosis (245, 246, 260). Endothelial cell apoptosis is modeled in tissue culture by sensitizing the cells. In this study, we used human endothelium sensitized by immortalization (EA.hy926 cells) or by pharmacological treatment (HUVEC treated with LY294002 in 1% serum) to undergo apoptosis.

The use of an endothelial cell line attempts to balance the advantage of stable phenotypic characteristics, with the unavoidable tumor-like properties associated with immortalization. The cell line used in this study was chosen because of its human derivation, and its long record of use since first described in 1983 (232). Multiple endothelial characteristics have been described including the presence of Weibel-Palade bodies (261), secretion of von Willebrand factor (262), upregulation of cell adhesion molecules following treatment with TNF (249-251), uptake of acetylated low-density lipoprotein (250), tube formation in Matrigel™ (263), and retained expression of caveolae (264). Several functional studies have been conducted that include an examination of leukocyte interactions (250, 252, 253, 265, 266). Various methods are used to immortalize endothelial cells, including fusion with cancer cells, introduction of SV40 large T antigen and/or telomerase, but all methods suffer from some loss of primary endothelial characteristics (250), and in this EA.hy926 cells are no exception. For example, EA.hy926 cells treated with bacterial lipopolysaccharide do not express IL-8 (but express IL-6, MCP-1, and GM-CSF) and in contrast to treatment with TNF, do not upregulate adhesion molecules on exposure to lipopolysaccharide or IL-1β (250). When a direct comparison was made (267), EA.hy926 cells were found to express many of the

mRNAs present in HUVEC, as well as additional genes that regulate the cell cycle and apoptosis. These additional genes were introduced by hybridization (since the chromosome number in EA.hy926 cells is expanded to 80 (249)), and this probably explains the susceptibility of the cells to apoptosis induced by death ligands such as FasL (268) and TRAIL (present study). In our view, this is fortuitous, because immortalization sensitizes EA.hy926 cells to apoptosis, allowing the impact on retained endothelial characteristics, such as activation, to be explored. Indeed, as a general approach, virtually all *in vitro* studies of endothelial cell apoptosis employ sensitization. Sensitization generally uses an intervention that alters signaling pathways that regulate cell survival. In the case of EA.hy926 cells, this intervention was hybridization with A549 cells, although the precise molecular alterations have not been described. In the case of primary cultures, most studies expose cells to reduced serum levels to deplete growth factors, and combine this with an agent of interest (often pathogenic) or agents that target specific signaling pathways (242-244)(85, 269-271).

We wished to determine whether a different method of sensitization applied to primary cultures would also unmask endothelial cell activation in response to TRAIL. To do this, we sensitized primary HUVEC by using LY294002 in reduced (1%) serum, because two independent studies, including our own, showed that this treatment sensitizes the cells to TRAIL (242-244). We show that, under these conditions, TRAIL both induces apoptosis (Figure 3.7) and also results in activation, inducing an increase in neutrophil adhesion to the cells (Figure 3.8). In contrast to EA.hy926 cells, TRAIL treatment of sensitized HUVEC did not result in increased surface expression of either E-selectin or ICAM-1, and so the mechanism by which TRAIL increases neutrophil adhesion in this model has not yet been determined. Indeed, both LY294002 and TRAIL caused a small

but statistically significant reduction in ICAM-1 labeling. We speculate that this may indicate a change in the conformation and binding avidity of ICAM-1 independent of surface expression, reducing the binding of the monoclonal antibody used for flow cytometry. Altered avidity could mediate increases in neutrophil adhesion.

There were some differences in the responses seen in HUVEC in this study, compared to Dr Easton's previous work, which highlights the chief disadvantage of using primary cultures, which is the variability between primary cultures from different sources. In this study, cell numbers were reduced when placed in 1% serum alone, and additionally after 6 h of exposure to LY294002 (Figure 3.7). In Dr. Easton's previous study (244), 6 hour treatments with 1% serum and LY294002 had no effect. Our previous study also showed that TRAIL increased HUVEC numbers, an observation otherwise interpreted as a proliferative response by native cells (242); however, no increase was noted in this study (Figure 3.2). Thus HUVEC from different sources show differences in sensitivity to serum withdrawal and inhibitor treatment, as well as in their cellular responses to TRAIL.

The central conclusion of this study is that human vascular endothelium is activated by TRAIL when sensitized to undergo apoptosis. Sensitized endothelium may serve to model those physiological and pathological states in which endothelial cell apoptosis plays a role. For example, multiple stimuli have been reported to inhibit angiogenesis with concurrent inhibition of phosphatidylinositol 3-kinase (272), and this may partly occur by induction of endothelial cell apoptosis (273). Angiogenesis depends upon endothelial cell proliferation as well as vessel regression through apoptosis. Endothelial cell apoptosis contributes to atherosclerosis, by increasing lipid deposition, and activation contributes to inflammatory infiltration of atherosclerotic plaques (245). A

recent report (274) in a mouse model, however, shows that the administration of exogenous TRAIL inhibits the development of atherosclerosis by inducing apoptosis in infiltrating macrophages, and by promoting smooth muscle formation. Thus TRAIL may exert different effects depending on dose, disease stage and the cells targeted. It has also been suggested that endothelial cells undergoing apoptosis become activated as a general response (275). LY294002 (20 μM) alone induced apoptosis (Figure 3.7) and increased neutrophil adhesion (Figure 3.8), showing a link between apoptosis and activation using a stimulus different to TRAIL. This study also expands the concept that death ligands, such as FasL (reviewed in (276)) and TRAIL, regulate apoptosis and inflammatory activation simultaneously in particular target cells.

3.5. Acknowledgement

This study was funded by the Heart and Stroke Foundation of Canada, the Multiple Sclerosis Society of Canada and the Nova Scotia Health Research Foundation (grants to A.E.). P.-L.C. was funded by a George Loh Scholarship through the Department of Pathology, Dalhousie University.

Figure 3.1. Surface Expression of TRAIL, FasL and Their Cognate Receptors Detected by Flow Cytometry in HUVEC (A) and EA.hy926 Cells (B).

Open histograms indicate fluorescent labeling with receptor-specific monoclonal antibodies; shaded histograms show background labeling with isotype-matched control antibody in the same cells. Data are representative of 4 separate experiments.

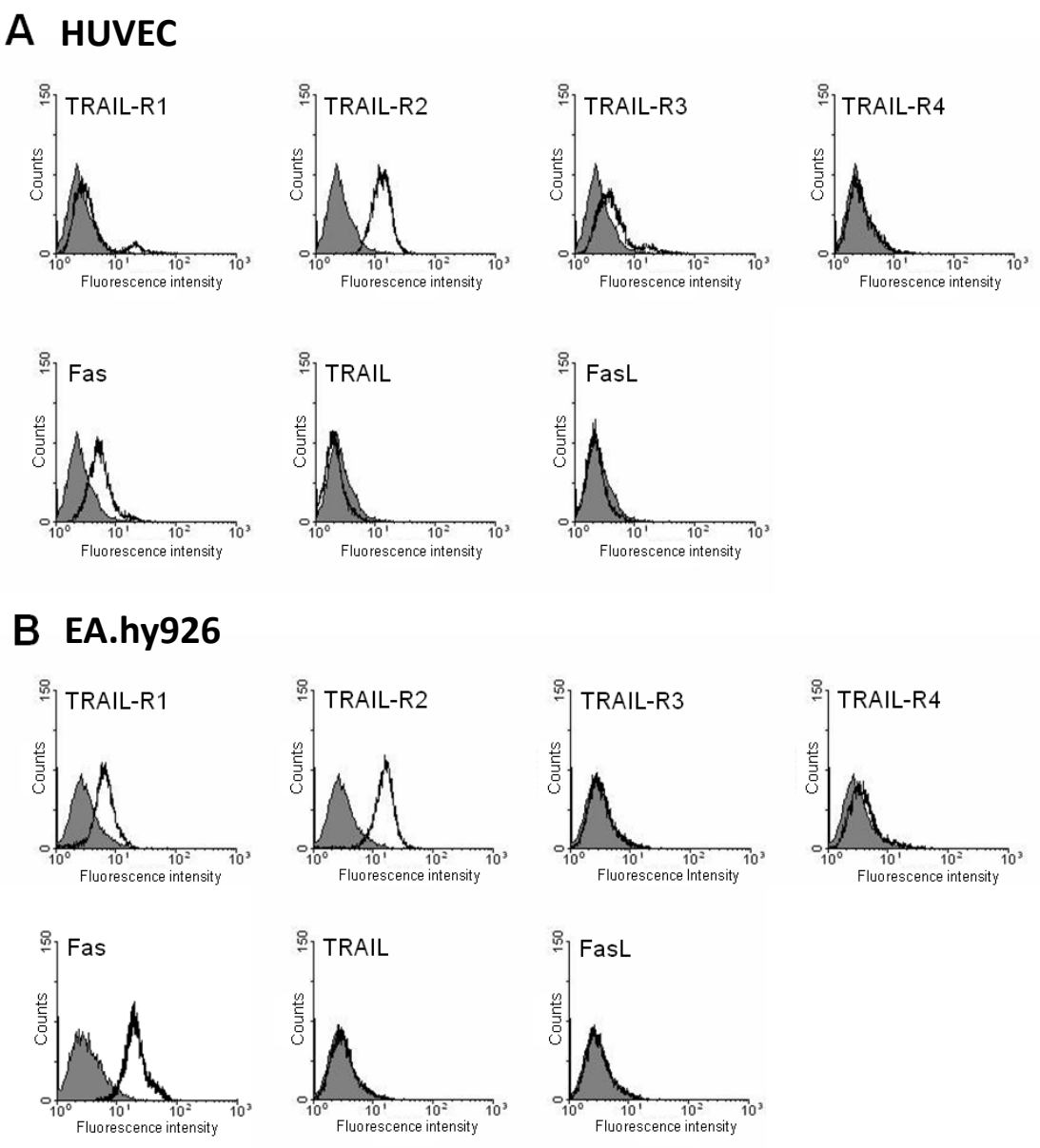
A HUVEC
TRAIL-R1
TRAIL-R2
TRAIL-R3
TRAIL-R4
Fas
TRAIL
FasL
Counts
Fluorescence intensity
B EA.hy926
TRAIL-R1
TRAIL-R2
TRAIL-R3
TRAIL-R4
Fas
TRAIL
FasL
Counts
Fluorescence intensity

Figure 3.1.

Figure 3.2. Effect of TRAIL and FasL on EA.hy926 Cells and HUVEC Numbers, Measured by Acid Phosphatase Assay.

Cells, either EA.hy926 (A, C) or HUVEC (B), were treated with the indicated doses for 24 hours. TRAIL was applied alone or in combination with the caspase-3 inhibitor Z-DEVD-FMK (50 µM, CI-3) or caspase-8 inhibitor Z-IETD-FMK (50 µM, CI-8). Means ± SEM (n = 16). Significance is compared to the untreated control (NS = Not significant, * * $p < 0.01$, * * * $p < 0.001$), or between TRAIL alone and TRAIL plus CI-3 ($p < 0.05$). B. No significant difference versus control was found.

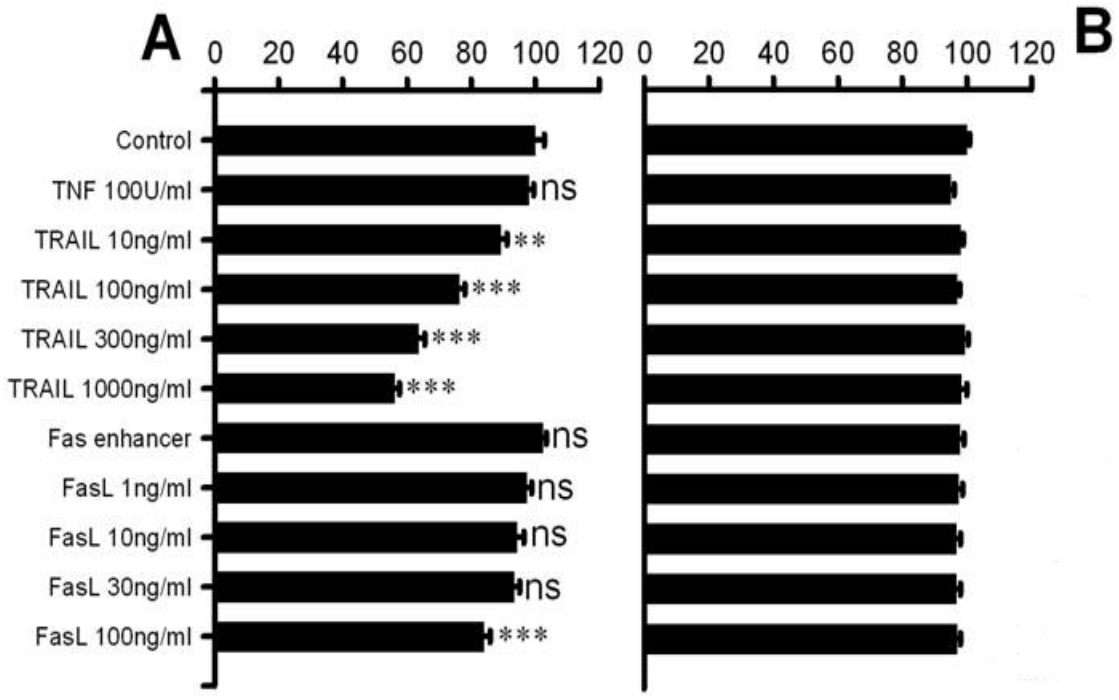
Cell Number (% of control)
EA.hy926
HUVEC
A
B
0 20 40 60 80 100 120
0 20 40 60 80 100 120
Control
TNF 100U/ml
TRAIL 10ng/ml
TRAIL 100ng/ml
TRAIL 300ng/ml
TRAIL 1000ng/ml
Fas enhancer
FasL 1ng/ml
FasL 10ng/ml
FasL 30ng/ml
FasL 100ng/ml
ns
**
***
***
***
ns
ns
ns
ns
***

Figure 3.2.

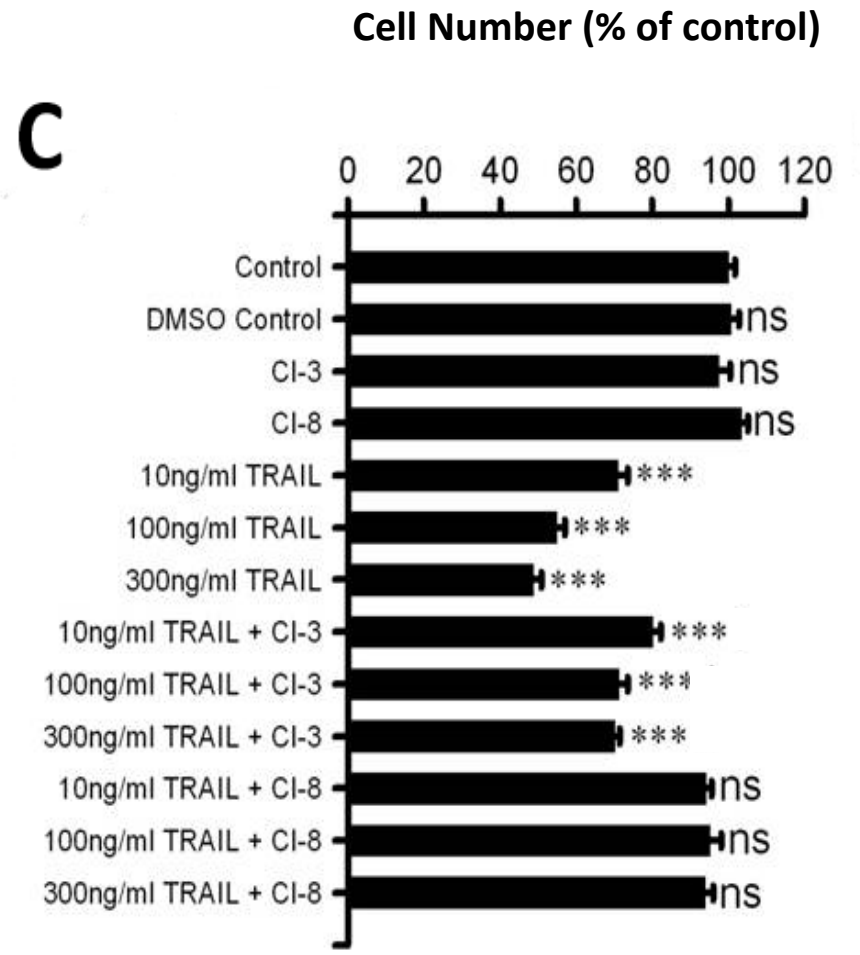
Cell Number (% of control)
C
0
20
40
60
80
100
120
Control
DMSO Control
CI-3
CI-8
10ng/ml TRAIL
100ng/ml TRAIL
300ng/ml TRAIL
10ng/ml TRAIL + CI-3
100ng/ml TRAIL + CI-3
300ng/ml TRAIL + CI-3
10ng/ml TRAIL + CI-8
100ng/ml TRAIL + CI-8
300ng/ml TRAIL + CI-8
ns
ns
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Figure 3.2.

Figure 3.3. Effect of TRAIL, TNF and FasL on Apoptosis of EA.hy926 Cells Measured by Annexin V-FITC Labeling.

A. Early apoptosis as percent of cells with high annexin V staining and low PI labeling. Control = vehicle alone. Means ± SEM (n = 4). * $p < 0.001$ TRAIL treatments vs. control. B. Representative scatter plots of the raw data for cells treated for 6 h with TRAIL (100 ng/ml) or vehicle alone (control). The quadrant markers determine early apoptosis from the percentage (indicated) of cells in the lower right quadrants.

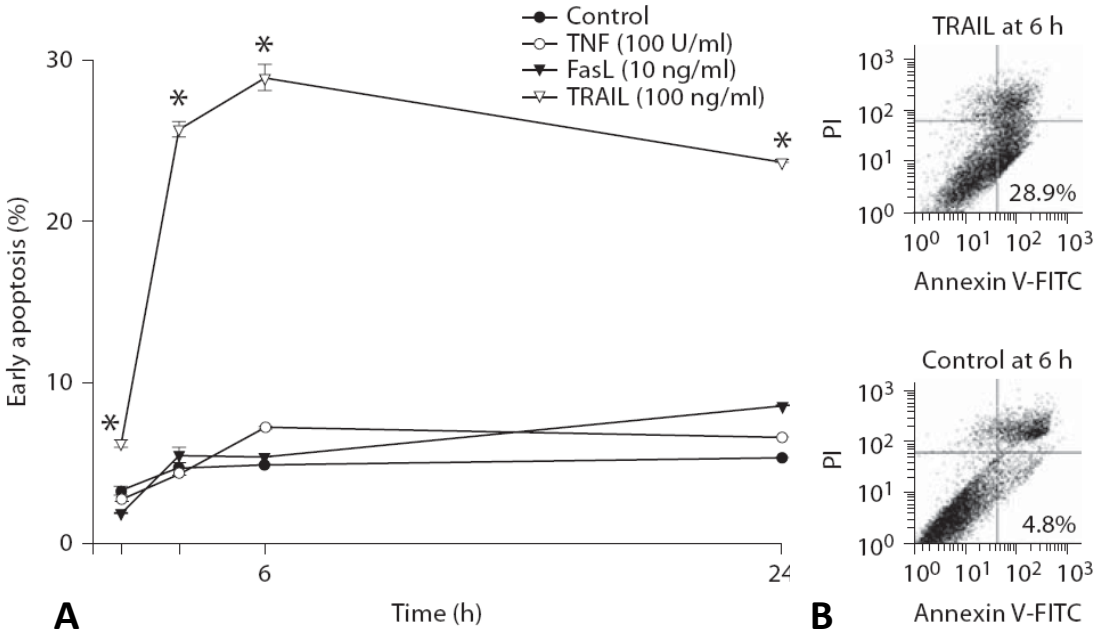
Control
TNF (100 U/ml)
FasL (10 ng/ml)
TRAIL (100 ng/ml)
Early apoptosis (%)
30
20
10
0
6
24
Time (h)
A
TRAIL at 6 h
PI
28.9%
Annexin V-FITC
Control at 6 h
4.8%
B

Figure 3.3.

Figure 3.4. Effect of TRAIL and FasL on Neutrophil Adhesion to EA.hy926 Cells, Assessed by Calcein Labeling.

Cells were treated with the indicated doses for 24 h unless otherwise indicated. Ligands were applied alone (A) or in combination with inhibitors (B, C). B. Treatments (all 50 µM) with the pan-caspase inhibitor Z-VAD-FMK (CI-pan), caspase-3 inhibitor (CI-3) Z-DEVD-FMK, or caspase-8 inhibitor (CI-8) Z-IETD-FMK. C. Treatments with blocking antibodies to TRAIL (anti-TRAIL), to TRAIL-R1 and TRAIL-R2 (anti-R1 and -R2) and to CD18 (anti-CD18). Means ± SEM (A, n = 40; B, C, n = 8). Significance is compared to the untreated control (NS = Not significant, * * $p < 0.01$, * * * $p < 0.001$)

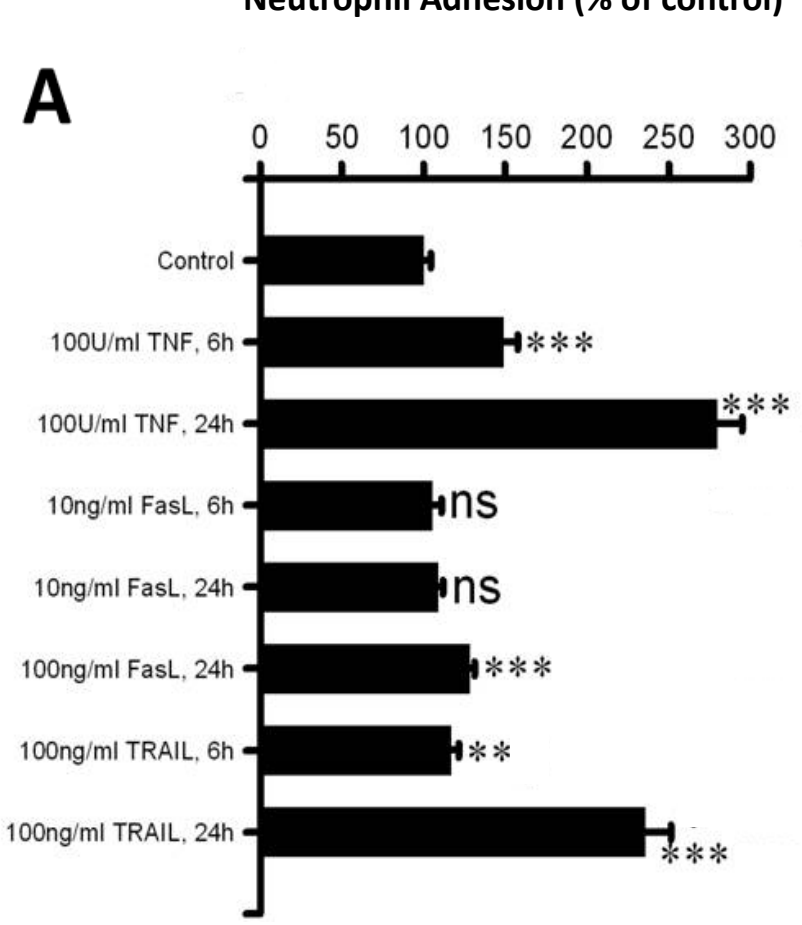
Neutrophil Adhesion (% of control)
A
0
50
100
150
200
250
300
Control
100U/ml TNF, 6h
***
100U/ml TNF, 24h
***
10ng/ml FasL, 6h
ns
10ng/ml FasL, 24h
ns
100ng/ml FasL, 24h
***
100ng/ml TRAIL, 6h
**
100ng/ml TRAIL, 24h
***

Figure 3.4.

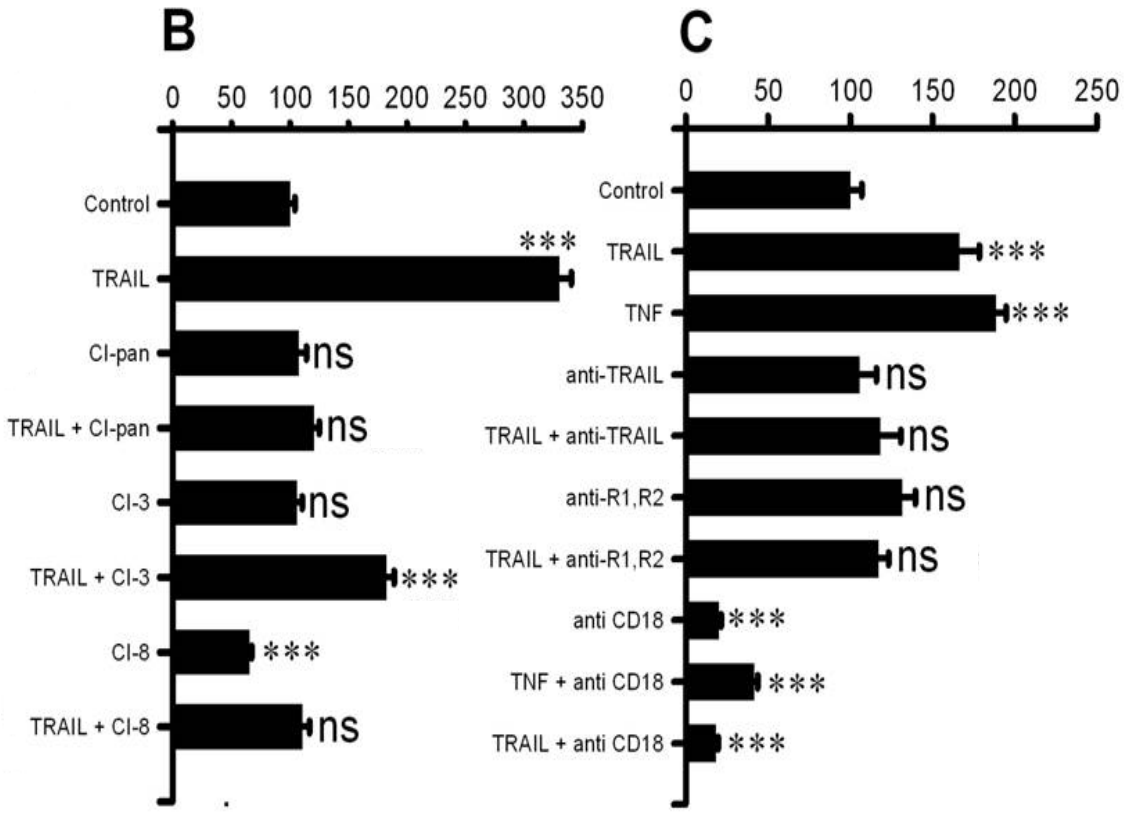
Neutrophil Adhesion (% of control)
B
0 50 100 150 200 250 300 350
Control
TRAIL
***
CI-pan
ns
TRAIL + CI-pan
ns
CI-3
ns
TRAIL + CI-3
***
CI-8
***
TRAIL + CI-8
ns
C
0 50 100 150 200 250
Control
TRAIL
***
TNF
***
anti-TRAIL
ns
TRAIL + anti-TRAIL
ns
anti-R1,R2
ns
TRAIL + anti-R1,R2
ns
anti CD18
***
TNF + anti CD18
***
TRAIL + anti CD18
***

Figure 3.4.

Figure 3.5. NF-κB Activation in EA.hy926 Cells Treated with TNF, TRAIL, and FasL.

Cells were treated with the indicated doses for 1, 3, 6, and 24 h, and nuclear proteins extracted. The concentration of p50 that bound to the NF-κB consensus binding site is shown, normalized (%) to the untreated control. Means ± SEM, n = 4 per time point. NS = Not significant, * $p < 0.05$, * * $p < 0.001$, vs. untreated control.

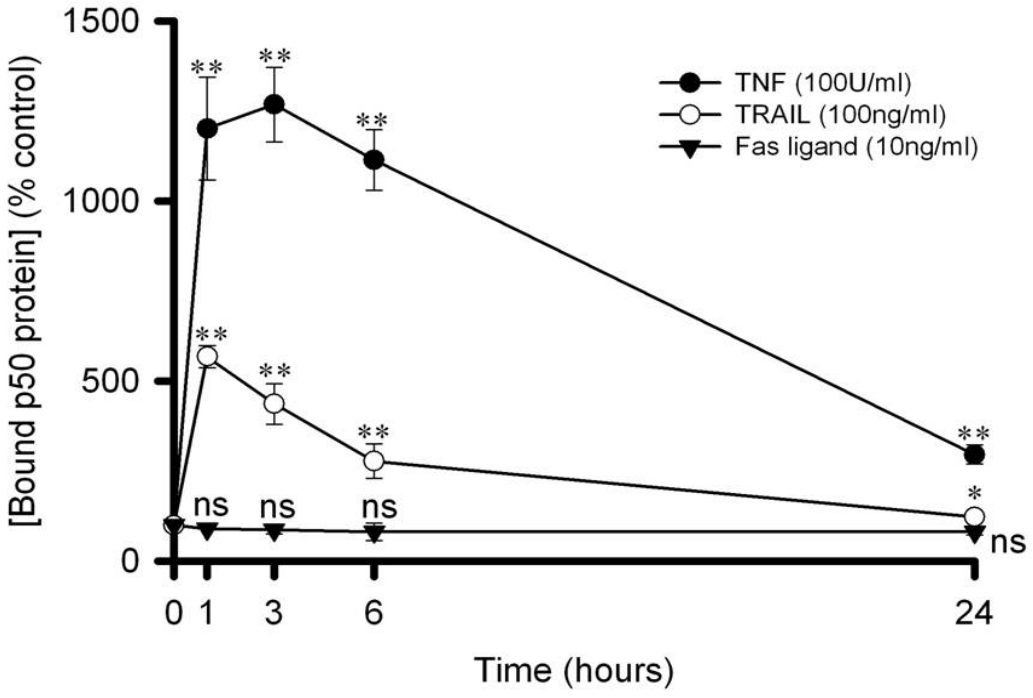
1500
1000
500
0
[Bound p50 protein] (% control)
TNF (100U/ml)
TRAIL (100ng/ml)
Fas ligand (10ng/ml)
**
**
**
**
**
**
**
**
*
ns
ns
ns
ns
0 1 3 6 24
Time (hours)

Figure 3.5.

Figure 3.6. Surface Expression of ICAM-1 Detected by Flow Cytometry in EA.hy926 Cells Following Treatments with TRAIL, FasL, and TNF.

Open histograms indicate fluorescent labeling with ICMA-1-specific monoclonal antibodies; shaded histograms show background labeling with isotype-matched control antibody in the same cells. Percent counts within the range specified by the marker (M) is shown on each panel, which contains < 1% of cells labelled with the isotype control. Data are representative of 4 separate experiments.

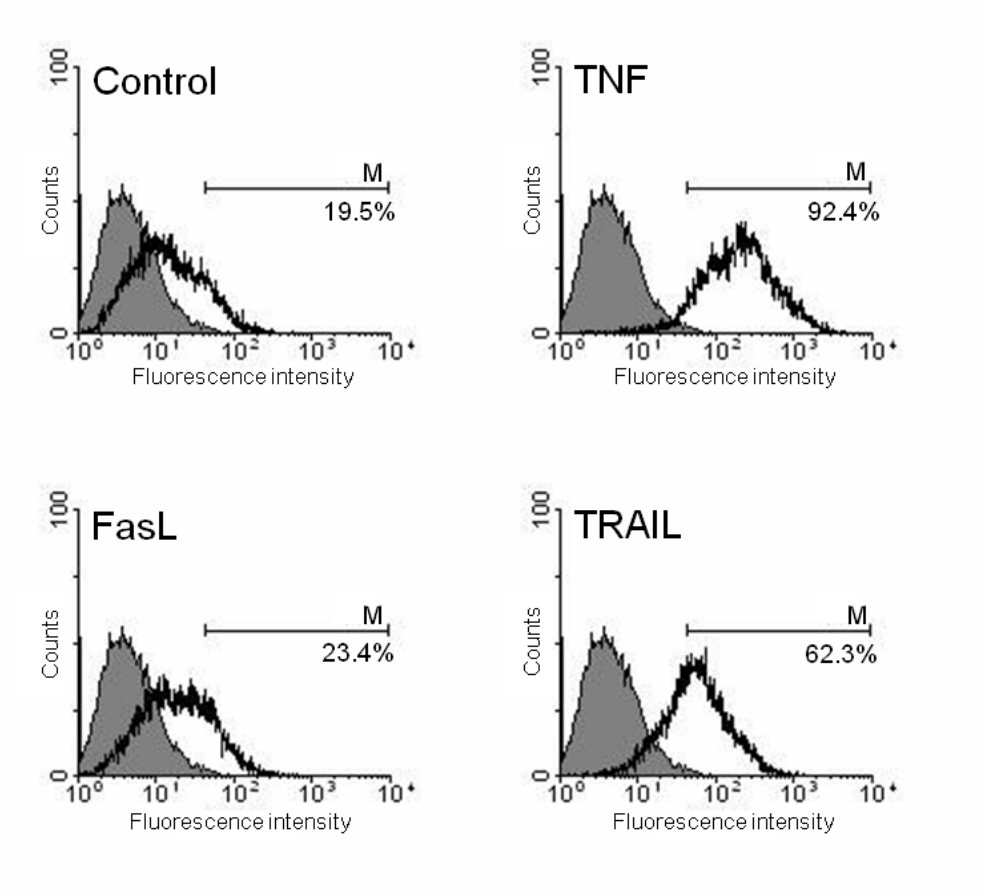
Control
M
19.5%
Counts
Fluorescence intensity
TNF
M
92.4%
Counts
Fluorescence intensity
FasL
M
23.4%
Counts
Fluorescence intensity
TRAIL
M
62.3%
Counts
Fluorescence intensity

Figure 3.6.

Figure 3.7. HUVEC Sensitized to Undergo Apoptosis by Treatment with LY294002 Alone, or in Combination with TRAIL (100 ng/ml).

All treatments were applied in reduced (1%) serum medium for 6 h. A. The caspase-3 inhibitor Z-DEVD-FMK (CI-3) or the caspase-8 inhibitor Z-IETD-FMK (CI-8; both 50μM) were co-applied with TRAIL and 5μM or 20μM LY294002. Means ± SEM, n = 24. B. Early apoptosis (% of cells with high annexin-V, low PI labeling). Means ± SEM, n = 3. A., B. Significance is compared to the untreated control or to treatments linked by cross bars (NS = not significant, * $p < 0.05$, * * $p < 0.01$, * * * $p < 0.001$).

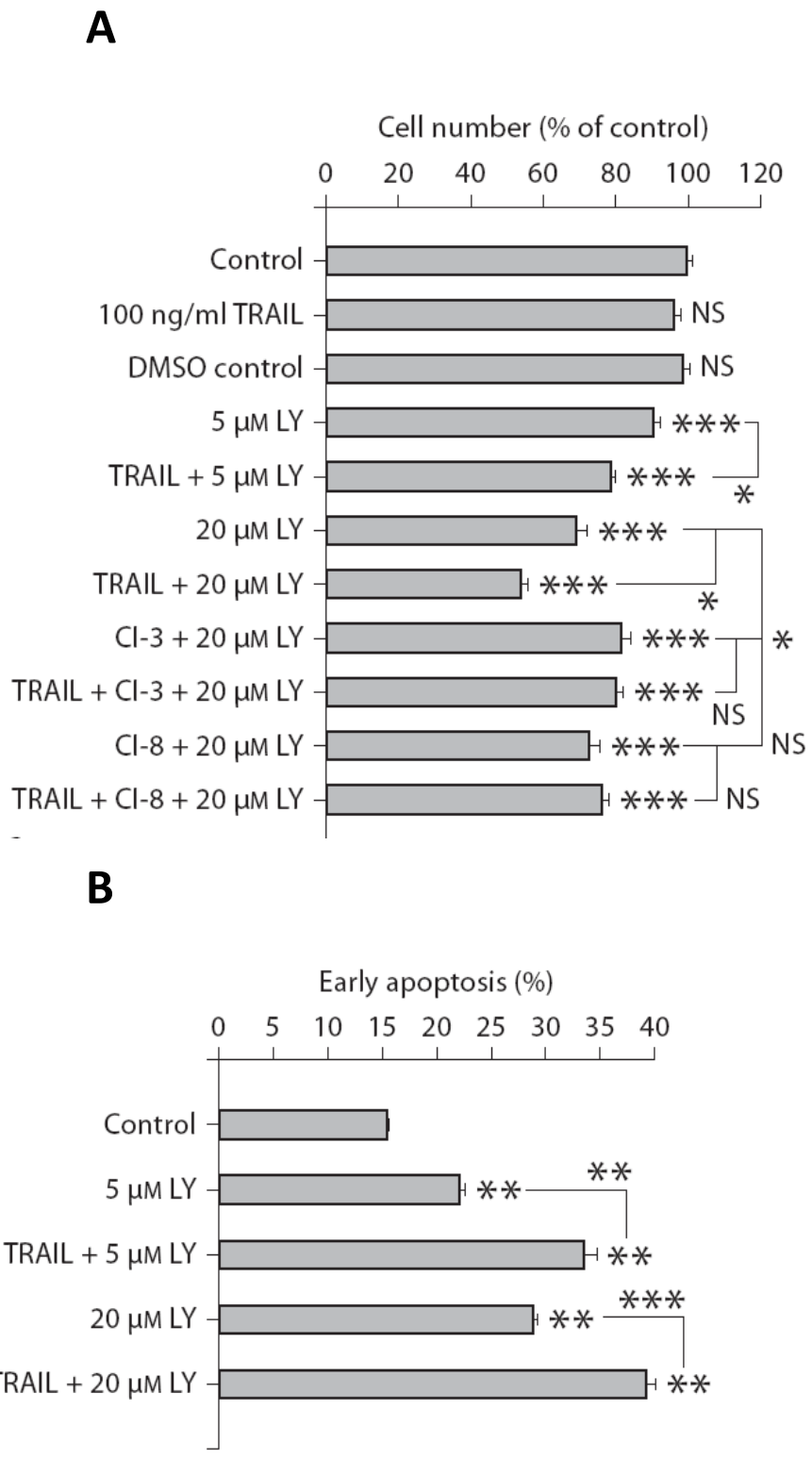
A
Cell number (% of control)
0
20
40
60
80
100
120
Control
100 ng/ml TRAIL
DMSO control
5 μM LY
TRAIL + 5 μM LY
20 μM LY
TRAIL + 20 μM LY
CI-3 + 20 μM LY
TRAIL + CI-3 + 20 μM LY
CI-8 + 20 μM LY
TRAIL + CI-8 + 20 μM LY
NS
***
*
B
Early apoptosis (%)
0
5
10
15
20
25
30
35
40
Control
5 μM LY
TRAIL + 5 μM LY
20 μM LY
TRAIL + 20 μM LY
**

Figure 3.7.

Figure 3.8. Neutrophil Adhesion to HUVEC.

Cells were treated in 20% serum containing medium with ligands for 24 h, at the indicated doses (A). Cells were also treated for 6 h in reduced (1%) serum medium with TRAIL and LY294002, at the indicated doses, either alone, or in combination (B). Means ± SEM, n = 8 (A) and n = 24 (B). NS = Not significant; * * * $p < 0.001$ vs. untreated control.

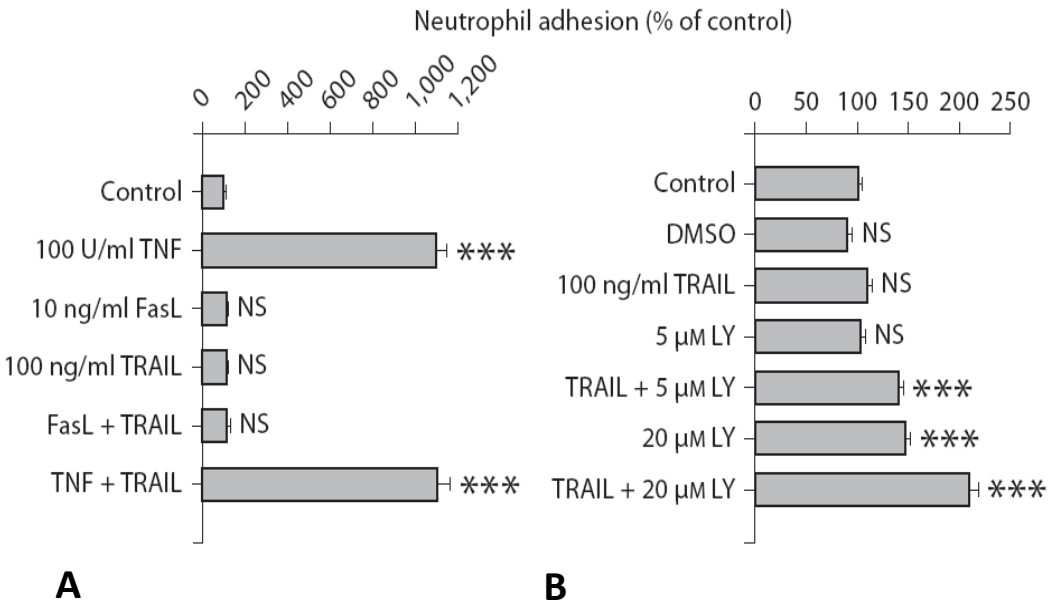
Neutrophil adhesion (% of control)
0
200
400
600
800
1,000
1,200
Control
100 U/ml TNF
10 ng/ml FasL
100 ng/ml TRAIL
FasL + TRAIL
TNF + TRAIL
NS
***
A
0
50
100
150
200
250
Control
DMSO
100 ng/ml TRAIL
5 µM LY
TRAIL + 5 µM LY
20 µM LY
TRAIL + 20 µM LY
NS
***
B

Figure 3.8.

CHAPTER 4 – Evidence that Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) Inhibits Angiogenesis by Inducing Vascular Endothelial Cell Apoptosis.

Pei-Lin Chen and Alexander S. Easton; Biochemical and Biophysical Research Communications (2010); 391: 936–941. (Published)

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4.1. Abstract

Tumor necrosis factor (TNF) and its related ligands TNF-related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL) play roles in the regulation of vascular responses, but their effect on the formation of new blood vessels (angiogenesis) is unclear. Therefore, we have examined the effects of these ligands on angiogenesis modeled with primary cultures of human umbilical vein endothelial cells (HUVEC). To examine angiogenesis in the context of the central nervous system, we have also modeled cerebral angiogenesis with the human brain endothelial cell line hCMEC/D3. Parameters studied were bromodeoxyuridine (BrdU) incorporation and cell number (MTT) assay (to assess endothelial proliferation), scratch assay (migration) and networks on Matrigel™ (tube formation). In our hands, neither TRAIL nor FasL (1, 10, and 100ng/ml) had an effect on parameters of angiogenesis in the HUVEC model. In hCMEC/D3 cells by contrast, TRAIL inhibited all parameters (10-100ng/ml, 24h). This was due to apoptosis, since its action was blocked by the pan-caspase inhibitor z-VAD-FMK (50µM) and TRAIL increased caspase-3 activity 1h after application. However, FasL (100ng/ml) increased BrdU uptake without other effects. We conclude that TRAIL has different effects on *in vitro*

angiogenesis depending on which model is used, but that FasL is generally ineffective when applied *in vitro*. The data suggest that TRAIL primarily influences angiogenesis by the induction of vascular endothelial apoptosis, leading to vessel regression.

4.2. Introduction

Angiogenesis, or the formation of new blood vessels, is an essential component of many physiological and pathological processes including embryogenesis, wound healing, formation of blood supply to tumors or ischemic tissues, and as a driver of inflammatory processes. New vessels promote inflammation by increasing the sum total of inflammatory mediators produced by vascular endothelial cells and by increasing the surface area for leukocyte interactions. In the central nervous system (CNS), angiogenesis may play a role in diseases as diverse as glioma, multiple sclerosis and stroke (277-279).

Angiogenesis is studied by using *in vitro* models that examine the many processes that vascular endothelial cells participate in during the formation of new vasculature. These processes include endothelial proliferation, migration and tube formation. Also relevant is an understanding of how each of these processes are limited through induction of endothelial apoptosis, which leads to vessel regression. Inhibition of angiogenic vessels may prove a key strategy in limiting pathological angiogenesis, by cutting off blood supply to tumors, or by reducing harmful inflammation.

Vascular endothelial apoptosis in this context may be induced by death ligands. Death ligands are inducers of caspase dependent apoptosis through cognate binding to death receptors, and include members of the tumor necrosis factor (TNF) family of ligands including TNF itself, tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL). TRAIL induces apoptosis by binding to its death receptors

TRAIL-R1 and TRAIL-R2. TRAIL also binds to two decoy receptors TRAIL-R3 and TRAIL-R4, as well as the soluble receptor osteoprotegerin (238). The binding of TRAIL to its death receptors results in activation of caspase-8. Through the extrinsic-death receptor mediated pathway, this causes direct cleavage of caspase-3 into active subunits that execute apoptosis (66). Caspase-8 also activates the intrinsic-mitochondrial pathway, a more complex sequence of events involving mitochondrial membrane depolarization with release of cytochrome c which recruits Apaf1, ATP, and caspase-9 to form an apoptosome. Here the cleavage product of caspase-9 results in cleavage and activation of caspase-3 to execute apoptosis (280).

In this study, we have modeled angiogenesis using two *in vitro* models. Most work on *in vitro* angiogenesis has been conducted in human umbilical vein endothelial cells, but previous reports on the action of TRAIL are contradictory (281, 282). Therefore we examined the effects of both TRAIL and FasL in this model. There are to our knowledge, no previous reports on the effect of these ligands in models of cerebral angiogenesis. So, cerebral angiogenesis was modeled with hCMEC/D3 cells, a recently described human brain endothelial cell line (233).

4.3. Results

4.3.1. Effects of TRAIL, Fas Ligand and TNF in HUVEC

The result of the BrdU incorporation, cell number and migration assays, is shown in Figure 4.1. As a positive control, 24 h treatment with vascular endothelial growth factor (VEGF, 10ng/ml) and basic fibroblast growth factor (bFGF, 10, 50 ng/ml) induced a significant increase in BrdU incorporation (Figure 4.1-A). bFGF at these doses also induced a significant increase in cell numbers (Figure 4.1-B). VEGF and bFGF (both 100 ng/ml) also significantly increased cell migration over 24 h (Figure 4.1-C). TRAIL, FasL

(both 1, 10, and 100 ng/ml) and TNF (10, 100 U/ml) had no effect on these parameters (Figure 4.1-A.B.C). Tube formation was also analyzed in HUVEC (Figure 4.2). Representative images are shown in Fig. 4-2A. The data are expressed as the average length or average area of the tubular structures. Under control conditions, average area increased slightly over 24 h, while average length remained stable. VEGF (50 ng/ml) induced significant increases in both parameters over 24 h (Figure 4.2-B, C). FasL (1, 10, and 100ng/ml), TRAIL (1, 10, and 100 ng/ml) and TNF (1, 10, and 100 U/ml) had no effect (the effect of individual doses is shown in Figure 4.2-B, C). These data show that TRAIL, FasL, and TNF have no effect on parameters of angiogenesis modeled with HUVEC.

4.3.2. Effect of TRAIL, Fas Ligand, and TNF in hCMEC/D3 Cells

Flow cytometry was carried out to detect receptors for TRAIL and FasL on hCMEC/D3 cells. The fluorescence intensity of labeling with specific antibodies was plotted as a function of cell number (Appendix II, Figure S.1). The isotype control antibody staining was gated to give a value of ~10% of the cells (9.93 ± 0.17%, mean ± SEM, n = 9). This allows the relative increase in staining for other antibodies to be expressed as % of cells within the set range. Compared to ca. 10% staining in control cells, ~90% of cells stained for TRAIL-R2 (90.76 ± 2.00%, n = 5, $P < 0.001$ compared to isotype control). This was considerably greater than staining for TRAIL-R3 (17.20 ± 0.31%, n = 5, $P < 0.001$ compared to control), with no significant increase above control for either TRAIL-R1 (12.12 ± 0.25%, n = 5) or TRAIL-R4 (9.75 ± 1.00%, n = 4). The Fas receptor was also detected in a high percentage of cells (63.98 ± 1.79%, n = 5, $P < 0.001$), but there was no detectable Fas ligand (FasL) or TRAIL (means under 3%, not significantly increased). There was also expression of the VEGF receptors VEGF-R1 (20.1 ± 0.36%, n = 4, $P < 0.001$) and VEGF-R2 (66.18 ± 0.36%, n = 4, $P < 0.001$).

The hCMEC/D3 line has an ordinary requirement for medium containing multiple growth factors. We therefore anticipated that single growth factors would be insufficient to act as positive controls. Indeed, VEGF and bFGF (10, 50, and 100 ng/ml) applied singly or in combination with the co-factor heparin (50 ng/ml plus 100 U/ml heparin, had no effect on BrdU incorporation, cell numbers or migration (Appendix II, Figures S.2, S.3, S.4) (effect of 100 ng/ml shown in Figure 4.3). However, introduction of 5% FBS to cells rendered quiescent (in serum free medium containing 2% BSA) stimulated a strong positive responses, that served as positive controls (Figure 4.2-A, B, C).

Quiescent cells responded differently to TRAIL and FasL. TRAIL (1–100 ng/ml) induced a dose dependent reduction in BrdU incorporation (Figure 4.3-A.), that was inhibited by co-treatment (100 ng/ml TRAIL) with the pan-caspase inhibitor Z-VAD-FMK (50μM). A similar reduction was observed in cell numbers and migration, which were also blocked by Z-VAD-FMK co-treatment (Figure 4.3-B, C). In contrast, FasL (1, 10ng/ml) had no effect on BrdU uptake, while 100 ng/ml had a small stimulatory effect (Figure 4.3-A). TNF (10, 100 U/ml) mimicked the inhibitory effect of TRAIL on BrdU incorporation (Figure 4.3-A). Both FasL and TNF had no effect on cell numbers or migration (Figure 4.2-B).

To confirm the impression that TRAIL induced apoptosis in the cells, caspase-3 activity was measured. TRAIL induced a significant increase in caspase-3 activity over untreated cells at 1 h (2.10 ± 0.35 vs control 0.10 ± 0.01, arbitrary fluorescence units, $n = 4$, $P < 0.001$ compared to control), with values returning to baseline by 3 h (0.73 ± 0.23, $n = 4$) and 6 h (0.50 ± 0.22, $n = 4$).

Tube formation on Matrigel™ was assessed for periods between 3 and 24 hours. Representative images are shown in Figure 4.4-A. These images show that the tubular structures increased in both length and area but reduced in number as the assay

progressed. The individual regions were therefore analyzed in terms of average length and average area (Figure 4.4.B, C). Untreated cells (control data) showed a progressive increase in both parameters over time. This was accelerated by the introduction of 5% FBS as a positive control (Figure 4.4-B, C). 50 ng/ml doses of VEGF and bFGF had no significant effect on either parameter while TRAIL (100 ng/ml) reduced both parameters, inducing a statistically significant reduction in average length at 6 hours (Figure 4.4-B.). FasL (10 ng/ml) and TNF (100 U/ml) had no effect (Figure 4.4-B, C). The data confirm that tube formation in hCMEC/D3 cells is inhibited by TRAIL, and unaffected by other ligands.

4.4. Discussion

4.4.1. Effect of TRAIL on HUVEC

TRAIL was initially reported to promote apoptosis in cancer cells without harming normal cells, and it remains a promising candidate for novel anti-cancer therapy (239). Physiologically, its best characterized function is in tumor surveillance (283). In the vasculature, TRAIL is expressed by vascular smooth muscle (259) and may function in a paracrine function to regulate vascular endothelial functioning by inducing endothelial production of nitric oxide and prostanoids (284). In an inflammatory context, TRAIL is upregulated by virtually all immune cells including neutrophils, T and B lymphocytes, natural killer cells, dendritic cells and monocytes (285).

There are different outcomes in previous studies of TRAIL and angiogenesis in peripheral endothelial cells. In HUVEC, TRAIL was reported to induce activation of Akt and ERK leading to endothelial proliferation (242) and to increase cell migration and tube formation without altering endogenous endothelial cell expression of VEGF (286). TRAIL had no effect on angiogenesis in the rat aortic ring model (286). TRAIL was reported to inhibit cancer related angiogenesis in an implant of PC-3 tumor cells in mice,

with a reduction in local VEGF expression (287). Finally, in contrast to the previous finding that TRAIL is stimulatory (281), TRAIL was found to inhibit tube formation, cell numbers and endogenous VEGF expression in HUVEC in another study (282). The pro-angiogenic action of TRAIL (281) was induced by 10 ng/ml TRAIL linked to a histidine tag, while the anti-angiogenic effect of TRAIL (282) was induced by higher doses (100–400 ng/ml). Tagged forms of TRAIL may behave differently from untagged forms, as for instance when histidine tagged TRAIL induced apoptosis in hepatocytes that was not seen with the untagged moiety (288, 289). The data in this study lead us to conclude that both TRAIL and FasL have no effect on parameters of angiogenesis modeled in HUVEC (Figures 4.1 and 4.2). We speculate that this could relate to our use of untagged TRAIL instead of histidine tagged TRAIL (281), since other conditions (doses of TRAIL,10 ng/ml in (281), 100 ng/ml in (282), presence of serum in both studies) have been replicated in this work.

4.4.2. Actions of TRAIL and FasL in hCMEC/D3 Cells

This is to our knowledge, the first study to examine the actions of TRAIL and FasL in a model of cerebral angiogenesis. In hCMEC/D3 cells, TRAIL induced a dose dependent reduction in BrdU uptake, cell numbers, migration and tube formation (Figures 4.3 and 4.4). The effect of TRAIL was inhibited in combination with the pan-caspase inhibitor Z-VAD-FMK (Figure 4.3). To confirm that TRAIL inhibited these processes through induction of apoptosis, TRAIL was found to induce an early (1 h) increase in caspase-3 activity. TNF also reduced BrdU uptake (Figure 4.3) but had no effect on migration (Figure 4.3) or tube formation (Figure 4.4). In contrast, Fas ligand had minimal effects, inducing a small but significant increase in BrdU uptake (Figure 4.3), without effect on other parameters (Figures 4.3 and 4.4).

The propensity of hCMEC/D3 cells to undergo apoptosis in response to TRAIL is not

the result of serum withdrawal, since TRAIL was also effective in reducing BrdU uptake, cell numbers and migration when 5% FBS was present (Figure 4.3 and Appendix II, Figures S.2, S.3, and S.4). Also, FasL did not induce an apoptotic response in the cells, so there is no general propensity for hCMEC/D3 cells to undergo apoptosis in response to death ligands. However, a study in primary cultures of human brain endothelial cells found that TRAIL does not induce apoptosis (290). This suggests that the human brain endothelial cells used in this study have been sensitized to TRAIL by the process of immortalization. This is not irrelevant to an understanding of the role that TRAIL may play in regulating cerebral angiogenesis. Other evidence indicates that TRAIL only induces apoptosis in vascular endothelial cells when they have been previously sensitized. This may be relevant to understanding the balance between pro- and anti-angiogenic factors in limiting angiogenesis. Thus, TRAIL induces apoptosis in HUVEC only when there is inhibition of phosphatidylinositol 3-kinase and its downstream activation of the serine/threonine kinase, Akt (242, 244). TRAIL (but not FasL) also induces apoptosis in unmodified EA.hy926 cells, an immortalized cell line derived from HUVEC. In this context the endothelial cells become pro-adhesive for neutrophils (291). TRAIL promotes apoptosis of human dermal microvascular endothelial cells when they are grown on a substrate that inhibits integrin signaling (292). Angiogenesis induced by VEGF acts through Akt activation and can be blocked when Akt is inhibited by various anti-angiogenic factors such as angiotensin II and thromboxane A2 (293, 294). Thus, factors that inhibit angiogenesis could sensitize vascular endothelium to undergo apoptosis by TRAIL, leading to vessel regression. However, TRAIL would not be expected to induce apoptosis in the normal microvasculature.

4.5. Conclusions

The data in this study suggest that TRAIL (with supportive evidence for TNF) may inhibit cerebral angiogenesis by inducing apoptosis in appropriately sensitized cerebral endothelial cells, and so exert a potential anti-inflammatory role in the human CNS. Fas ligand may be neutral, or even promote cerebral angiogenesis. The combination of our findings in HUVEC and hCMEC/D3 suggests that TRAIL principally influences angiogenesis by inducing apoptosis in vascular endothelial cells, leading to vessel regression. In contrast to other in vitro studies (242, 281), we did not find evidence of a pro-angiogenic role for TRAIL.

4.6. Acknowledgments

The study was funded by the Canadian Institutes of Health Research, the Nova Scotia Health Research Foundation, and the Multiple Sclerosis Society of Canada. P.-L.C. was funded by a George Loh Scholarship through the Department of Pathology, Dalhousie University.

Figure 4.1. Effects of Multiple Factors on Parameters of Angiogenesis in the HUVEC.

Data are shown for BrdU incorporation (A), cell number analysis by MTT assay (B) and cell migration by scratch assay (C). Cells were treated (24 h) with vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), TNF-related apoptosis inducing ligand (TRAIL), Fas ligand (FasL), and tumor necrosis factor (TNF). Numbers indicate doses in ng/ml except for TNF where dosage is U/ml. Data are normalized to the untreated control, and shown as mean ± standard error of the mean (SEM). Significance is compared to the untreated control; only significant differences are marked (*P < 0.05, **P < 0.01, ***P < 0.001). In (A, B), n = 24 for untreated cells and n = 12 for other groups. In (C), n = 12 for untreated cells, n = 6 for other groups.

HUVEC

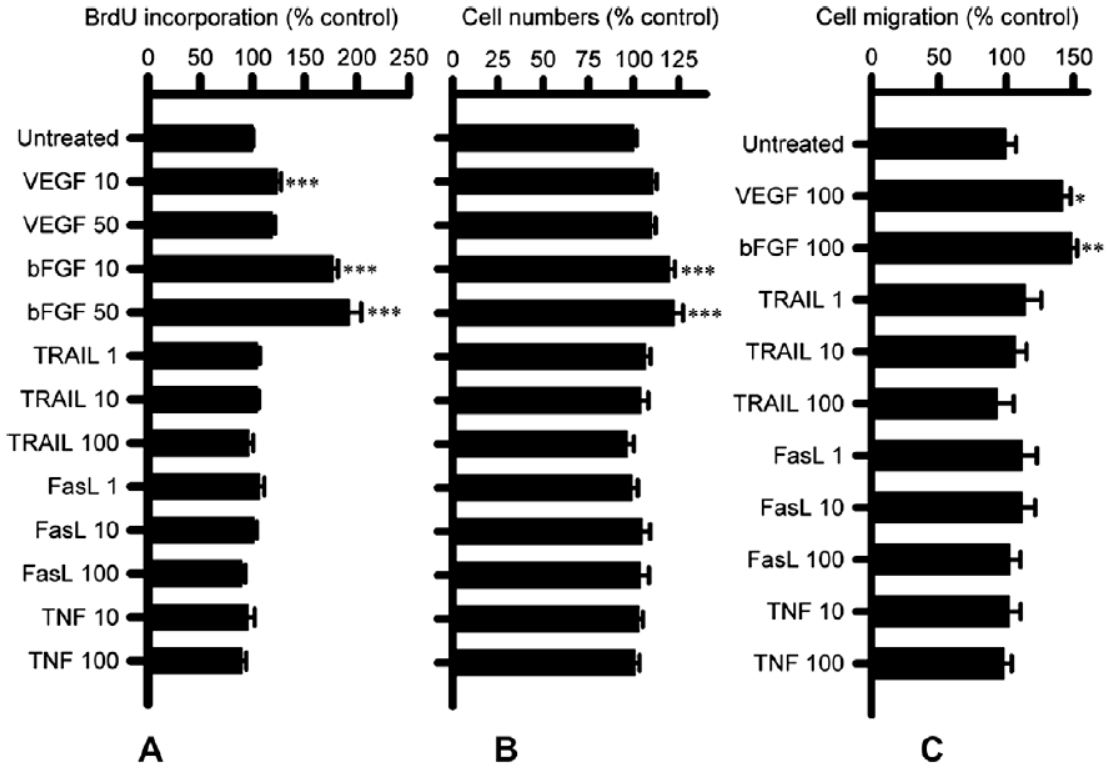

Figure 4.1.

Figure 4.2. Tube Formation Assay on Matrigel™ in HUVEC Treated with Growth Factors and Indicated Ligands.

Doses are ng/ml except TNF (U/ml). Representative images are shown (A) taken at 20x magnification (scale bar = 500 µm). Data (mean ± SEM) are expressed as average length (B) or average area (C) of tubular structures over time. Relative changes are shown (expressed in arbitrary units). Significance is compared to the untreated control and only significantly different results are shown (**P < 0.01, ***P < 0.001). In (B and C), n = 8 for controls and n = 4 for all other groups.

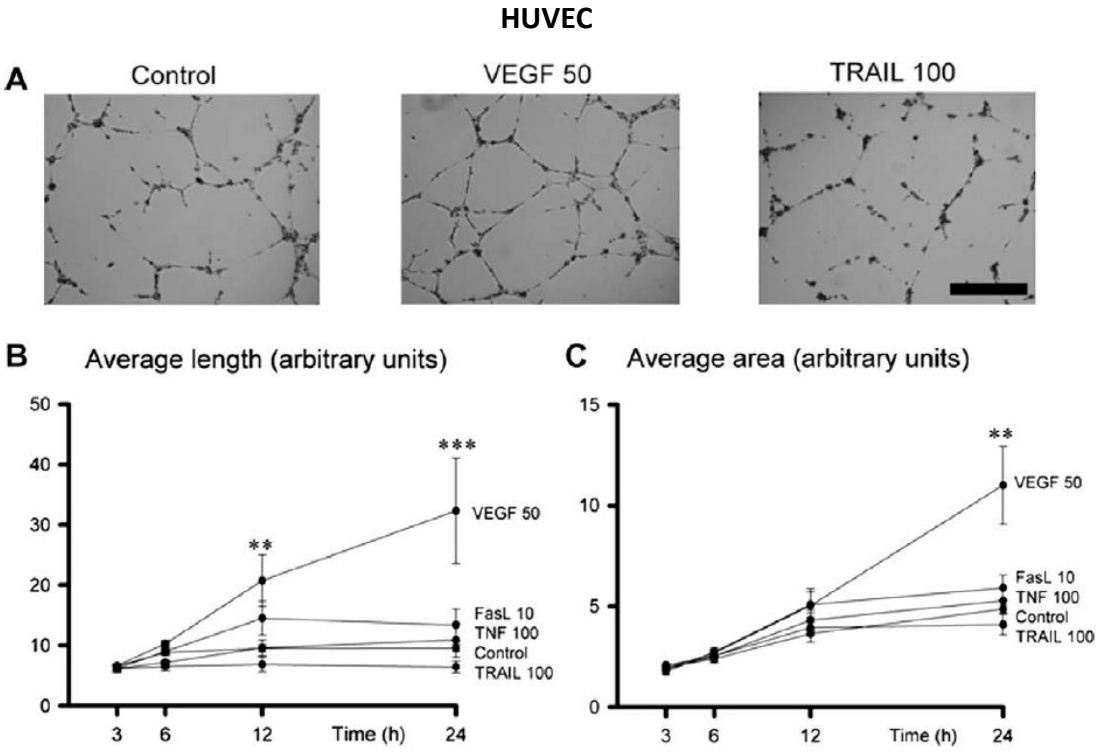
HUVEC
A
Control
VEGF 50
TRAIL 100
B
Average length (arbitrary units)
50
40
30
20
10
0
**
***
VEGF 50
FasL 10
TNF 100
Control
TRAIL 100
3
6
12
Time (h)
24
C
Average area (arbitrary units)
15
10
5
0
**
VEGF 50
FasL 10
TNF 100
Control
TRAIL 100
3
6
12
Time (h)
24

Figure 4.2.

Figure 4.3. Effects on Parameters of Cerebral Angiogenesis Modeled with hCMEC/D3 Cells.

Data are shown for BrdU incorporation (A), cell number analysis (B), and cell migration (C). Cells were treated (24 h) with growth factors and indicated ligands. In some experiments, TRAIL was combined with the caspase inhibitor Z-VAD-FMK (50 μM, CI). Numbers indicate doses in ng/ml except for TNF (U/ml). Data are normalized to the untreated control, and shown as mean ± SEM. Significance is compared to the untreated control except where indicated by cross bars; only significant differences are marked (***$P < 0.001$). In (A), n = 32 for untreated cells, n = 8–12 for 5% FBS and growth factors and n = 4–20 for other ligands. In (B), n = 60 for untreated cells, n = 26 for 5% FBS, n = 24 for growth factors, and n = 12–36 for other ligands. In (C), n = 79 for untreated cells, n = 63 for 5% FBS, and n = 15–40 for other ligands.

hCMEC/D3 cells

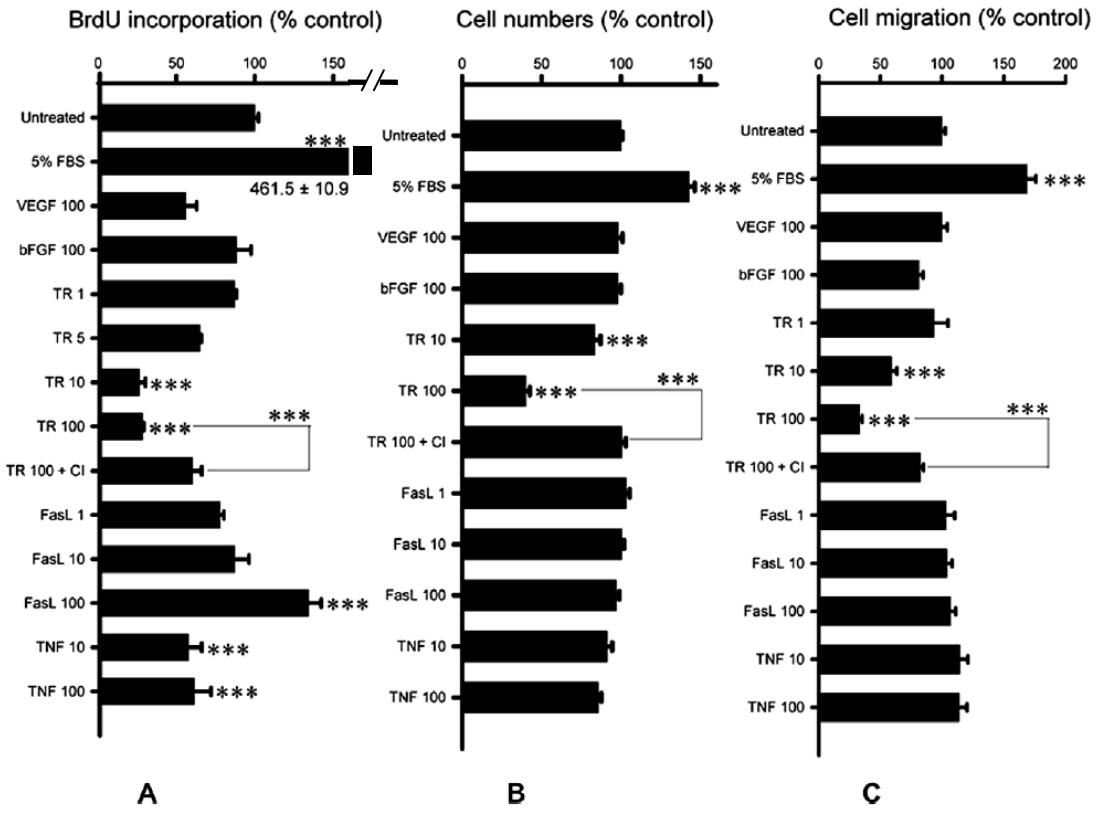

Figure 4.3.

Figure 4.4. Tube Formation Assay on Matrigel™ in hCMEC/D3 Cells Treated in Serum (5% FBS) and with Indicated Ligands.

Doses are ng/ml except for TNF (U/ml). Representative images are shown (A) taken at 20x magnification (scale bar = 500 µm). Data (mean ± SEM) are expressed as average length (B) or average area (C) of tubular structures over time. Relative changes are shown (expressed in arbitrary units). Significance is compared to the untreated control and only significantly different results are shown (*P < 0.05, **P < 0.01, ***P < 0.001). In (B, C), n = 8 for controls and n = 4 for all other groups.

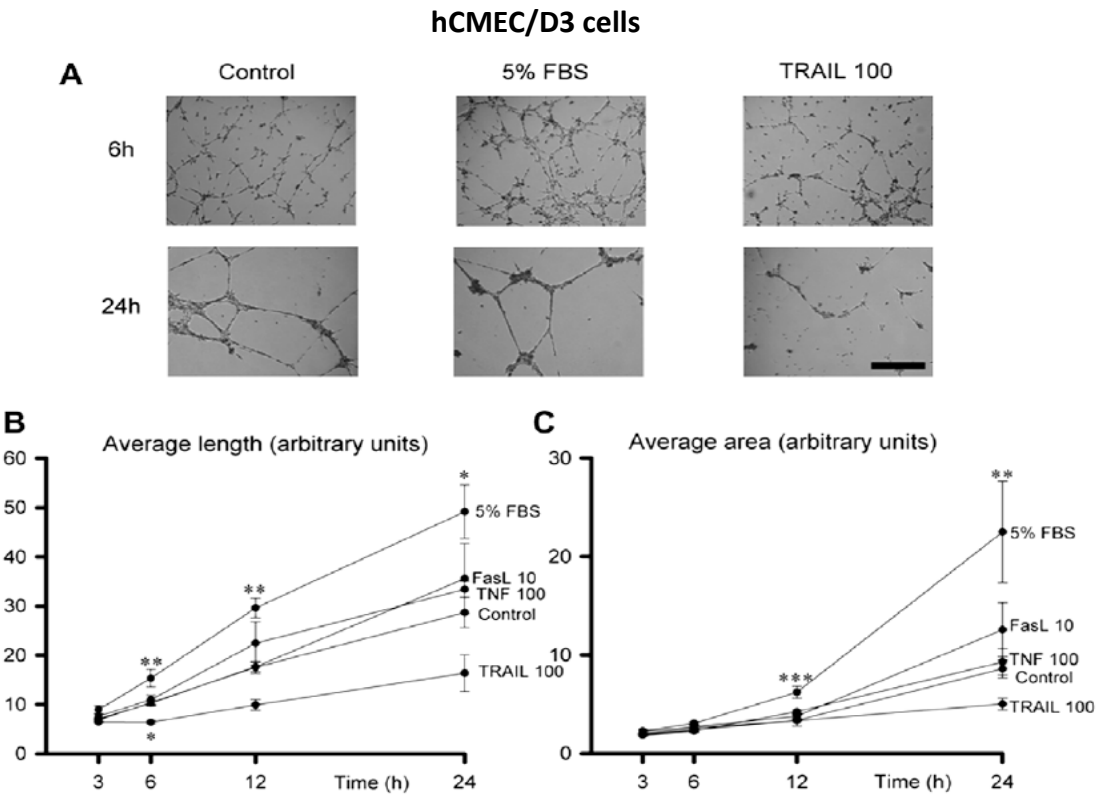
hCMEC/D3 cells
A
Control
5% FBS
TRAIL 100
6h
24h
B
Average length (arbitrary units)
60
50
40
30
20
10
0
3
6
12
24
Time (h)
5% FBS
FasL 10
TNF 100
Control
TRAIL 100
C
Average area (arbitrary units)
30
20
10
0
3
6
12
24
Time (h)
5% FBS
FasL 10
TNF 100
Control
TRAIL 100

Figure 4.4.

CHAPTER 5 – Anti-angiogenic Effects of Resveratrol on Cerebral Angiogenesis.

Pei-Lin Chen and Alexander S. Easton; (accepted by the Current Neurovascular Research in December, 2010; E-published ahead of print on Jan 5th, 2010)

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5.1. Abstract

Angiogenesis is implicated in diseases of the central nervous system, and its modulation represents an attractive therapeutic strategy. This is the first report of the effects of resveratrol (*trans*-3,4',5-trihydroxystilbene) on cerebral angiogenesis, using an *in vitro* model. Processes associated with angiogenesis were studied in cerebral vascular endothelial cells, using the human brain endothelial cell line hCMEC/D3 and primary bovine brain microvessel EC (BBMEC). Comparisons were made to human umbilical vein EC (HUVEC). In cerebral cultures, resveratrol (24h) induced a dose dependent reduction in BrdU incorporation and cell numbers (MTT assay) between 10-100μM, while lower doses (100nM-5μM) had no effect. Cell migration (scratch assay) was inhibited between 10-100μM depending on cell type. Doses between 10-100μM reduced average tubule length on Matrigel™, while higher doses (50, 100μM) also inhibited process formation around explanted rat aortic rings (*ex vivo* assay). Cell cycle analysis in hCMEC/D3

(propidium iodide) showed reduced progression to the G2/M phase, with a maximal effect at 25µM. Resveratrol did not induce apoptosis in hCMEC/D3, based on the absence of caspase-3 activity. Cytotoxicity (LDH release) was induced by resveratrol (50,100µM) in hCMEC/D3 and HUVEC, peaking at ~15% in hCMEC/D3 and ~35% in HUVEC. Cytotoxic effects were not detected in BBMEC. Resveratrol (10-50µM) inhibited phosphorylation of the serine/threonine kinase Akt, by Western blot (15min, 1h) with a prolonged inhibition (24h) for 25µM. In conclusion, this study shows inhibitory effects of resveratrol on cerebral angiogenesis, using an *in vitro* model. This is discussed in terms of dosage, *in vivo* equivalence and therapeutic potential.

5.2. Introduction

Resveratrol (*trans*-3,4',5-trihydroxystilbene) is a natural phytoalexin found in the skin of grapes, and other plant sources including berries, plums, peanuts and pines (295). Interest in resveratrol derives from its presence in red wine, and the inverse correlation between red wine consumption and cardiovascular health. Because this was noted in studies carried out in southern France, despite a diet rich in saturated fats, the effect is known as the 'French paradox' (206). Since these initial observations, there has been a burgeoning of studies looking not only at the cardiovascular effects of resveratrol, but also its effects on cancer and aging.

Angiogenesis is the formation of new blood vessels from pre-existing vessels. The process of angiogenesis is orchestrated by the balance of pro and anti-angiogenic factors, and involves vascular endothelial cells in the processes of proliferation, migration and tubule formation. Angiogenesis occurs in a physiological as well as a pathological context.

For instance, it is involved in embryonic development, menstruation and wound healing (1, 20). In the setting of disease, angiogenesis has mixed effects. In cancer, angiogenesis is harmful, because it is associated with tumor growth and the promotion of metastasis (296-301). In ischemic heart disease and stroke, angiogenesis is believed to be protective, promoting the restoration of blood flow to injured tissues, or limiting the effects of subsequent ischemia (302-307).

The impact of resveratrol on angiogenesis has been assessed in tissue culture as well as animal models. Previous work was carried out in large vessel endothelial cells, such as human umbilical vein endothelial cells (HUVEC, (181, 308-311)), bovine pulmonary artery (312) and aortic (310, 313) endothelial cells and porcine aortic endothelial cells (16). Far fewer studies have been carried out in smaller vessels, including human coronary arteriolar endothelial cells (314) and human microvascular endothelial cells (315). In general, these studies have shown an inhibitory effect of resveratrol on parameters of angiogenesis. Effects have been noted in the micromolar range. The exceptions are coronary vessel endothelium, where resveratrol promotes tube formation (314), and a recent study in HUVEC that describes pro-angiogenic effects for 5μM resveratrol but inhibitory effects at 20μM (311). A variety of different steps in the molecular regulation of angiogenesis have been studied *in vitro*, including those regulating endothelial cell proliferation (16, 181, 311, 312), endothelial cell attachment to neighbouring cells via VE-cadherin (308), and endothelial cell adhesion to the substratum via αvβ3 integrins (310). As mentioned, resveratrol promotes tube formation in human coronary arteriolar endothelial cells (314), suggesting that its effects are organ specific. The same study showed that resveratrol promotes angiogenesis after

myocardial ischemia in rat heart, which supports its cardioprotective effects (314). Dosage appears to be a critical factor in its *in vivo* actions. In a study of angiogenesis in rat ischemic limbs, low doses of red wine polyphenols promoted angiogenesis while higher doses were inhibitory (316). Several *in vivo* assays, including the corneal micropocket assay, skin Matrigel™ plug and chick chorioallantoic membrane (CAM) assay, have also documented an inhibitory effect on angiogenesis (16, 315, 317), while 5µM promoted vessel formation in the chick CAM assay (311). Resveratrol also inhibits vessel formation induced by cancer cells *in vitro* (318) and following treatment of *in vivo* cancer implants (228).

The impact of resveratrol on angiogenesis in the central nervous system (CNS) is incompletely understood. CNS angiogenesis plays a role in the promotion of neoplasia. High grade gliomas induce vascular proliferation as a hallmark characteristic. Angiogenesis is also induced following cerebral ischemia, and may play a role in restoring blood flow to tissue around a stroke, and help to protect this tissue from subsequent ischemic injury (302, 319). There is now evidence that angiogenesis is induced in inflammatory diseases such as multiple sclerosis, and its animal model experimental allergic encephalomyelitis (8, 9), but in this context angiogenesis may prove harmful, since new vessels can support ongoing inflammation both by generating pro-inflammatory mediators and acting as conduits for the recruitment of pathogenic leucocytes. However, *in vivo* studies have been lacking. In one study, resveratrol inhibited glioma associated angiogenesis (228). In a study of ischemic stroke, rats treated with resveratrol at the time of stroke induction, showed a smaller reduction in blood vessel density on the side of the stroke, compared to control animals, suggesting that

resveratrol either protects vessels from elimination, or actively promotes angiogenesis in the CNS (320). There are to date no studies of the actions of resveratrol on CNS angiogenesis modeled *in vitro*.

This is the first study to examine the actions of resveratrol on cerebral angiogenesis using cerebrovascular endothelial cultures. Vascular endothelial cell proliferation, migration and tubule formation were examined as surrogate measures of angiogenesis. Mechanisms were also studied, with a focus on apoptosis, cytotoxicity and cell cycle progression, and downstream effects on the key signaling molecule Akt.

5.3. Results

5.3.1. Impact of Resveratrol on BrdU Incorporation and Cell Numbers

The effect of resveratrol was tested in vascular endothelial cell cultures from the CNS (hCMEC/D3 and BBMEC) and periphery (HUVEC). To explore differential effects between lower and higher doses, the dose range was between 100nM and 100µM. The result of BrdU incorporation and cell number assays is shown in Figure 5.1. In CNS cultures (hCMEC/D3 and BBMEC), there was a significant reduction in BrdU incorporation for doses between 25-100µM (10µM in BBMEC). Resveratrol 10µM reduced cell numbers in hCMEC/D3 ($P<0.05$), while higher doses (25, 50, 100µM) reduced cell numbers in both hCMEC/D3 and BBMEC. Lower doses (100nM, 1µM) had no significant effect. In HUVEC, higher doses of resveratrol (25, 50, 100µM) induced significant reductions in BrdU incorporation, while lower doses had no significant effect. Higher doses (25, 50, 100µM) caused significant reductions in HUVEC cell numbers.

5.3.2. Effect of Resveratrol on Cell Migration

Endothelial cell migration was assessed in the different endothelial types, using the scratch assay. Images of the denuded zone are shown in Figure 5.2-A, after 24h treatment with 1-100µM resveratrol, and the data is summarized in Figure 5.2-B. Migration was significantly inhibited by 10µM resveratrol in hCMEC/D3 and HUVEC, while 1µM had no effect. Higher doses of resveratrol (25, 50, 100µM) inhibited migration in all cell types.

5.3.3. Possible Explanations for the Impact of Resveratrol

A reduction in BrdU incorporation, cell numbers and migration can occur if resveratrol induces apoptosis, induces non-specific toxicity (necrosis), or inhibits cell cycle progression and proliferation. In Figure 5.3-A, the LDH release assay was used to assess cytotoxicity. In hCMEC/D3 and HUVEC, there were significant increases in LDH release at the two higher doses of resveratrol (50, 100µM), with no significant impact in BBMEC at any dose. Cytotoxicity reached at 15.67 ± 1.02% in hCMEC/D3 treated with 100µM resveratrol, and 34.51 ± 1.81% in HUVEC. This compares with cell number reductions of about 50% for the same dose and duration of resveratrol treatment (to 50.52 ± 1.96% in hCMEC/D3 and 47.91 ± 9.47% in HUVEC). At 50µM, the toxicity data is 7.10 ± 1.33% in hCMEC/D3 and 17.66 ± 2.11% in HUVEC, compared to cell number reductions of around 20% in hCMEC/D3 (to 79.27 ± 1.43%) and around 35% in HUVEC (to 64.11 ± 2.29%). To assess the possible contribution of apoptosis to the effects of resveratrol in hCMEC/D3, caspase-3 activity was measured between 1-6h for doses of resveratrol between 10-100µM, using TNF-related apoptosis inducing ligand (TRAIL,

100ng/ml) as a positive control, based on our previous assessment (321). The data are presented in Fig. 5.3-B, where all doses of resveratrol (50, 100µM are shown) were without significant effect. Cell cycle progression in hCMEC/D3 was assessed with propidium iodide staining. Figure 5.3-C shows representative flow cytometry plots that estimate the proportion of cells in the G1/G0, S and G2/M phases of the cell cycle. Compared to vehicle, resveratrol (25µM) arrested progression to the G2/M phase, with an increase in S phase fraction, absent G2/M fraction and a reduction in the G1/G0 fraction. The data is summarized in Figure 5.3-D. Effects begin to be seen for 10µM resveratrol, with an increase in S phase fraction, while 25µM abolishes cells in G2/M, with significant reductions for 50 and 100µM. G1/G0 fraction was significantly increased by 50 and 100µM resveratrol.

5.3.4. Effects of Resveratrol on Tubule Formation

Tubule formation was assessed by plating hCMEC/D3 cells on Matrigel™ , and by measuring tubule formation around everted rat aortic rings exposed to different doses of resveratrol. Figure 5.4-A shows the effect of 10, 25 and 100µM resveratrol on tubule formation in Matrigel™ after an incubation period of 16h, in which there is a reduction in length of individual tubules compared to vehicle alone. The tubules were quantified by image analysis (Figure 5.4-B) as changes in average length at 4 and 16h. Significant reductions in average length were detected for 10 and 100µM resveratrol at 4h, with significant reductions at 16h for doses between 10-100µM. Resveratrol at 100nM and 1µM had no significant effect. To further assess the impact of resveratrol on tubule formation, an *ex vivo* assay was carried out in rat aortic rings (Figure 5.5-A, B). A well

defined plexus develops around the rings in vehicle treated wells by day 5 (Figure 5.5-A). The area of this plexus was significantly reduced by 50µM resveratrol, while its formation was blocked at 100µM.

5.3.5. Effects of Resveratrol on Key Signaling Molecules

There was a dose dependent reduction in the phosphorylated form of Akt (p-Akt) relative to total protein, on exposure to 10, 25 and 50µM resveratrol for different durations (15min, 1h, 24h). Treatment for 15min and 1h induced a significant reduction in levels of p-Akt for 25 and 50µM while 10µM was effective only after 15min. For the 24h treatments, only 25µM induced a significant reduction in levels of p-Akt (Figure 5.6.-A, B). These dose-durations of resveratrol treatment had no effect on relative levels of phosphorylated forms of the MAP kinases ERK1/2 (Appendix II, Figure S.8.A).

5.4. Discussion

5.4.1. Principal Findings

This study presents the first *in vitro* evidence on the actions of resveratrol on angiogenesis in the CNS, and draws useful comparisons with a widely used peripheral model (HUVEC). Resveratrol inhibited vascular endothelial cell proliferation in both hCMEC/D3 cells (derived from human brain primary endothelium) and primary bovine brain derived cultures. Its effects on BrdU incorporation and cell number reduction were only significant for doses in the micromolar range (25, 50, 100µM with variable effects at 10µM, Figure 5.1). Resveratrol also inhibited cell migration and tubule formation (Figures 5.2, 5.4, 5.5). The effect on migration was significant for the same dose range

(10-100μM). Tubule formation in Matrigel™ was also inhibited for 10-100μM, while 50 and 100μM inhibited process formation around explanted rat aortic rings. Lower doses (100nM, 1μM) did not induce significant stimulatory in either central or peripheral endothelium. The mechanisms of action of resveratrol were also explored. The data show significant cytotoxicity for higher doses of resveratrol (50, 100μM), particularly in HUVEC; however, the magnitude does not account for the total reduction in cell number at these doses. There was no evidence for apoptosis, and propidium iodide staining showed that resveratrol (10-100μM) inhibited cell cycle progression, with arrested progression to G2/M for 25 μM (Figure 5.3). Resveratrol (10, 25, 50μM) also inhibited phosphorylation of Akt, with a prolonged effect after 24h treatment with the 25μM dose (Figure 5.6).

5.4.2. The Significance of Angiogenesis

Angiogenesis refers to the formation of new blood vessels from pre-existing vasculature. The process of angiogenesis involves the detachment of pericytes from the capillary wall, allowing endothelial cells to proliferate and invade the surrounding basement membrane and interstitial matrix as sprouts. These migrate outwards, forming tubular structures that eventually canalize and establish new capillary networks (302). The regulation of angiogenesis is tightly controlled by the balance of pro-angiogenic factors (like vascular endothelial growth factor, VEGF) and anti-angiogenic factors (such as angiostatin), and the outward invasion of vascular sprouts depends on matrix metalloproteinases (MMPs). The endothelial processes linked to angiogenesis are regulated by several signaling molecules including the mitogen activated protein (MAP) kinases, phosphatidylinositol 3 (PI3)-kinase and activation of the serine/threonine kinase

Akt/protein kinase B, and through transcription factors such as Nuclear factor kappa B (NF-kB), HIF-1α and AP-1. These regulate multiple cellular processes including proliferation, production of growth factors and proteases and endothelial cell apoptosis (272, 322).

Angiogenesis has beneficial roles in physiology and disease. It is involved in diverse physiological processes including embryogenesis, wound healing and the normal menstrual cycle (1, 20). Angiogenesis is stimulated following ischemic injury to the myocardium or the central nervous system (CNS), in myocardial infarction and stroke, respectively (319, 323). In these contexts, it is believed to restore blood supply to injured tissue, which may promote recovery and protect from additional injury. Angiogenesis may also play a detrimental role in disease. The additional vasculature can facilitate unwanted inflammatory responses, both by generating pro-inflammatory mediators, and by providing routes for the egress of inflammatory cells into the tissues. In the context of the CNS, this may relevant in inflammatory conditions such as meningitis or multiple sclerosis. Cancer cells recruit a chaotic vasculature in comparison to the normal pattern of blood supply. Vessels are not differentiated into arterioles or venules, and blood supply is inefficient, so that tumor tissue is relatively hypoxic (324). The interruption of this vasculature can inhibit cancer growth and metastasis, although many of the agents currently in clinical trials have met with significant drug resistance, since cancer cells develop means of evading the influence of particular factors or the need to depend on a blood supply (325). In this context, the use of phytochemicals such as resveratrol to inhibit cancer associated angiogenesis has been promoted along several lines.

Resveratrol inhibits multiple signaling pathways in cancer cells, and the doses required to inhibit cancer growth in animals are well tolerated (172, 181-184).

5.4.3. Resveratrol and Angiogenesis

The actions of resveratrol on angiogenesis have been studied both in tissue culture and using *ex vivo* explants, and *in vivo* assays. Tissue culture studies have mostly been carried out in large vessel endothelial cells (EC), including human umbilical vein EC (HUVEC) (242-244), bovine pulmonary artery EC (312), bovine aortic EC (310, 313), porcine aortic EC (16) and human coronary arteriolar EC (314). In the majority of these studies, the effect of resveratrol was to inhibit angiogenesis related processes. The effective dose ranges from 1-100μM, and the mechanism of action includes reduced endothelial production of MMP-2 (309), inhibition of endothelial cell binding to the αvβ3 integrin receptor (310), reduced ROS dependent Src-dependent phosphorylation of VE-cadherin (308), reduced phosphorylation of MAP kinases (16), upregulation of p21 causing cell cycle arrest in the G1 phase (312), and reductions in phosphorylation of Akt (181, 311, 326) linked in one study to downstream activation of FOXO transcription factors (326). In exploring actions of resveratrol on cancer associated angiogenesis, resveratrol reduced production of VEGF, MMP-2 and MMP-9 by the myeloma cell line RPMI 8226, and reduced its ability to stimulate *in vitro* angiogenesis in HUVEC (318). Fewer studies have explored the actions of resveratrol on microvascular endothelial cells, although these likely represent the substrate for sprouting angiogenesis *in vivo*. In human microvessel EC, resveratrol inhibited proliferation, and showed activation of elongation factor 2 kinase (315). Resveratrol also inhibits angiogenesis in the *ex vivo* aortic explant assay (309). Several *in vivo* assays, including the corneal micropocket assay,

skin Matrigel™ plug assay and chick chorioallantoic membrane (CAM) assay have also documented an inhibitory effect of resveratrol on angiogenesis (16, 315, 317). Although most of these studies imply that resveratrol inhibits angiogenesis, this may be organ specific. Studies in the heart have shown that resveratrol promotes angiogenesis after myocardial ischemia, and promotes tube formation in human coronary arteriolar EC (314). A recent study also documents a selective effect for 5μM resveratrol, which promoted angiogenesis both in HUVEC and the chick CAM assay, while 20μM had the opposite effect, suggesting some variability in its reported effects in different peripheral assays (311). Its *in vitro* effects include activation of thioredoxin-1, hemoxygenase-1 and VEGF (315). Little is presently known about the actions of resveratrol on angiogenesis in the CNS. In a study of ischemic injury (stroke), mice were given resveratrol orally (50mg/kg/day) at the time of stroke induction (using nylon suture occlusion of the middle cerebral artery followed by reperfusion 2h later) or with a delay of 24h. Mice were treated for 7 days, and assessed for blood vessel density in the vicinity of the stroke. Control mice showed a reduction in cortical vessel counts, however the reduction was mitigated in mice treated with resveratrol on stroke induction. This was associated with tissue production of VEGF and MMP-2 (320). This implies that resveratrol can protect pre-existing vessels from loss as the result of ischemic injury, perhaps by countering vascular endothelial apoptosis, but does not define its independent effects on cerebral angiogenesis. Also, it is significant that delayed administration was ineffective.

5.4.4. Resveratrol Dosage and Metabolism

Dosage is also a critical consideration in these studies. It is difficult to translate the

in vitro findings to the *in vivo* setting (172, 181-184). Resveratrol is extensively metabolized *in vivo* to conjugated forms in the gut and liver. Levels of free resveratrol associated with red wine consumption are in the nM range, while oral administration of higher doses are associated with levels of the order of 1-10μM (176, 327). *In vivo* studies of angiogenesis in post-ischemic limbs have suggested that red wine polyphenols, of which resveratrol is one, may promote angiogenesis at low doses, but inhibit angiogenesis at higher doses (316). In an attempt to address these differences, studies have examined the effects of low (nM) doses of resveratrol in HUVEC. Gene microarray studies using 100nM resveratrol showed induction of endothelial nitric oxide synthase (eNOS) and downregulation of endothelin-1 (183) and other work showed that 50nM resveratrol increases production of NO via activation of eNOS (328). These studies are of interest, because low dose polyphenols also appeared to stimulate angiogenesis via eNOS stimulation (316), and VEGF may induce angiogenesis via NO (329). It is not clear whether nM doses of resveratrol also promote angiogenesis in the *in vitro* models used to date, but we did not detect pro-angiogenic effects for doses as low as 100nM. The requirement for higher doses to see inhibitory effects may also reflect the artificial conditions used. For instance, cell cultures are routinely exposed to hyperoxic conditions (room air) which increase oxidative stress. As resveratrol is a known antioxidant, perhaps higher doses are necessary to reduce this oxidative stress and unmask more typical effects on angiogenesis related processes. Also, many studies deprive cells of serum, and so increase environmental stress through withdrawal of growth factors.

5.4.5. Present Findings

Given the lack of information on the effect of resveratrol on cerebral angiogenesis,

this study was carried out using cultures of human and bovine brain capillary endothelial cells, and comparisons made with HUVEC. We have previously reported the use of the human brain endothelial cell line hCMEC/D3 as a model of cerebral angiogenesis (321). We report that resveratrol inhibits the processes of endothelial proliferation, migration and tube formation in a dose dependent manner, with maximal effects in the micromolar range (10-100μM, Figures 5.1, 5.2, 5.4, and 5.5). Lower doses (100nM, 1μM) did not significantly increase these responses. The effects of resveratrol cannot be entirely attributed either to cytotoxicity or apoptosis. However, cytotoxicity was significant when HUVEC were exposed to 100μM resveratrol (reaching ~ 35%, Figure 5.3). This effect was not assessed in many of the previous studies in HUVEC, and may have confounded some of the previous data. However, although present to a lesser extent in hCMEC/D3, cytotoxicity was absent in BBMEC, suggesting it is not the principal reason for the actions of resveratrol on CNS endothelium. Instead, resveratrol acts by reducing cell cycle progression (Figure 5.3) and is associated with reduced phosphorylation of the signaling molecule Akt (Figure 5.6). In our hands, in hCMEC/D3 cells, resveratrol had no effect on phosphorylation of MAP kinases (ERK1/2, p38 and SAPK/JNK; Appendix II Figure S8). This is similar to reports in HUVEC, in which resveratrol inhibited Akt phosphorylation at either 20μM (311) or at 10 and 25μM (181). The same doses either had no effect on ERK phosphorylation (181) or showed an inhibitory effect ((311), using 20μM). Lower doses (5μM, (311); 0.5μM, (181)) were reported to increase phosphorylation of both Akt and ERK, consistent with a selective pro-angiogenic effect (311), however we did not examine doses lower than 10μM in this study. The same dose range (0.5-25μM, (181)) had no effect on JNK phosphorylation in HUVEC. The actions of

resveratrol on Akt phosphorylation have been studied in a variety of cell types. Resveratrol inhibits Akt phosphorylation in vascular smooth muscle cells, with reduced hypertrophy induced by angiotensin II or epidermal growth factor (330). It also inhibits Akt phosphorylation in a variety of cancer cells (prostate, liver, glioma) (331-333). Downstream effects include reduced mTOR signaling and enhanced apoptosis of glioma cells (331). By contrast, resveratrol increases Akt phosphorylation in hepatic cells after traumatic injury, where it induces upregulation of hemoxygenase-1 as a protective response (334). Akt is upregulated in diabetic myocardial cells by resveratrol, leading to nitric oxide induced glucose uptake via the GLUT-4 transporter (335). The role of Akt is multipotent, but includes the regulation of cell cycle progression, whose effects have a major impact in a variety of CNS diseases such as stroke and Alzheimer's disease (336-338). The potential impact of resveratrol on these diseases is therefore wider than its effects on angiogenesis. However, the present study implies that resveratrol is inhibitory for angiogenesis in the CNS, unlike its distinct pro-angiogenic effect on the myocardium, or possibly in HUVEC at lower doses. As such, it may be effective against certain inflammatory diseases that could be exacerbated by angiogenesis. For instance, resveratrol was effective in reducing the severity of experimental allergic encephalomyelitis, a murine model of multiple sclerosis (339). At high doses *in vivo*, it was also effective in reducing blood vessel density in implanted rat gliomas (228). The high doses used to inhibit these processes *in vitro* would probably not correlate to levels of free resveratrol present as the result of red wine consumption, but begin to be significant if oral (pharmacological) dosing with higher doses of resveratrol is taken into account. In one study, resveratrol reached peak serum levels of around 5μM (340),

similar to the effective doses used in this and other studies. It is also likely that *in vitro* dosing has a poor correlation to *in vivo* levels.

5.5. Conclusion

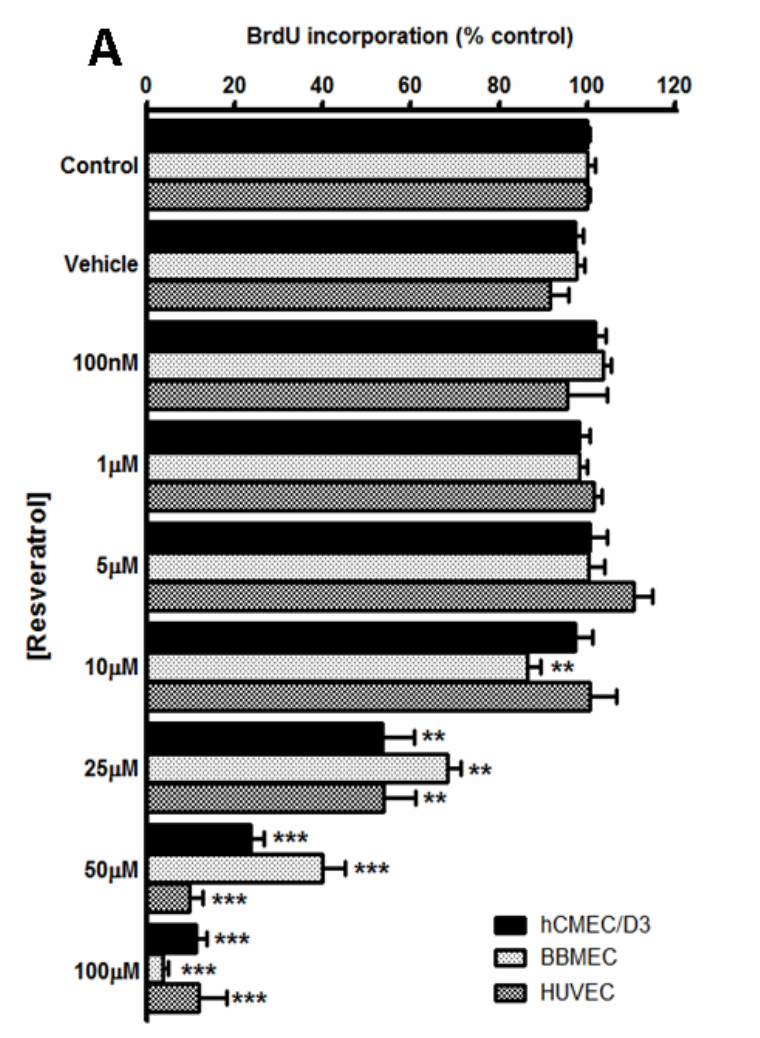
We present the first report on the effects of resveratrol on *in vitro* processes linked to angiogenesis in the CNS. Resveratrol has a broad inhibitory effect, when applied in the micromolar range. The protective effects of resveratrol on the cardiovascular system, and in stroke, needs to be balanced against its possible inhibitory effect on angiogenesis. Dosage, timing of administration and context are likely to prove critical in assessing the use of resveratrol as a therapy for diseases of the CNS. However, resveratrol may play a useful role as adjunctive therapy in CNS inflammatory diseases such as multiple sclerosis, where inhibition of angiogenesis might be beneficial.

5.6. Acknowledgement

This work was funded by the Nova Scotia Health Research Foundation (grant to AE). P.-L.C. was funded by a George Loh Scholarship from the Department of Pathology, Dalhousie University.

Figure 5.1. Impact of Resveratrol on BrdU Incorporation and Cell Number.

Cells were treated for 24h in complete medium (with serum, Control), with vehicle (0.1% DMSO) or resveratrol at indicated doses. Effects of resveratrol on cell proliferation (DNA synthesis) assessed by BrdU incorporation (A) or cell number analyzed by MTT assay (B). Data are normalized to the medium only control and shown as mean ± standard error of the mean (SEM). Significance is compared to the vehicle control; only significant differences are marked (*P<0.05, **P<0.01, ***P<0.001). In A, n=10-25 for control, 4-11 for vehicle, and 3-15 for resveratrol. In B, n=11-29 for control, 6-8 for vehicle and 3-19 for resveratrol. This indicates replicate experiments; each experiment pools data from 4 wells per treatment.


A
BrdU incorporation (% control)
0
20
40
60
80
100
120
[Resveratrol]
Control
Vehicle
100nM
1µM
5µM
10µM
25µM
50µM
100µM
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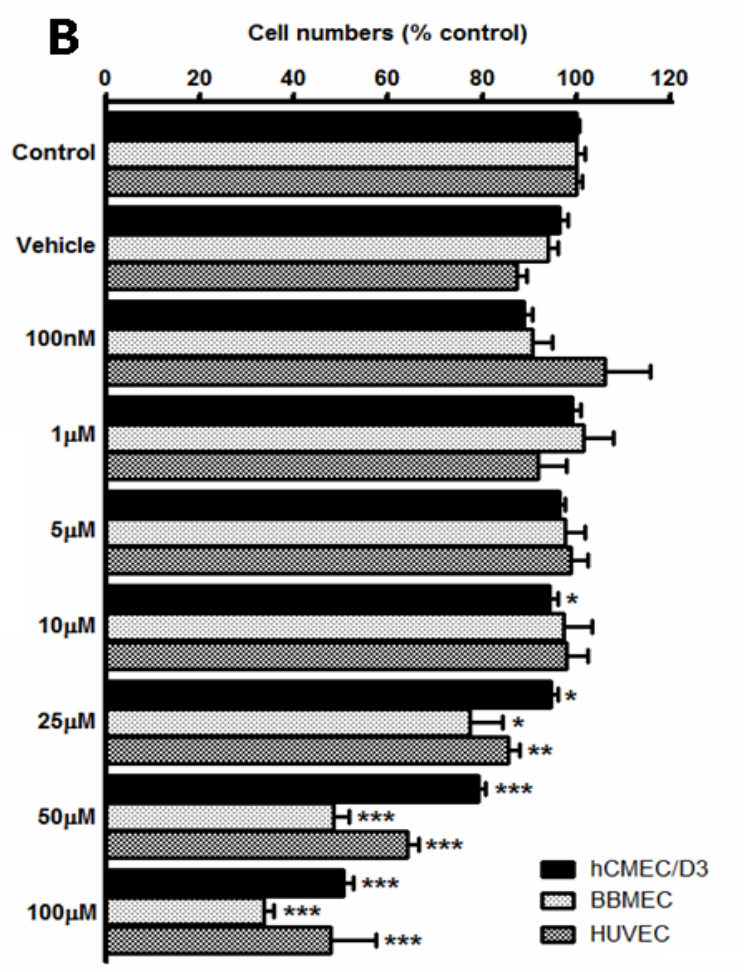
hCMEC/D3
BBMEC
HUVEC

Figure 5.1.


B
Cell numbers (% control)
0
20
40
60
80
100
120
Control
Vehicle
100nM
1µM
5µM
10µM
25µM
50µM
100µM
*
*
**
***
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***
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***
hCMEC/D3
BBMEC
HUVEC

Figure 5.1.

Figure 5.2. Effect of Resveratrol on Migration Assessed by Scratch Assay.

Cells were treated for 24h with medium (Control), 0.1% DMSO (Vehicle) or indicated doses of resveratrol. A. Shows representative images of the denuded zone, with filling after 24h in control wells. Increases in resveratrol dosage reduce the number of cells entering the zone. Images were taken at 20x magnification (scale bar = 500 µm) B. Summarizes the data, expressed as mean ± SEM, normalized to the control. Significance is compared to vehicle; only significant differences are marked (*P<0.05, **P<0.01, ***P<0.001). In B, n=4-22 for control, 4-10 for vehicle, and 4-14 for resveratrol. This indicates replicate experiments; each experiment pools data from 4 wells per treatment.

hCMEC/D3

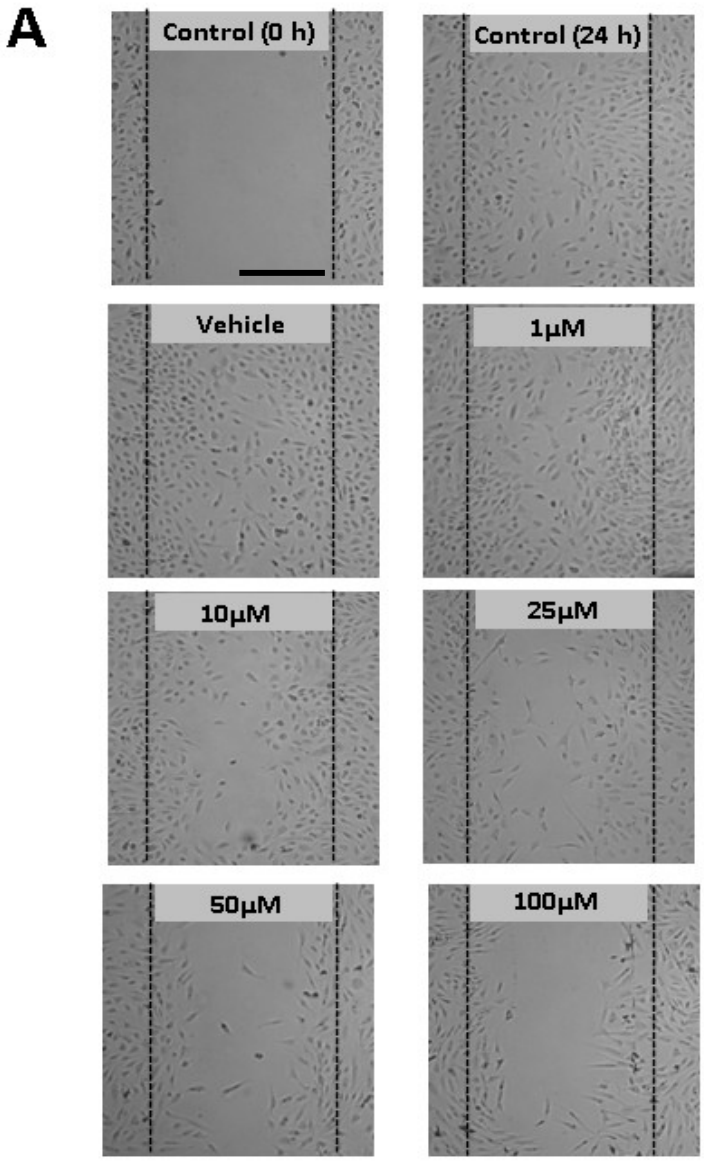
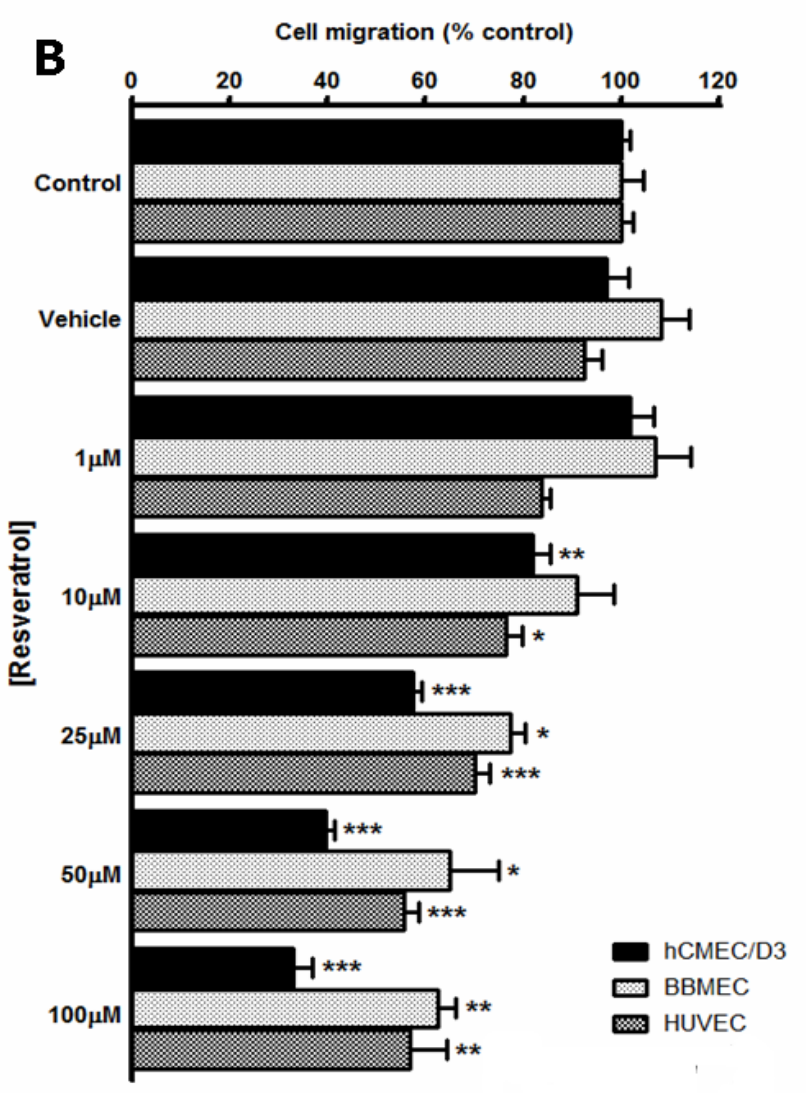


Figure 5.2.


B
Cell migration (% control)
0
20
40
60
80
100
120
Control
Vehicle
1µM
10µM
25µM
50µM
100µM
[Resveratrol]
**
*
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*
***
***
*
***
***
**
**
hCMEC/D3
BBMEC
HUVEC

Figure 5.2.

Figure 5.3. Possible Mechanisms of Action of Resveratrol.

A. Cytotoxicity assessed from LDH release, expressed as % maximal release from lysed cells. Cells were treated for 24h with vehicle (0.1% DMSO) or indicated doses of resveratrol. B. Apoptosis assessed from caspase-3 activity assay. hCMEC/D3 were treated for indicated times with TRAIL (100ng/ml, positive control), vehicle or resveratrol at indicated doses. C. Representative scatter plots showing content of propidium iodide. hCMEC/D3 cells have been allotted to different phases of the cell cycle as indicated. D. Summary data for proportion of cells in each phase of the cell cycle showing the effect of vehicle (V) or indicated doses of resveratrol on hCMEC/D3 cells. In each plot, data is mean ± SEM, and significance is calculated relative to the vehicle control. Only significant results are marked (*P<0.05, ***P<0.001). In A, n=8 (vehicle) or 16 (resveratrol, each dose). In B, n=3 per treatment. In D, n=10-13 for each bar.

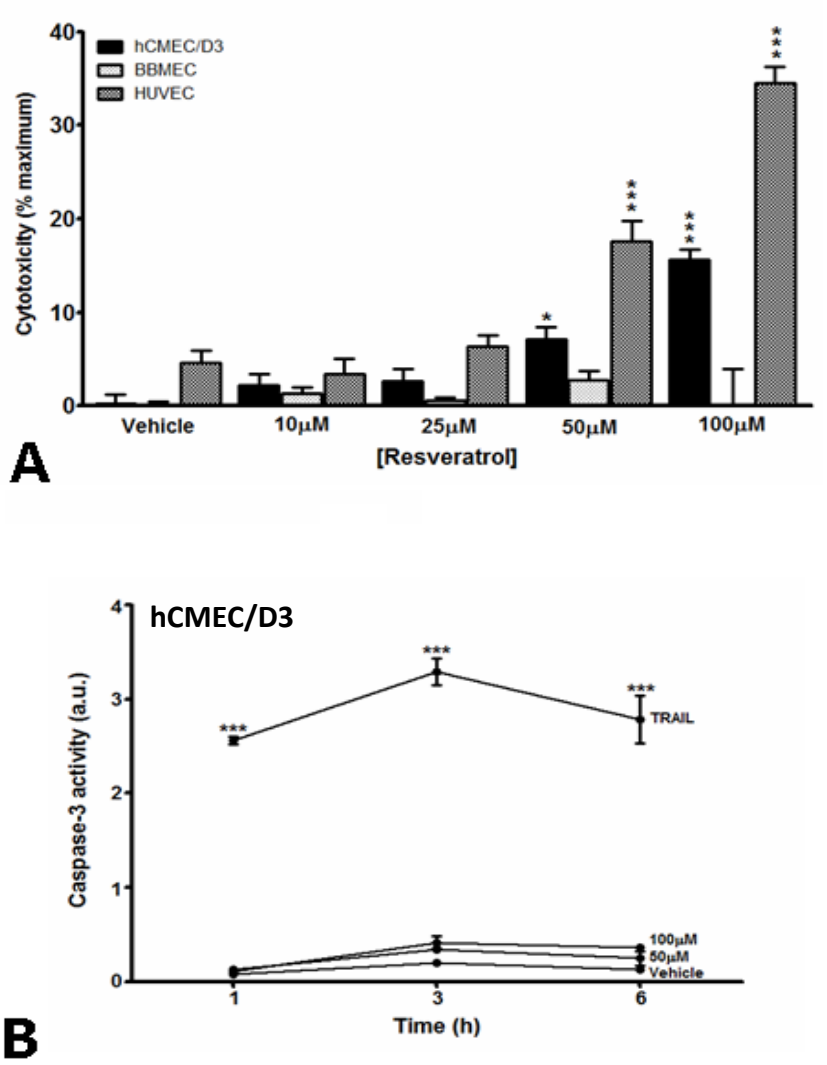
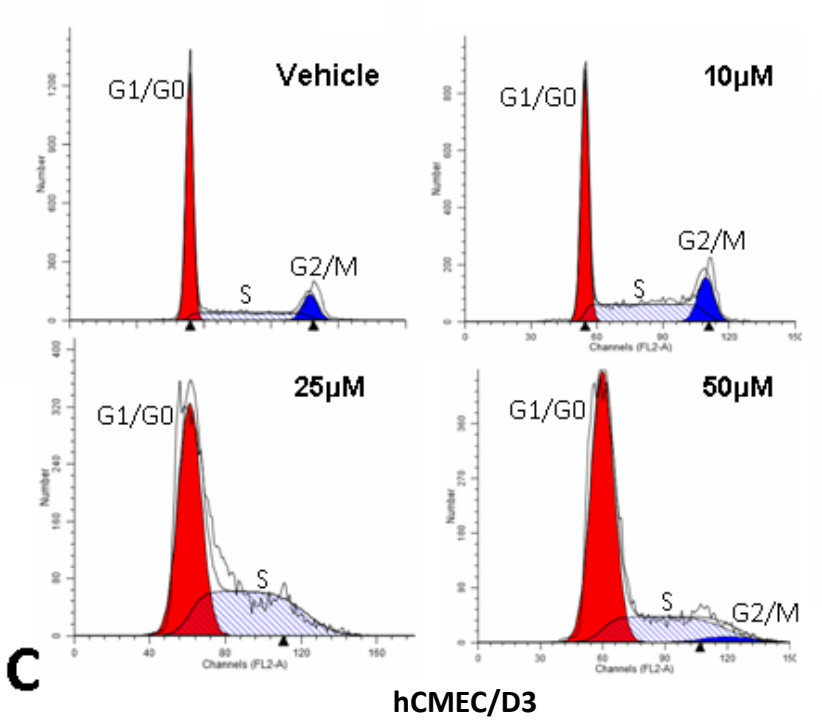
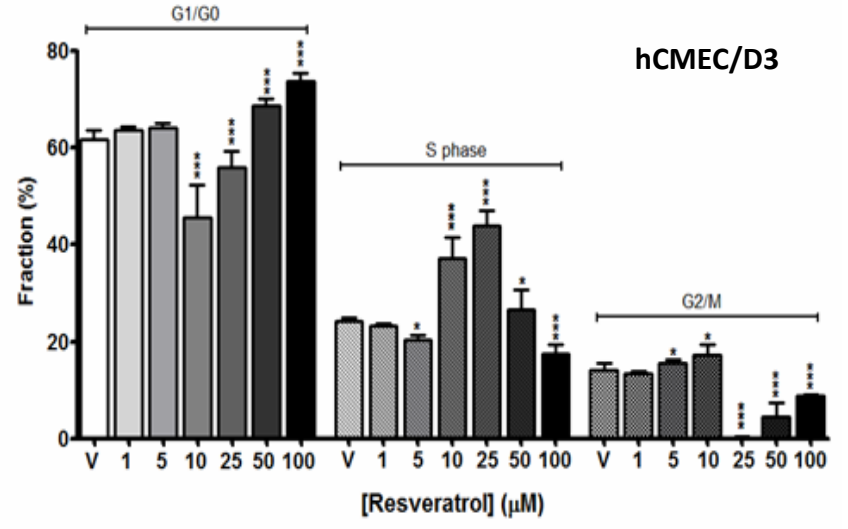

A
hCMEC/D3
BBMEC
HUVEC
Cytotoxicity (% maximum)
40
30
20
10
0
Vehicle
10μM
25μM
50μM
100μM
[Resveratrol]
B
hCMEC/D3
Caspase-3 activity (a.u.)
4
3
2
1
0
TRAIL
100μM
50μM
Vehicle
1
3
6
Time (h)

Figure 5.3.



C

hCMEC/D3



D

Figure 5.3.

Figure 5.4. Tubule Formation Assay on Matrigel™ in hCMEC/D3 Cells Treated with Vehicle or Indicated Doses of Resveratrol.

A. Shows representative images of the networks formed after 16h, with a reduction in tubule length for 10 and 100μM resveratrol. Images were taken at 20x magnification (scale bar = 500 μm)B. Shows summary data for average tubule length at 4 and 16h, expressed as mean ± SEM. Data is normalized to the vehicle treated wells at 4h. Significance is relative to vehicle at each time point; only significant results are marked (*P<0.05, **P<0.01, ***P<0.001). N=6 (vehicle) and 9 for each treatment group. Vehicle, 0.1%DMSO.

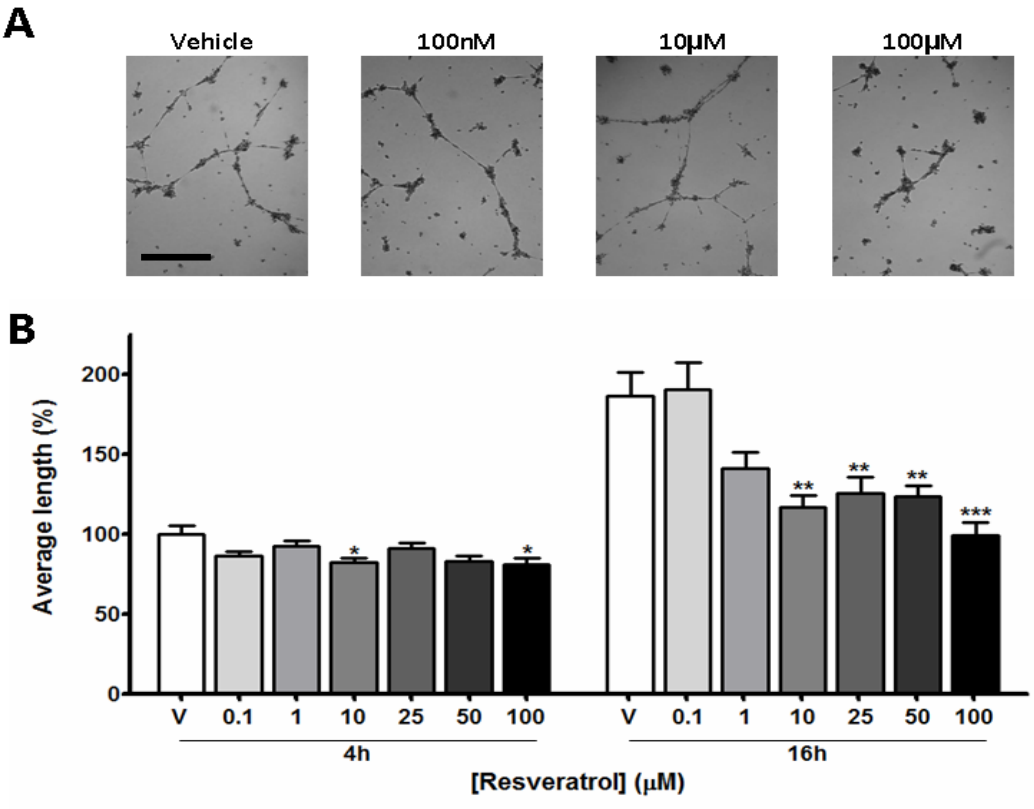

A
Vehicle
100nM
10µM
100µM
B
Average length (%)
200
150
100
50
0
V
0.1
1
10
25
50
100
4h
16h
[Resveratrol] (µM)
*
**
***

Figure 5.4.

Figure 5.5. *Ex Vivo* Angiogenesis Assay using Explanted Rat Aortic Rings.

A. Shows representative images of the network forming around explants treated with vehicle (0.1% DMSO) or indicated doses of resveratrol after 5 days. The network is delimited by a dashed line superimposed on the images. Images were taken at 4x magnification (scale bar= 500 µm) B. Summary data showing network area normalized to the area of the explant as a function of time in days. Data is expressed as mean ± SEM for indicated treatments, and significance shown relative to vehicle; only significant results are marked (*P<0.05, **P<0.01). For each group, data is taken from 3-5 separate explants.

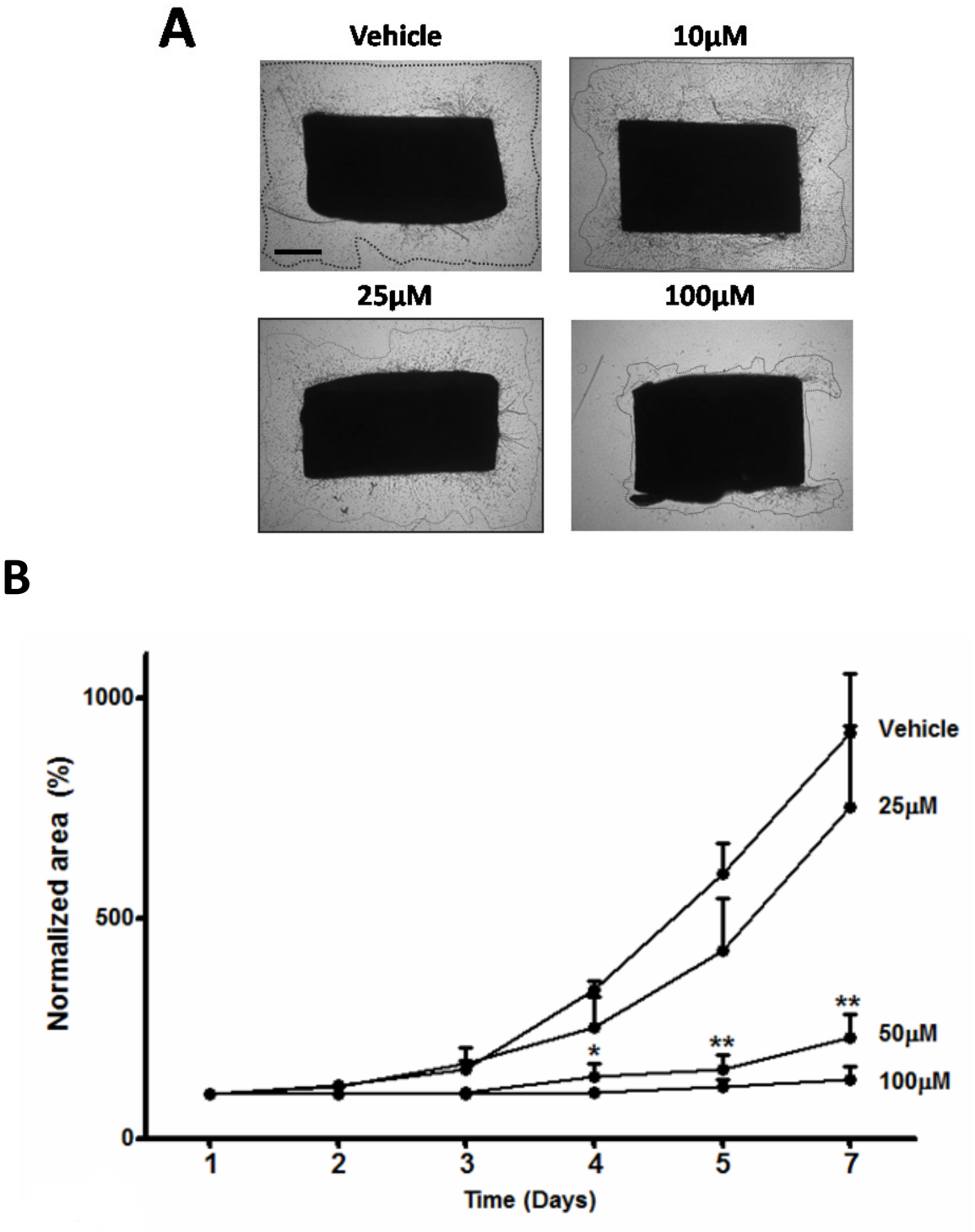

A
Vehicle
10µM
25µM
100µM
B
Normalized area (%)
1000
500
0
1
2
3
4
5
7
Time (Days)
Vehicle
25µM
50µM
100µM
*
**
**

Figure 5.5.

Figure 5.6. Resveratrol Induces a Reduction in the Phosphorylated Form of Akt Relative to Total Akt Levels by Western Blot on hCMEC/D3 Cells.

A. Representative blots showing effect of different treatment durations (15min, 1h, 24h) with vehicle (0.1% DMSO) or indicated doses of resveratrol (10, 25, 50µM). V: vehicle control. B. Summary data obtained by densitometry for 3-4 blots per treatment. Data is expressed as mean ± SEM and has been normalized to the vehicle treated group at each time point. Significance is relative to the vehicle, and only significant results are shown (*P<0.05, **P<0.01). p-Akt, phosphorylated form of Akt. β-actin was used as a loading control.

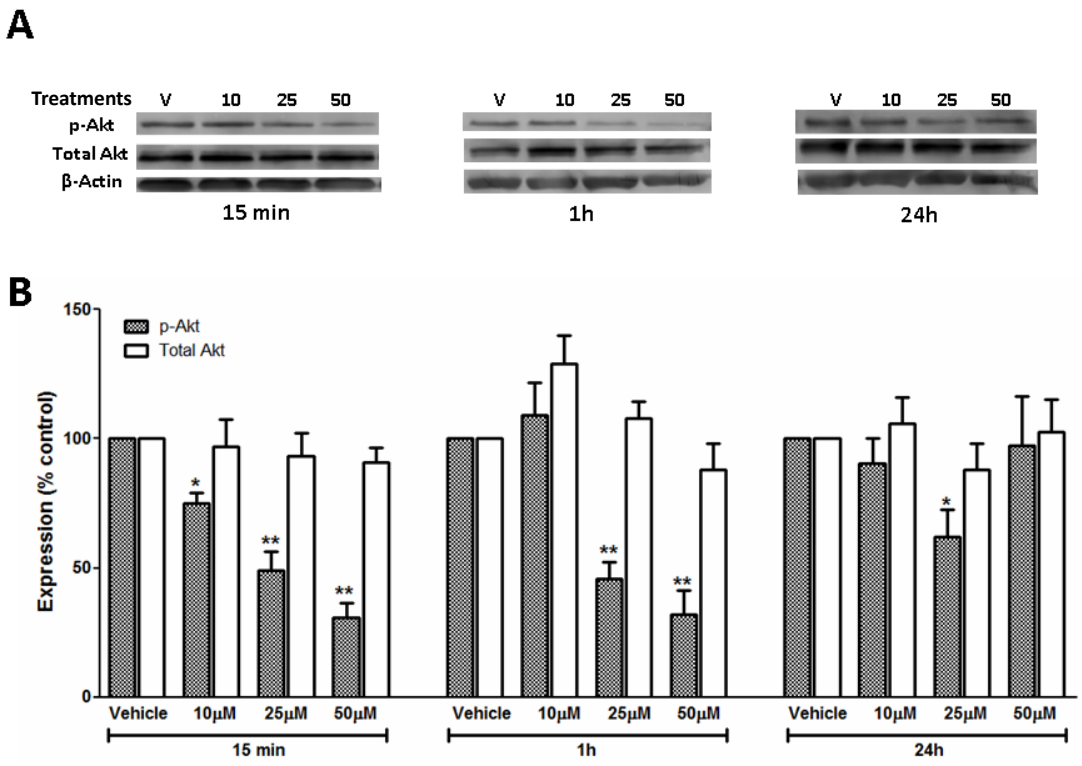

A
Treatments
V
10
25
50
p-Akt
Total Akt
β-Actin
15 min
1h
24h
B
p-Akt
Total Akt
Expression (% control)
150
100
50
0
*
**
**
**
**
*
Vehicle
10μM
25μM
50μM
15 min
1h
24h

Figure 5.6.

CHAPTER 6 – DISCUSSION AND CONCLUSIONS

6.1. Overview and Principal Findings

The research outlined in this thesis is focused on the modulatory molecules that underlie the cross talk between inflammation and angiogenesis, especially in the CNS, with a focus on regulation of vessel regression as a way of limiting angiogenesis. Molecules from both endogenous and exogenous sources were examined for their ability to regulate vascular endothelial activation, as well as *in vitro* surrogates for vessel formation and regression. The purpose of the study is to gain an insight into the basic mechanisms that regulate these vascular responses, with an ultimate aim of establishing rationales for potential clinical use in limiting angiogenesis.

The endogenous molecules chosen in this study were TRAIL and FasL from the TNF cytokine superfamily. These factors were chosen because we hypothesized that death ligands could regulate vessel regression by inducing endothelial apoptosis. In this way, they form a link between the immune system and angiogenesis. The literature shows that TNF is a key factor in bridging the processes of inflammation and angiogenesis (39, 117, 119). The role of other members from the same family in the link is not clear, despite the knowledge that levels of these molecules are elevated in many inflammatory conditions and immune diseases.

In addition, an exogenous agent was also included because modulation of endogenous molecules could have adverse physiological effects, particularly over the long term. For example, TNF plays a crucial role in cancer and autoimmune diseases and anti-TNF therapy is used currently to treat these conditions (131). However, TNF is also required for the proper function of the immune system; the anti-TNF treatment can

therefore be harmful and lead to immune dysregulation (131). For this reason, phytochemicals derived from fruits and vegetables that are safe to consume might be good therapeutic alternatives, with all the benefits attributed to anti-TNF and other immune modulating therapies. We chose to study resveratrol as the literature shows its potential modulatory role in peripheral endothelium and relatively low toxicity (308, 315). Specifically we hypothesized resveratrol would inhibit cerebral angiogenesis as this has not been previously examined.

Another fundamental aim of this research was to incorporate both commonly used primary human and animal endothelial cell models and to compare these to immortalized cell lines of both peripheral and CNS origin. This can assist in interpretation of data in the literature as many CNS related studies rely on data obtained from peripheral large vessel endothelial cells to reach their conclusions.

The first part of the work (chapter 3) investigated the role of TRAIL and FasL in both endothelial activation and apoptosis. This study showed that FasL and TRAIL did not induce or activate normal human endothelial cells. However, when endothelial cells were sensitized to undergo TRAIL induced apoptosis, TRAIL was able to activate cells simultaneously and promote neutrophil adhesion. This implied that process of vessel regression is enhanced by the direct interactions between endothelial cells and immune effector cells (neutrophils). One way by which neutrophils might induce regression is through production of death ligands (341, 342). Therefore, the second part of the work (chapter 4) focused on the impact of the death ligands, TRAIL and FasL, in the multistep processes of angiogenesis. While these ligand showed modulatory effect on the human peripheral endothelial cell model (HUVEC), they had a significant inhibitory impact on a

human brain endothelial cell model (hCMEC/D3) by inducing apoptosis. The work was finally directed to exploring the effects of resveratrol on cerebral angiogenesis related processes since this had not been previously studied. It was found that resveratrol had anti-angiogenic properties in both primary (BBMEC) and immortalized (hCEMC/D3) brain endothelial cell models as well as primary human peripheral endothelial cells (HUVEC). However, resveratrol does not induce apoptosis but instead inhibits endothelial proliferation. This emphasizes that vessel regression can be controlled by different mechanisms.

6.2. General Direction of the Work

As the work progressed, it became clear that there were limitations in examining how TRAIL and FasL were able to stimulate neutrophil adhesion in endothelial models *in vitro*. Although, positive data was obtained in a HUVEC derived cell line (EA.hy926) it became clear that similar results were not seen in primary HUVEC, or in hCMEC/D3. This emphasizes the limitations of EA.hy926 cells as a model (discussed more fully in chapter 3). When we examined hCMEC/D3 cells, we found that TRAIL treatment did not result in changes in ICAM-1 expression (see Appendix II, Figure S.5). This was despite its ability to induce apoptosis in the cell line, as shown in chapter 4. Also, we examined neutrophil adhesion in the cells in response to TRAIL, but could not obtain consistent results. Given these limitations, we decided to examine the literature on angiogenesis, since this is an important aspect of endothelial activation, and one in which apoptosis may play a key inhibitory role. We discovered that the role of TRAIL and FasL was confusing (281, 282), which opened up new avenues for us to explore. The research group has a longstanding

interest in cerebrovascular endothelial function, and the blood-brain barrier (244, 343-351). Therefore, we were interested in exploring this using appropriate cell cultures. However, our initial attempts to culture primary brain endothelium from a variety of sources (human, bovine, porcine, rat, and mice) were unsuccessful. Another factor to consider is the difference in response between cells of human versus laboratory animal origin. Also, reagents such as antibodies and the specificity of different ligands is often not adapted to animal models. Sources of primary human brain endothelial cells have become extremely difficult to access, however, the hCMEC/D3 cell line had been described in 2005 (233) and were made widely available to other researchers. We were able to obtain these in 2006 from Dr. Weksler's group. This prompted us to move into studies in this cell line, both to examine cerebral function in a human derived cell, and to begin studies in a relatively novel model.

6.3. Limitations and Future Directions

In the following sections, the rationale of choosing the models and methods used, their limitations, the strengths and weaknesses, and the challenges encountered are discussed. In addition, alternative or additional methods that complement the current findings are provided as future work.

6.3.1. Use of the hCMEC/D3 Cell Line

hCMEC/D3 is an immortalized cell line that has been genetically engineered to possess the ability to divide indefinitely, theoretically, without changing its parental phenotype. Primary cell cultures are generally believed to be a more physiological model system than cell lines. However, after cells are extracted, the supporting cells and tissues

are separated from the cells of interest and this causes them to lose factors necessary to maintain their characteristics. For example, after several passages, brain endothelial cells show an increase in permeability (352), a reduction in levels of transporters and enzymes (353), and they alter their phenotype markedly on exposure to astrocyte secreted factors (354-356). However, cell lines tend to be more stable, and less dependent on these variables. The type of cell line also varies in its capacity to express different characteristics. For example, permeability across hCMEC/D3 is much lower than permeability across *in situ* endothelium or some primary culture models (28, 233).

The complexity of CNS endothelium is particularly challenging to represent *in vitro* (357, 358). However, there are several reports on hCMEC/D3 suggesting that it can represent different aspects of cerebral function. These include studies of permeability (233, 355, 359, 360), tight junction biology (233, 361, 362), cytokine function (233, 363), transporter function (364-367), but no studies of angiogenesis to date. Another critique of using hCMEC/D3 cells is that the blood-brain barrier depends on interactions with surrounding cells, so that monocultures may lose certain characteristics. To this end, other studies have tried to mimic these conditions by using co-culture of endothelial cells with other cells such as astrocytes (354, 355, 357, 358) or pericytes (357, 368). In addition, shear stress due to blood flow is also present *in vivo*, and some groups have cultured hCMEC/D3 cells in the presence of shear stress (the "dynamic *in vivo*" flow based conditions) and found reductions in permeability (tightening the barrier)(355) as a result (355). To what extent this limits our modeling of angiogenesis is open to question. We were unable to confirm the results in a primary human brain endothelial cell culture. As well, *in vivo* experiments would be necessary to verify the findings because a

reductionist approach was utilized throughout this study (since one cell type and one modulatory agent were examined at a time).

6.3.2. Limitations of Using Large Vessel Endothelial Cells

HUVEC is one of the easiest sources of human endothelial cells to obtain and the most popular human endothelial cell model used *in vitro*. They are relatively easy to maintain compared to microvascular endothelial cells isolated from adult skin or lung. However, HUVEC is of large vessel origin, while many of the vascular responses associated with angiogenesis and inflammation occur in microvascular endothelium (369-372). There is also a question of how representative HUVEC is of function in mature endothelial cells, since they are derived from the umbilical cord, where their function is limited in duration. Although HUVEC possess many endothelial cell specific properties, evidence shows that endothelial cells from different vascular beds display diversity at morphological, functional, biochemical, and molecular levels (369, 370, 372). To address the drawback of using HUVEC, we attempted studies in microvascular endothelium from a peripheral source (dermal HMVEC). Data on the effect of resveratrol and TNF family ligands on these cells is shown in Appendix II, Figure S.6. In general, the results parallel those observed in other primary cultures.

6.3.3. Lack of Response to Growth Factors

A puzzling aspect of the response of hCMEC/D3 cells was their general lack of responsiveness to a variety of pro-angiogenic growth factors. As an angiogenesis related study, growth factors are used as a positive control. Also, we were interested to examine responses to VEGF, because the $VEGF_{165}$ isoform is most often implicated in cerebral angiogenesis (116, 373). For instance, in human autopsy brains after stroke, there is

upregulation of VEGF at 3-4 days at the edge of the infarct which coincides with an increase in new vessel formation (374, 375). VEGF is expressed by choroid plexus, astrocytes and neurons (116). On brain endothelial cells, there are two known VEGF receptors, R1 and R2, and interaction with R2 mediates angiogenesis (116, 376). Expression of R2 is increased by ischemia at the infarct margin (377, 378). R1 acts as a decoy receptor that can bind to VEGF and prevent it from binding to R2 (379-381). Binding of VEGF is enhanced by neuropilins and heparin as a co-factor (376, 382, 383). We showed that hCMEC/D3 express both VEGF-R1 and VEGF-R2 (Appendix II, Figure S.1).

Within the limits of our assay, we were not able to demonstrate responses to most of the growth factors that were tested. Thus, treatment of cells for 24h with VEGF in medium lacking serum or in the presence of 5% FBS did not increase cell uptake of BrdU, cell numbers, cell migration or tube formation (Appendix II, Figures S.2, S.3, and S.4). There was a small but significant increase in BrdU uptake in response to PDGF, but EGF, NGF and bFGF were similarly ineffective (Figure S.2). The cells did however show a marked proliferative response when placed in 5% FBS comparing to those in no serum medium. Since heparin can act as a co-factor for VEGF responses (384), we also combined VEGF with heparin but this did not result in significant increases in BrdU uptake or cell proliferation (Figures S.2 and S.3). As a positive control, we repeated these assays in HUVEC, which responded with significant increases in BrdU incorporation on treatment with the growth factors (Figure 4.1).

The literature on the actions of VEGF includes many studies in HUVEC (282, 385, 386) but there are relatively few studies of the actions of VEGF on brain endothelial cells, either of human or animal origin. Zhao *et al.* (387) reported that VEGF induced a

significant increase in tube formation by human brain primary endothelial cells. The ligand was applied for 2h without serum, but tube formation was assessed in medium containing 3% serum for the next 24h. There was a significant increase in tube formation in human primaries but this was significantly enhanced in the cells obtained from the cavernous malformations. Chow *et al.* (388) reported the effect of VEGF in the rat brain derived endothelial cell line RBE4. In the presence of 10% FBS, VEGF promoted tube formation after treatment for 6 days, but this was only significant with 0.01 and 0.1ng/ml but not with higher doses (1, 10, 20ng/ml). In experiments that studied changes in permeability after treatment with VEGF in porcine brain EC (389), VEGF had no effect on permeability unless the cells were exposed to hypoxia or antioxidants. Taken together, these studies suggest that the response to VEGF depends on activation of the endothelium (e.g. by hypoxia) and that other growth factors (present in serum) may need to be present for VEGF to induce a response. This was the case in HUVEC, but we did not see responses in our serum treated cells. However, it is possible that the effect of many growth factors masks an additive effect of VEGF under these circumstances. Perhaps additional activation, or other durations of treatment would be required to induce a response. However, given the lack of response, it may be difficult to use hCMEC/D3 in order to model pro-angiogenic effects, since positive control data is not easy to obtain. This may reflect the maximal growth rate of immortalized cell lines. Hence we tried to render the cells quiescent using serum deprivation with 2% BSA, but under these conditions, the cells also failed to respond to growth factor stimulation. (Appendix II, Figure S.2, S.3, and S.4) In addition, we tested human VEGF and bFGF in bovine primary brain EC, and did not see responses (Appendix II, Figure S.7). However,

this could reflect a species difference, and so still requires further investigation.

6.3.4. Limitations of Activation and Apoptosis Assays

Although neutrophil adhesion assays are in common use to assess endothelial cell activation, there are several limitations. For instance, as mentioned, the use of shear stress during the assay would make it more physiological (390, 391). Also, we could have examined a more extensive time course for adhesion molecule expression, since these can vary considerably between different types of adhesion molecules. As for the approach to sensitize cells to apoptosis, LY294002 is not an entirely specific inhibitor of PI3 kinase, so its ability to sensitize cells could have been complemented by using more specific inhibitors or knockdown experiments with siRNA.

Expression of the FasL, TRAIL and their receptors was examined by flow cytometry. Flow cytometry was exclusively relied upon to assess expression of cell surface proteins. However, additional confirmation with Western blot analysis or cell surface ELISA would be preferable, especially where negative data was obtained. For instance, there are some reports of FasL expression on the surface of endothelial cells (158, 392), an observation we were not able to confirm. In order to control for the possibility that trypsinization cleaves FasL from the cell surface, a control was carried out using a non-enzymatic cell dissociation solution. However, this treatment did not alter the results (data not shown). Since FasL did not result in apoptosis, an additional control was carried out in which FasL induced apoptosis in Jurkat cells (Dr. A Issekutz personal communication). In addition, it would also have been preferable to confirm the presence of apoptosis using more than one assay in each cell type (e.g. combining an annexin

V-FITC method with a caspase-3 activity measurement and single stranded DNA analysis).

6.3.5. Limitations of Angiogenesis Assays

We are also aware of several limitations of modeling angiogenesis *in vitro*. In many of these assays, cell numbers are assessed without knowledge of the underlying processes, and there is considerable overlap in effect. For instance, a reduction in BrdU uptake or MTT can either reflect increased cell death (apoptotic or necrotic) or reduced proliferation. However, we did attempt to quantify the relative contribution of each of these processes. Many of the assays involve treating cells when they are subconfluent, in order to see proliferative effects, but this may not reflect responses in a mature culture, where cells are contact inhibited and therefore less likely to proliferate or undergo apoptosis. Moreover, the substratum used (collagen) was not varied in the experiments, although this may be an important variable in determining endothelial responses (368, 393). For instance, the substratum varies secretion of biomolecules (393). Collagen has particular molecular domains are known to alter endothelial proliferation, such as the alpha2(IV)NC1 domain (394). Also, particular extracellular matrix proteins such as fibronectin influence spreading and proliferation by altering integrin clustering on endothelial cells (395).

The migration assay is also limited because it does not distinguish between reduced migration versus reduced proliferation, since fewer cells may migrate into the artificial wound simply due to a reduction in available cell numbers. This could be addressed by using transmigration assays across simple filters or membranes. We attempted one such assay, in which we placed endothelial cells on Matrigel™ coated filters (a Boyden

chamber based assay) and counted the number of cells migrated through Matrigel™ during an incubation period of 4-48h. Due to the low invasive properties of the endothelial cells, differences in the number of migrated cells between treatments were too small to be statistically significant. These assays are often used to assess migration of cancer cells, but may be less useful for examining endothelial cells.

More difficulties were encountered when we attempted to assess tubule formation on Matrigel™. Many studies rely on simplified images of tube networks on Matrigel™ to demonstrate pro- or anti-angiogenic effects. However, we found that these networks were highly dependent on different variables, including thickness of Matrigel™, seeding density, duration, presence or absence of serum, pre-treatment of cells before application, degree of trypsinization, digital image quality, etc. For instance, after cells form solid networks (due to higher seeding density) they become more resistant to inhibitory treatments. When we included serum in our experiments (as a control), this promoted tube formation in the experiments shown in chapter 4, but in later work, serum appeared to be inhibitory. The reasons for this are unclear, but may be due to different batches of serum. Also, we faced difficulties in extracting meaningful data from a series of images. It was not clear how to relate the networks to the formation of a mature vascular plexus. Should we measure tube length, tube area, tube numbers, tube diameter etc? Studies have explored the use of image processing techniques looking at such parameters as vessel length, number of branch points and vessel densities after skeletonising and manipulating images of vessel networks in the chick CAM assay (396). This complexity is not usually reflected in most research reports. To further establish our effects, data could have been included with inhibitors of cytoskeletal proteins, such as

cytochalasin D (397), to determine their ability to inhibit migration. This could determine the relative contribution of migration and proliferation in the scratch assay. We also attempted to validate our findings using an *ex vivo* angiogenesis assay (rat aorta). However, this assay also has limitations, in that the tubules forming around the explants may not be exclusively endothelial in origin. For instance, it has been reported that smooth muscle cells as well as endothelial cells can grow out of the explants depending on the culture conditions (398). We could have done additional staining to confirm the endothelial nature of these tubes, such as Factor VIII and CD31. Furthermore, we could have used other *in vivo* assays of angiogenesis, such as the chick chorioallantoic membrane assay, and retinal angiogenesis assay. Lastly, to complement our angiogenesis work, we could also have studied expression of other modulators of angiogenesis such as MMPs.

In studying the signal transduction of resveratrol, we performed Western blot analysis for a variety of proteins in the MAP kinase family as well as NF-κB. Previous work has shown that resveratrol only inhibits NF-κB in HUVEC after overnight treatment but has no acute effect (399). Since this reduces phosphorylation of NF-κB subunits (p50/p65), we examined relative phosphorlation of p65 in hCMEC/D3 cells but found no effect of prolonged treatment (24 hours) (See Appendix II, Figure S.8-A, NF-κB; Figure S.8-B, ERK1/2). In many cases, resveratrol appeared to reduce the levels of absolute as well as phosphorylated forms of the proteins, so that absolute reductions were not seen. Also, there was some variability in response. Of all the proteins we studied, Akt was the most consistent. Cell cycle analysis was also limited by the inability to separate phases of the cell cycle with propidium iodide staining. For instance, we could not distinguish G0

from G1 or G2 from M phase. Additional work would be necessary to define this in greater detail, such as by using inhibitors or detecting the levels of cell cycle factors such as cyclins, cyclin-dependent kinases (CDKs), CDK regulatory proteins, cell division control (cdc) proteins, and Rb (retinoblastoma) by Western blot.

6.4. Future Directions

Many of the anti-inflammatory aspects of resveratrol remain to be studied. This includes its possible inhibitory effects on adhesion molecule expression in endothelial cells, and therefore effects on leukocyte adhesion and migration. Preliminary work shows that pre-treatment of hCMEC/D3 cells with resveratrol (50, 100μM) reduces the expression of ICAM-1 induced by TNF (see Appendix II, Figure S.9). This is similar to what was reported to human coronary arterial endothelial cells where resveratrol inhibited NF-κB induced expression of ICAM-1, VCAM, IL-6 and NOS (400). It would be interesting to study a range of adhesion molecules and chemokine expression in different *in vitro* models, and perhaps to study the interaction of other leucocytes, such as T cells, B cells or monocytes. Other aspects of interest include the antioxidant properties of resveratrol and its effects on vascular permeability.

To validate much of the *in vitro* work it would be necessary to carry out studies in animal models. Neutrophil migration can be assessed in different ways, for example by introducing chemokines or cytokines into the peritoneum and enumerating of migrating cells. In addition, experiments can be carried out at the single microvessel level, using both cerebral and peripheral sites. For instance, intravital microscopy can be used to image leukocyte interactions with microvessels on the brain surface (401) and peripheral

sites such as mesentery or cremaster muscle (402). These allow neutrophil adhesion and migration to the vessel wall to be directly recorded in real time. Moreover, in the case of TRAIL or FasL, genetically modified mice are available that have reduced expression of TRAIL (403, 404) and receptors (405)(406), and knockdown of both Fas(*lpr*) and FasL(*gld*) (407, 408). Therefore, complementary studies could be carried out in these models, to determine if TRAIL or FasL alters leucocyte interactions with sites of interest. Angiogenesis has been modeled *in vivo* by using the Matrigel™ plug assay (409-412), in which endothelial cell infiltration and vessel formation are assessed in subcutaneous implants of Matrigel™. Tissue site specific angiogenesis can also be assessed. For instance, it could be induced with adenoviral vectors expressing pro- or anti-angiogenic factors, such as VEGF and VEGF receptors (413-415). Many disease models are associated with angiogenesis, including stroke, EAE and cancer models. These offer an opportunity to assess angiogenesis in response to agents such as resveratrol or TRAIL. For instance, it would be interesting to determine whether resveratrol inhibits the cerebral angiogenesis associated with stroke or glioma, since this has yet to be established. In this respect, it would be important to distinguish between resveratrol pre-treatment or application at times after disease onset, since this has a direct bearing on its use as a therapy. Dosage effects are also critical in this context.

6.5. Concluding Remarks

The field of angiogenesis research has concentrated on the impact of hypoxia as a basic stimulus at the earlier stages of angiogenesis. However, less emphasis has been placed on factors that regulate vessel regression at the later stages of angiogenesis. The

role of the immune system in angiogenesis is often seen as a secondary factor in which immune cells respond to hypoxia and stimulate vessel formation. This work adds further evidence on how the immune system limits angiogenesis. We have explored endothelial apoptosis as a key mechanism and shown its relationship to neutrophil interactions and the effect of TNF related death ligands.

Cerebral angiogenesis has been less well studied than peripheral angiogenesis which reflects the difficulty in establishing valid models. This study characterized a novel human brain endothelial cell line, hCMEC/D3 as a model of cerebral angiogenesis. It was compared to more established models, using HUVEC and other cell types. While many questions remain unanswered, particularly *in vivo* or in disease models, the basic mechanisms that we have defined in response to TRAIL and resveratrol, provide a necessary foundation for future work. We suggest that the death promoting activity of TRAIL is critical to, and cannot be divorced from, its inflammatory effects, both on neutrophil adhesion as well as an inhibitor of angiogenesis. Fas ligand appears to have a much less active role. By contrast, we describe the first study showing that resveratrol can also inhibit angiogenesis in a cerebral model, but without inducing caspase-dependent apoptosis. Thus we have explored the complementary roles of apoptosis (induced by TRAIL) and cellular modulation (induced by resveratrol) in the regulation of cerebral angiogenesis.

Epilogue:

As my Chinese Kungfu Master, Dr. Hong Tao Tze, once told me, "(World) Peace needs love and love requires balance. If you view a cell like the world, and an organism like the universe, you will understand your pathology studies." Maybe I am still confused by his words. But one thing becomes clear to me is that both stimulating and inhibiting elements are essential to cell function, and it all requires a delicate balance -- the essence of Chinese philosophy of Yin and Yang!

"Life itself is a proper binge." -- Julia Child

APPENDIX I

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APPENDIX II: SUPPLEMENTARY MATERIALS

Figure S.1. Surface Expression of TRAIL, Fas Ligand and Their Cognate Receptors and the VEGF Receptors R1 and R2 in hCMEC/D3 Cells.

Open histograms indicate fluorescent labeling with receptor specific monoclonal antibodies; shaded histograms show background labeling with isotype-matched control antibody in the same cells. Data are representative of 4 separate experiments.

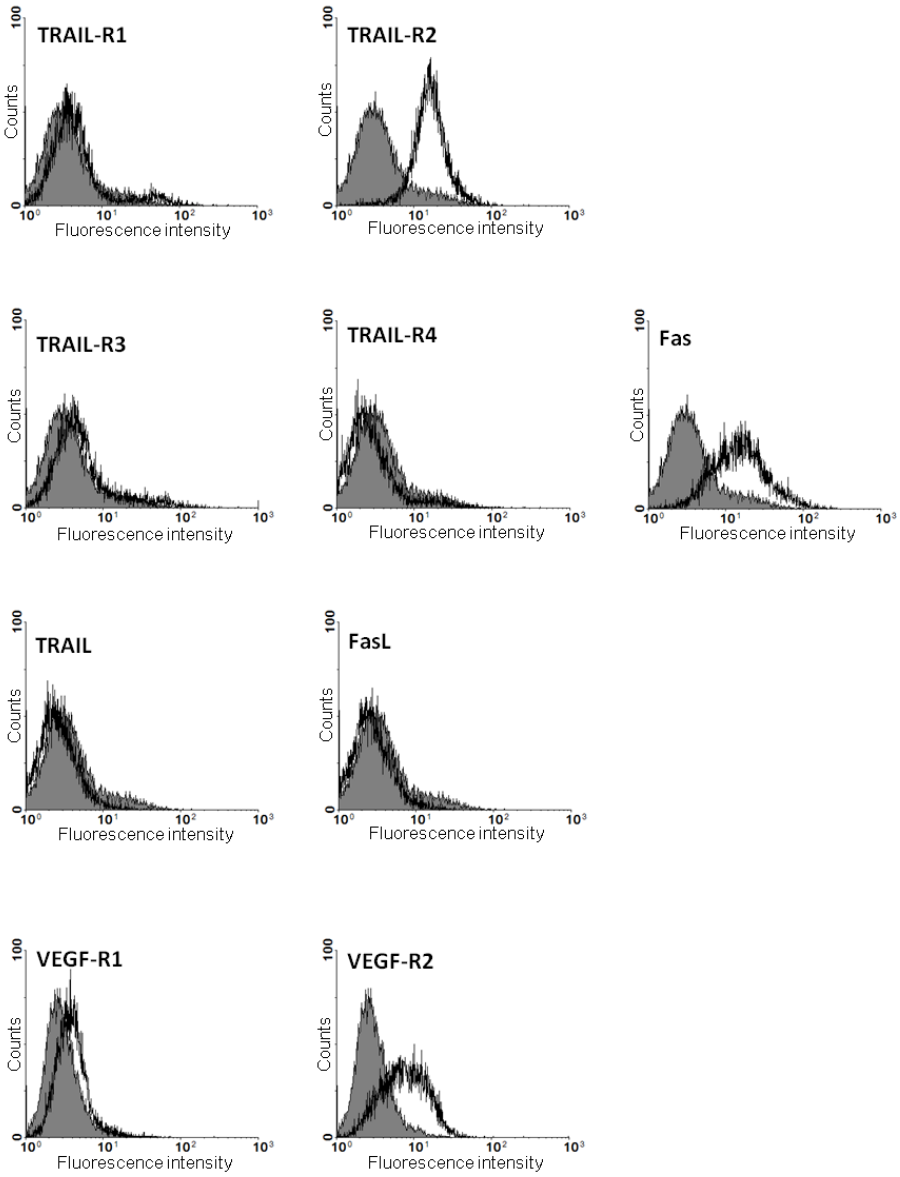


Figure S.1.

Figure S.2.: Effect of Growth Factors on BrdU Incorporation in hCMEC/D3 Cells.
Growth factor abbreviations are indicated in the text, doses are ng/ml. Data is shown (A) for quiescent cells (in serum free medium containing 2% BSA) or cells in 5% FBS (B), 24 h treatments. Data is normalized to the untreated control, and shown as mean ± SEM. Significance is compared to the untreated control; only significantly different bars are marked (*=P<0.05, **=P<0.01, ***=P<0.001). In (a), n=8 for untreated cells and n=3 for other groups. In (b), n=13 for untreated cells, n=6 for 2% BSA and n=2-4 for other groups; n= numbers of experiment done on a separate experimental day, and each experiment has 4 replications.

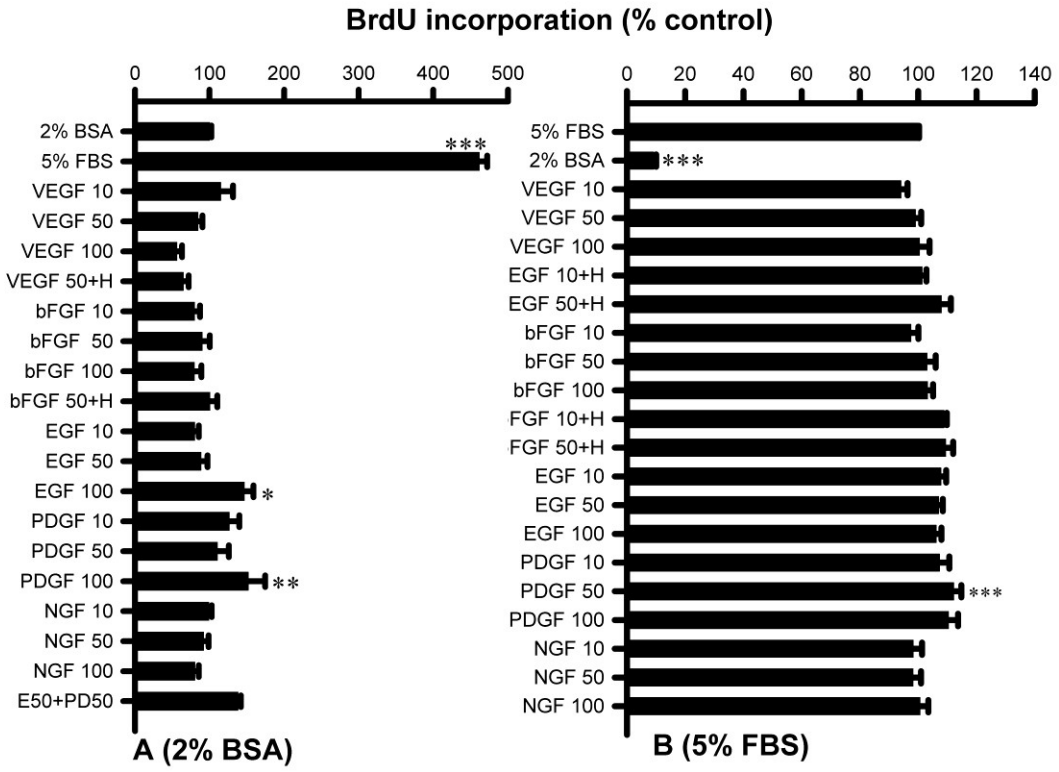


Figure S.2.

Figure S.3. Effect of Growth Factors and Heparin (H) on hCMEC/D3 Cell Numbers Assessed with the MTT Assay.

Cells were treated in serum free/ 2% BSA (A) or 5% FBS (B) medium for 24 hours. Significance is compared to the untreated control, only significantly different results are marked (***=P<0.001). In (a), n=15 for untreated cells, n=7 for 5% FBS, n=6 for 100ng/ml VEGF and bFGF, and n=4 for other groups. In (b), n=21 for untreated cells, n=12 for 2% BSA, n=7 for 100ng/ml bFGF, and n=2-5 for other groups. n= numbers of experiment done on a separate experimental day, and each experiment has 4 replications. Abbreviations in the text, doses in ng/ml. Data is normalized to the untreated control and expressed as mean ± SEM.

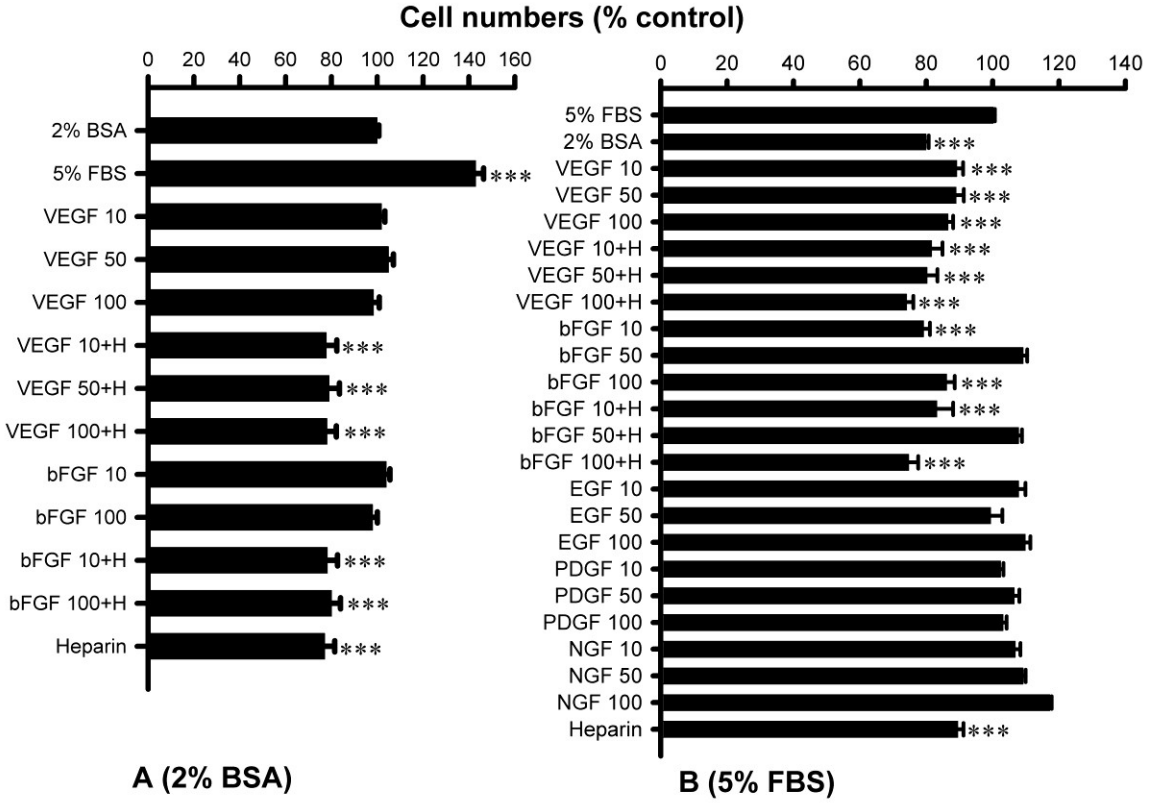


Figure S.3.

Figure S.4.: Cell Migration Assessed with Scratch Assay in hCMEC/D3 in Response to Growth Factors or TNF Family Ligands.

Data (mean ± SEM) is normalized to the untreated control in serum free/2%BSA (A) or 5% FBS (B) medium. Significance is compared to the untreated control, significantly different results only are marked (**=P<0.01, ***=P<0.001). In (A), n=20 for untreated cells, n=16 for 5% FBS, and n=4-10 for other groups. In (B), n=12 for untreated cells, n=12 for 2% BSA, n=3 for bFGF, and n=4-6 for other groups. n= numbers of experiment done on a separate experimental day, and each experiment has 4 replications. Use of the caspase inhibitor Z-VAD-FMK (50µmol/l) is indicated (CI). Other doses are ng/ml with the exception of TNF (U/ml).

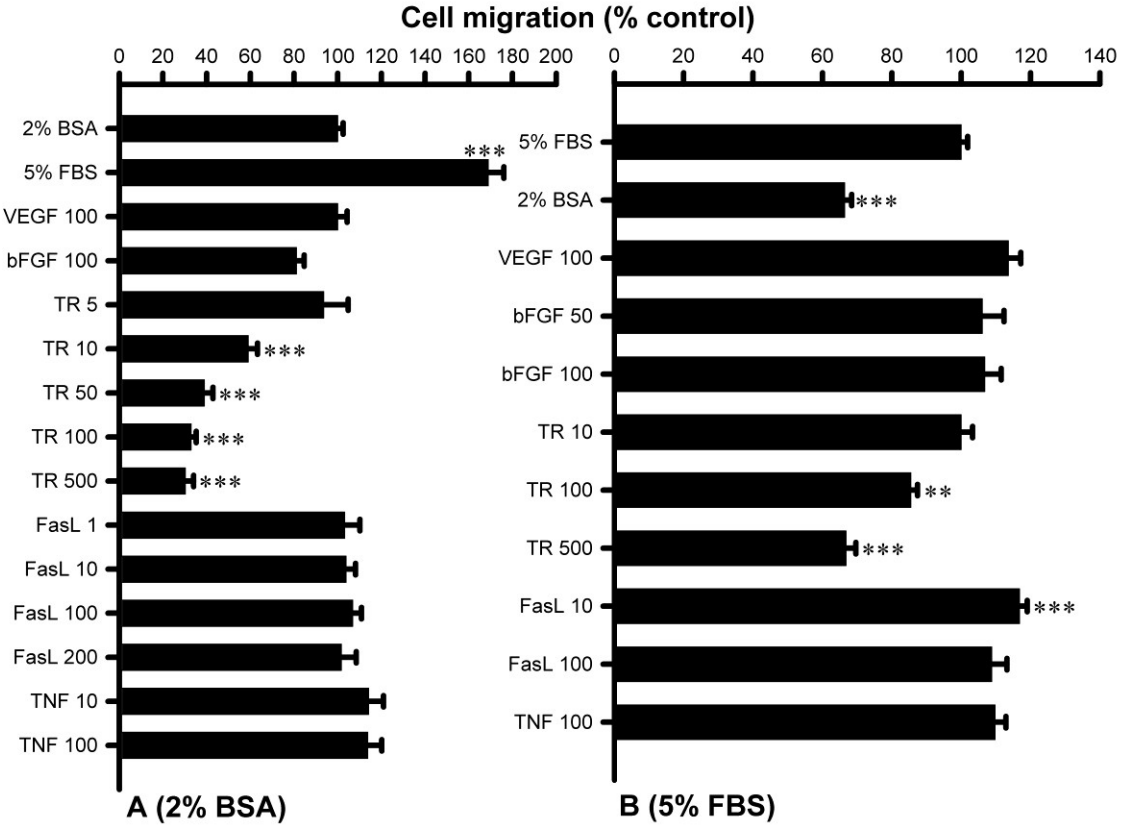


Figure S.4.

Figure S.5. ICAM-1 Expression in hCMEC/D3 Cells Following Treatments with TNF and TRAIL.

Open histograms indicate fluorescent labeling with ICAM-1-specific monoclonal antibodies; shaded histograms show background labeling with isotype-matched control antibody in the same cells. Results showed surface expression of ICAM-1 did not significantly alter on cells treated with TRAIL (100ng/ml) for 24 h. TNF (100U/ml) was a positive control and it induced an increase in ICAM-1 expression.

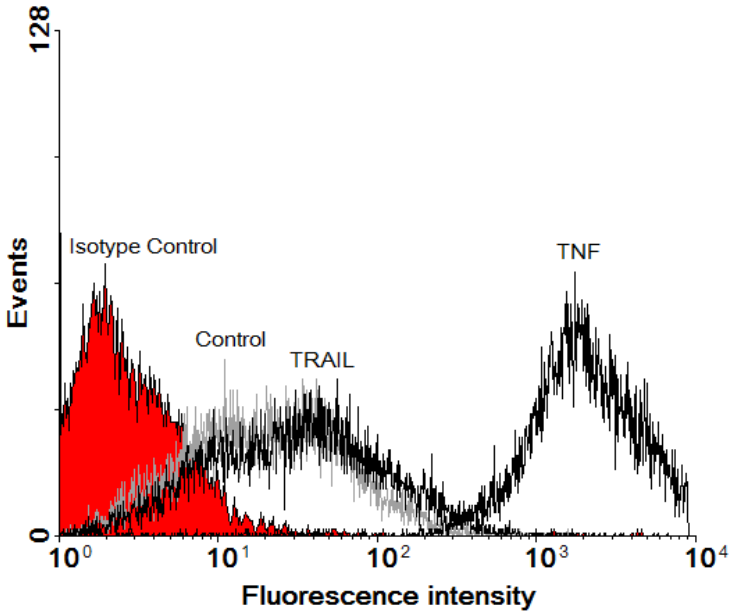


Figure S.5.

Figure S.6. Impact of Growth Factors, TNF Family Members and Resveratrol on HMVEC Cells.

A. BrdU assay; B. MTT cell proliferation assay; C. Scratch migration assay. Cells were treated for 24 hours in all three assays. Data is normalized to the untreated control, and shown as mean ± SEM. Significance is compared to the untreated control; only significantly different bars are marked (*=p<0.05, **=p<0.01, ***=p<0.001). In (a), n=2 for untreated cells and n= 1 for other groups. In (b), n=2 for untreated cells and n=1 for other groups. In (c), n=4 for untreated cells and n=2 for other groups; n= numbers of experiment done on a separate experimental day, and each experiment has 4 replications.

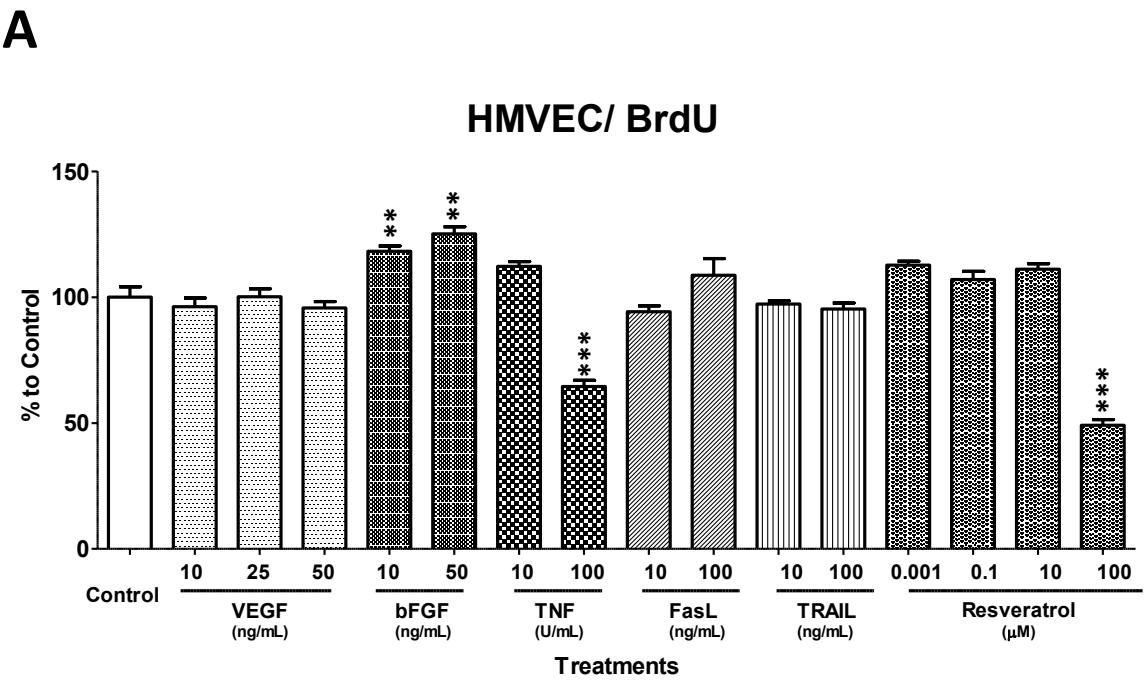
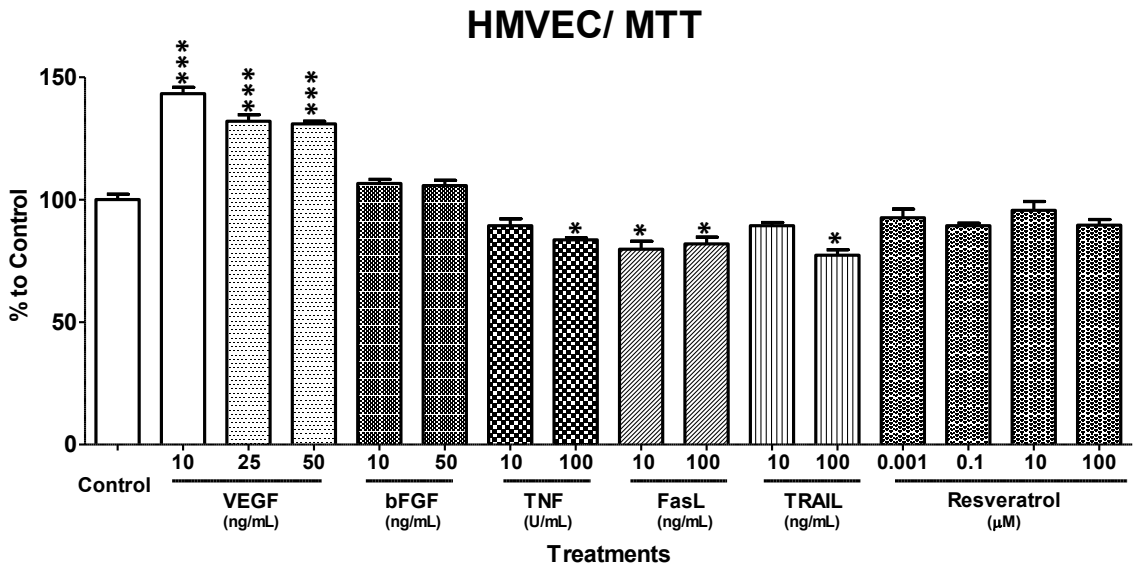


Figure S.6.

B


HMVEC/ MTT
% to Control
150
100
50
0
Control
10
25
50
10
50
10
100
10
100
10
100
0.001
0.1
10
100
VEGF
(ng/mL)
bFGF
(ng/mL)
TNF
(U/mL)
FasL
(ng/mL)
TRAIL
(ng/mL)
Resveratrol
(μM)
Treatments

C

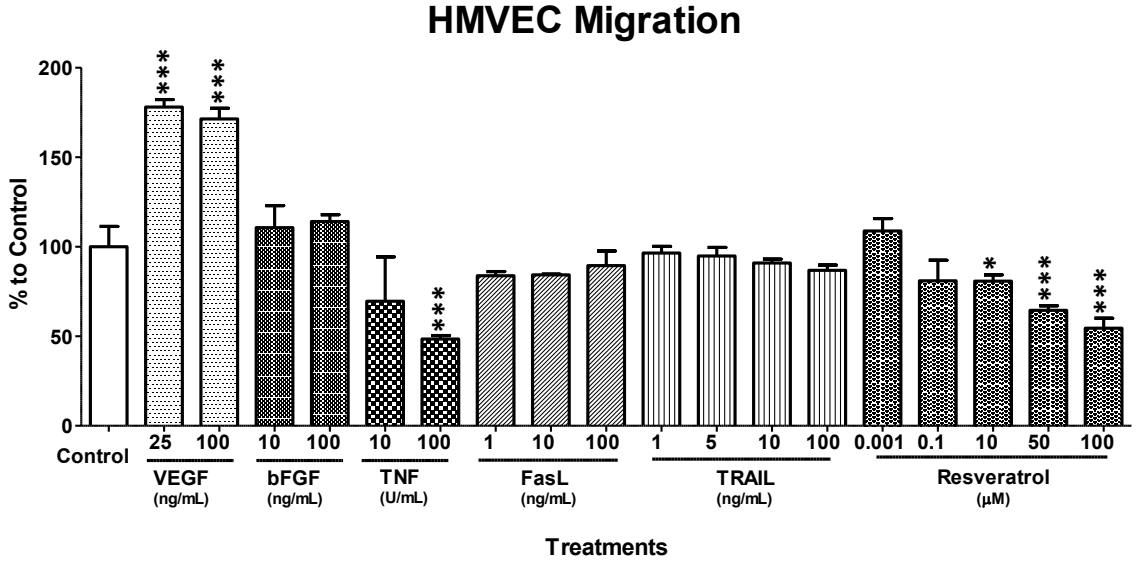

HMVEC Migration
% to Control
200
150
100
50
0
Control
25
100
10
100
10
100
1
10
100
1
5
10
100
0.001
0.1
10
50
100
VEGF
(ng/mL)
bFGF
(ng/mL)
TNF
(U/mL)
FasL
(ng/mL)
TRAIL
(ng/mL)
Resveratrol
(μM)
Treatments

Figure S.6.

Figure S.7. Impact of Growth Factors, TNF Family Members and Resveratrol on BBMEC Cells.

A. BrdU assay; B. MTT cell proliferation assay; C. Scratch migration assay. Cells were treated for 24 hours in all three assays. Data is normalized to the untreated control, and shown as mean ± SEM. Significance is compared to the untreated control; only significantly different bars are marked (*=p<0.05, **=p<0.01, ***=p<0.001). In (a), n=8 for untreated cells and n=3-6 for other groups. In (b), n=8 for untreated cells and n=3-8 for other groups. In (c), n=4 for untreated cells and n=2-4 for other groups; n= numbers of experiment done on a separate experimental day, and each experiment has 4 replications.

A

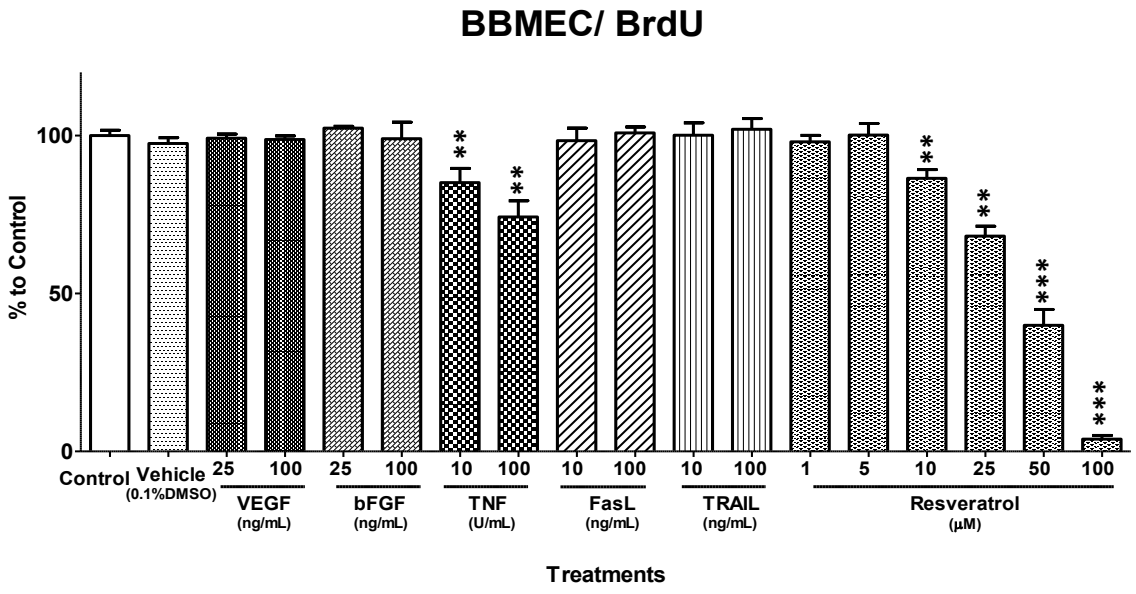
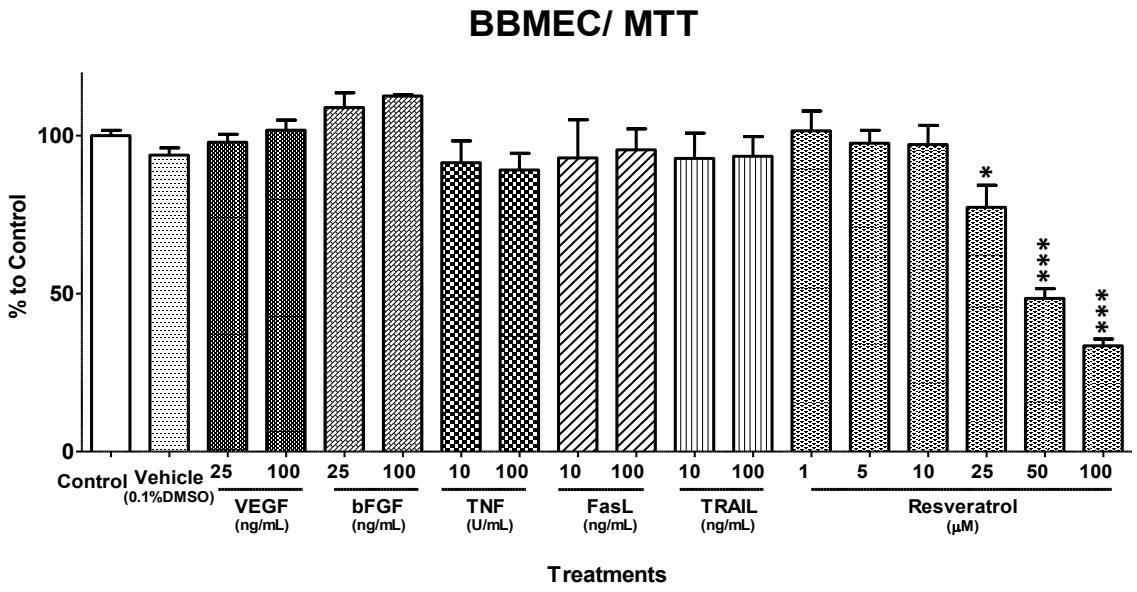


Figure S.7.

B


BBMEC/ MTT
% to Control
100
50
0
Control
Vehicle (0.1%DMSO)
25
100
VEGF (ng/mL)
25
100
bFGF (ng/mL)
10
100
TNF (U/mL)
10
100
FasL (ng/mL)
10
100
TRAIL (ng/mL)
1
5
10
25
50
100
Resveratrol (μM)
Treatments

C

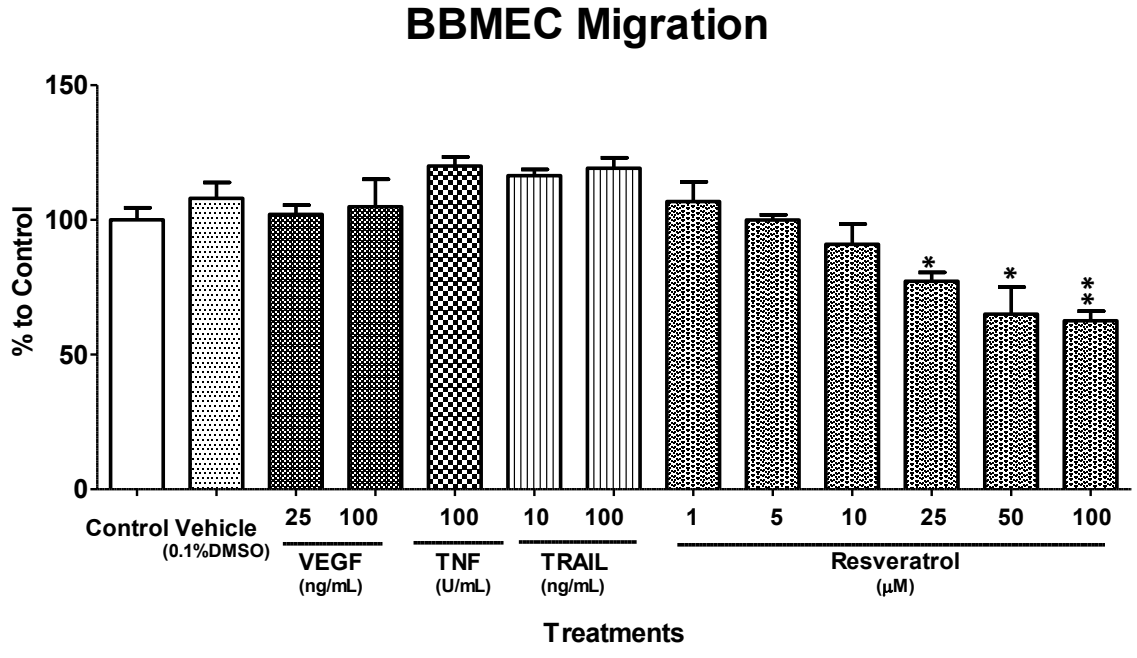

BBMEC Migration
% to Control
150
100
50
0
Control
Vehicle (0.1%DMSO)
25
100
VEGF (ng/mL)
100
TNF (U/mL)
10
100
TRAIL (ng/mL)
1
5
10
25
50
100
Resveratrol (μM)
Treatments

Figure S.7.

Figure S.8.Detection of NF-κB and MAPK Signaling Molecules in hCMEC/D3 Cells by Western Blotting.

A. Expression of a phosphorylated form of NF-κB (p-p65) and total cytosolic protein (p65) were detected by Western blotting analysis. Representative blots show the effect of different treatment durations (5min and 24h) with vehicle (0.1% DMSO) or indicated doses of resveratrol (10, 25, or 50μM). B. Summary data obtained by densitometry for 3 blots per treatment. Data is expressed as mean ± SEM and has been normalized to the vehicle treated group at each time point. Significance is relative to the vehicle (*P<0.05, **P<0.01). V=vehicle control.

A

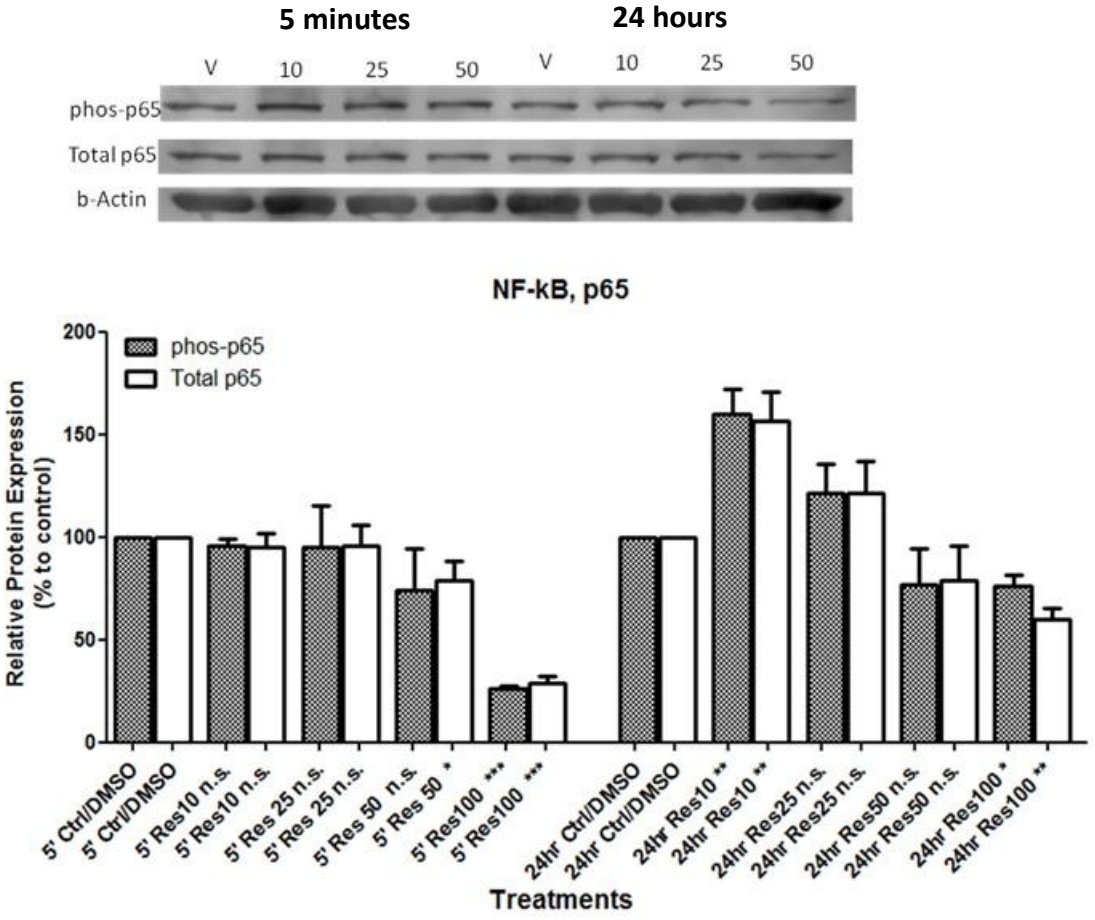


Figure S.8.

B. Expression of phosphorylated form of MAP kinase ERK1/2 (p-ERK1/2) and total ERK were detected by Western blotting analysis. Representative blots show the effect of different treatment durations (30 minutes and 24 hours) to untreated control (labelled as C), with vehicle (0.1% DMSO; labelled as V) or indicated doses of resveratrol (10, 25, 50, and 100µM). B. Summary data obtained by densitometry for 3 blots per treatment. Data is expressed as mean ± SEM and has been normalized to the vehicle treated group at each time point. Significance is relative to the vehicle (*p<0.05, **p<0.01, ***p<0.001).

B

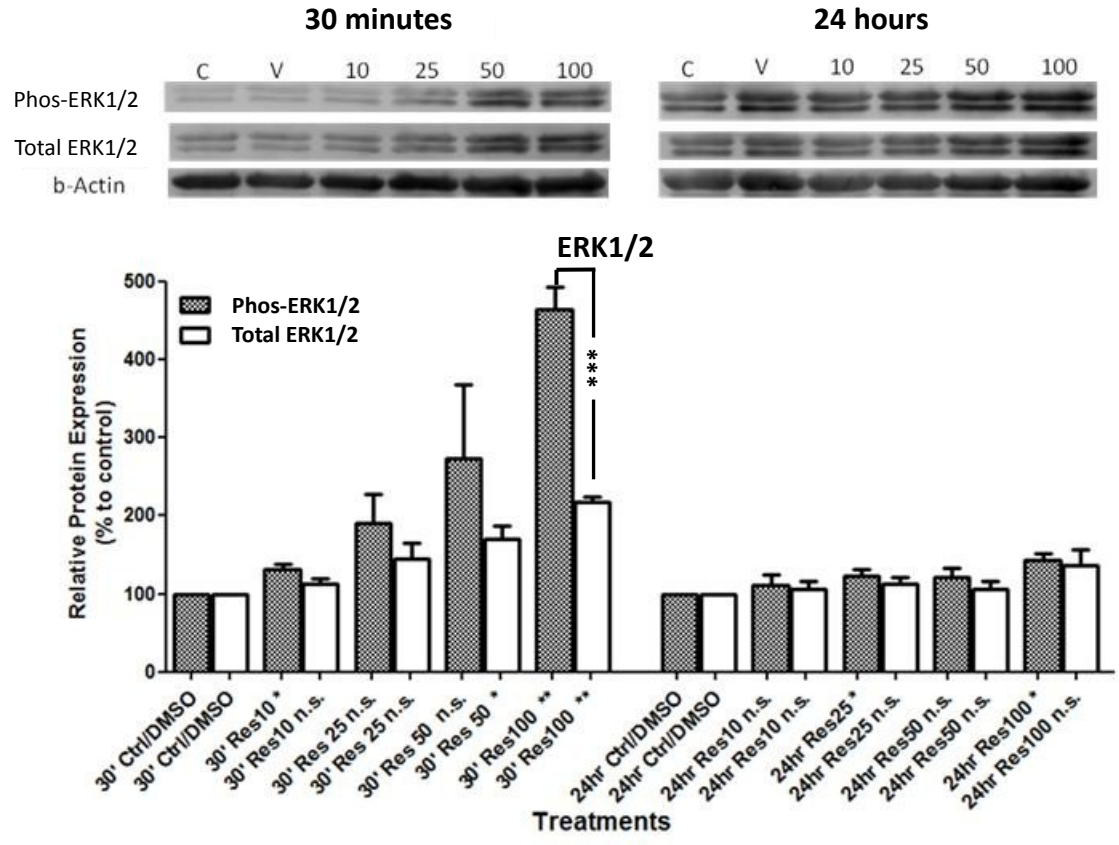


Figure S.8.

Figure S.9. Reduction in TNF-induced ICAM-1 Expression in hCMEC/D3 Cells Treated with Resveratrol.

ICAM-1 expression was detected by flow cytometry. For co-treatment experiments, hCMEC/D3 cells were treated with TNF (100U/ml) and resveratrol (50 or 100µM) or vehicle control (0.1%DMSO) for 24 hours. For pre-treatment experiments, cells were treated with resveratrol for 8 hours prior to addition of TNF for 24 hours. Data is an average of two samples obtained on a single day.

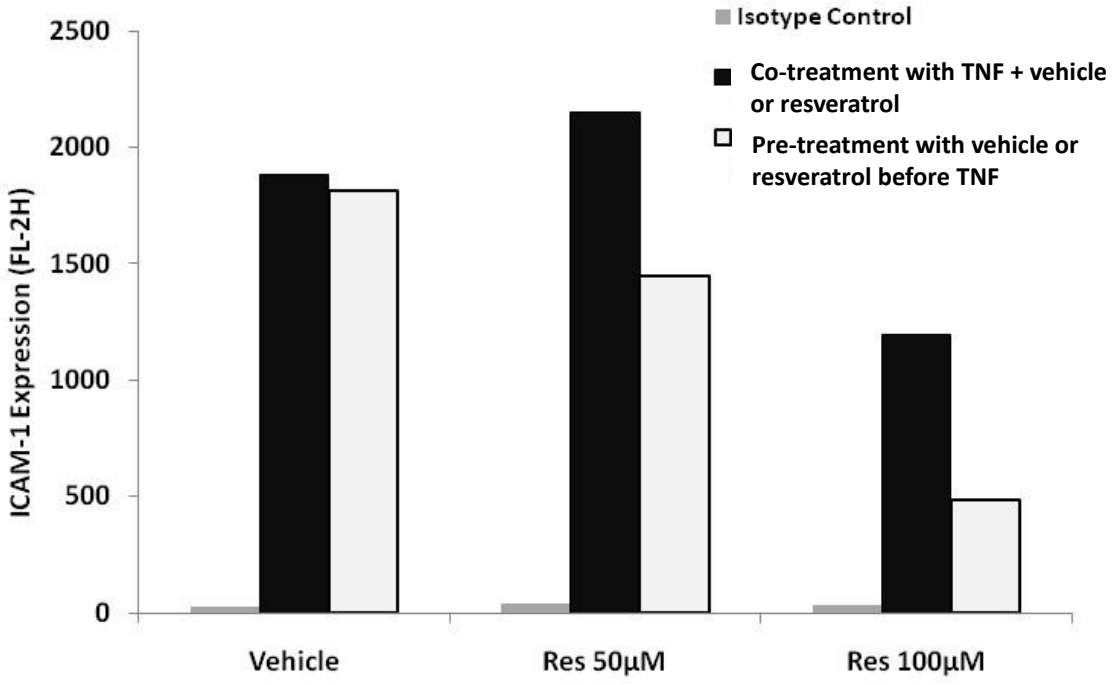


Figure S.9.

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